Chapter A
Cardiovascular Activity[1]

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A.1 Cardiovascular Analysis

A.1.1 In Vitro Methods

A.1.1.1 α_1-Adrenoreceptor Binding

PURPOSE AND RATIONALE

α_1-adrenoceptors are widely distributed and are activated either by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla. Receptor activation mediates a variety of functions, including contraction of smooth muscle, cardiac stimulation, cellular proliferation and activation of hepatic gluconeogenesis and glycolysis. In the CNS, the activation of α_1-adrenoceptors results in depolarization and increased neuronal firing rate.

The α-adrenoreceptor population of plasma membranes from rat heart ventricles consists only of the α_1-adrenoreceptor subtype. A constant concentration of the radioligand ^{3}H-prazosin (0.2–0.3 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from rat heart ventricles. If the test drug exhibits any affinity to α-adrenoceptors, it is able to compete with the radioligand for receptor binding äsites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug. The assay is used to evaluate the concentrationbinding characteristics of drugs atria the α_1-adrenoceptor.

PROCEDURE

Solutions

preparation buffer A:

$MgCl_2 \times 6H_2O$	5 mM
$MgCl_2 \times 6H_2O$	1 mM

D(+)-sucrose 250 mM

pH 7.4

preparation buffer B (= rinse buffer):

Tris-HCl 50 mM

$MgCl_2 \times 6H_2O$ 10 mM

pH 7.4

incubation buffer:

Tris-HCl 50 mM

$MgCl_2 \times 6H_2O$ 10 mM

ascorbic acid 1.6 mM

catechol 0.3 mM

pH 7.4

radioligand:

^{3}H-prazosin × HCl

specific activity

0.37–1.11 TBq/mmol

(10–30 Ci/mmol) (NEN)

Tissue Preparation

Male Sprague-Dawley rats (200–300 g) are sacrificed by decapitation and the dissected hearts are placed in ice-cold preparation buffer A. After removal of the atria, ventricles (approx. 30 g from 40 rats) are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/20 ml preparation buffer A), the homogenate is filtered through gauze, and centrifuged at 2000 *g* (4°C) for 10 min. The pellets are discarded; the supernatant is collected, and centrifuged again at 40,000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml preparation buffer B, homogenized by Ultra-Turrax and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in preparation buffer B, corresponding to 1 g ventricle wet weight/4 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at –77°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 *g* (4°C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of 1.0–1.5 mg/ml. After homogenization by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

For each concentration samples are prepared in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

total binding:

- 50 µl ^{3}H-prazosin (12 concentrations, 5×10^{-11}–5×10^{-9} M)
- 50 µl incubation buffer

non-specific binding:

- 50 µl ^{3}H-prazosin (4 concentrations, 5×10^{-11}–$\times 10^{-9}$ M)
- 50 µl phentolamine (10^{-5} M)

Competition Experiments

- 50 µl ^{3}H-prazosin (1 constant concentration, 2–3 × 10^{-10} M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (1.0–1.5 mg protein/ml). The samples are incubated for 30 min in a shaking bath at 25°C. The reaction is stopped by withdrawing the total incubation volume by rapid vacuum filtration over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding–non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-prazosin versus non-labeled drug by a computer-supported analysis of the binding data.

$$K_i = \frac{K_D\ ^3H \times IC^{50}}{K_D\ ^3H + [^3H]}$$

IC_{50} = concentration of the test drug, which competes 50% of specifically bound ^{3}H-prazosin in the competition experiment

[^{3}H] = concentration of ^{3}H-prazosin in the competition experiment.

$K_D\ ^3H$ = dissociation constant of ^{3}H-prazosin, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug. The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

MODIFICATION OF THE METHOD

Binding of ^{3}H-WB 4101 to α_1-adrenergic receptors in brain is used to test hypotensive activity as a possible side effect of neuroleptic drugs. The test E.5.1.6 is described in the chapter on neuroleptic activity.

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α_1-adrenoceptor similar to that found in the vas deferens.

SUBTYPES OF THE α_1-ADRENOCEPTOR

Several subtypes of the α_1-adrenoceptor have been identified by pharmacological means (α_{1A} and α_{1B}, α_{1C}, α_{1D}; α_{1H}, α_{1L} and α_{1N} adrenoceptors; Endoh et al. 1992; García-Sáinz et al. 1992, 1993; Ohmura et al. 1992; Regan and Cotecchia 1992; Satoh et al. 1992; Schwinn and Lomasney 1992; Veenstra et al. 1992; Aboud et al. 1993; Oshita et al. 1993; Vargas et al. 1993; Ruffolo et al. 1994; Minneman and Esbenshade 1994; Alexander et al. 2001) or by recombinant technology ($\alpha_{1a/d}$, α_{1b}, α_{1c} adrenoceptors). They correspond to the pharmacologically defined α_{1A}, α_{1B},and α_{1D} adrenoceptors in native tissues (Bylund et al. 1994, 1998; Hieble et al. 1995; Graham et al. 1996; Hieble and Ruffolo 1996; Alexander et al. 2001; Hawrylyshyn et al. 2004; Waitling 2006).

Binding of the radioligand [^{3}H]-prazosin to the α_{1A}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat submaxillary glands (Michel et al. 1989).

Binding of the radioligand [^{3}H]-prazosin to the α_{1B}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat livers (Adolfo et al. 1989).

According to Eltze and Boer (1992), the adrenoceptor agonist SDZ NVI 085 discriminates between α_{1A}- and α_{1B}-adrenoceptor subtypes in vas deferens, kidney and aorta of the rat and may therefore be used as a tool either to detect (rat vas deferens or kidney) or exclude (rat aorta) the functional involvement of "α_{1A}-adrenoceptors in smooth muscle contraction.".

Stam et al. (1998) found that (+)-cyclazosin, which behaves as a selective, high-affinity α_{1B}-adrenoceptor ligand in binding experiments, did not show the profile of a α_{1B}-adrenoceptor antagonist in functional tissues.

Decreased blood pressure response in mice deficient of the α_{1b}-adrenergic receptor was found by Cavalli et al. (1997).

Kenny et al. (1995) used the contractile response of rat aorta to adrenaline after the application of various α_1-adrenoceptor antagonists for characterization of a α_{1D}-adrenoceptor.

REFERENCES AND FURTHER READING

Aboud R, Shafii M, Docherty JR (1993) Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. Br J Pharmacol 109:80–87

Adolfo JA et al (1989) Species heterogeneity of hepatic α_1-adrenoceptors: α_{1A}-, α_{1B}-, and α_{1C}-subtypes. Biochem Biophys Res Comm 186:760–767

Ahlquist RP (1948) A study of the adrenotropic receptors. Am J Physiol 153:586–600

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Bylund DB, Eikenburg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR, Trendelenburg U (1994) IV. International Union of Pharmacology Nomenclature of Adrenoceptors. Pharmacol Rev 46:121–136

Bylund DB, Bond RA, Clarke DE, Eikenburg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR, Strosberg AD, Trendelenburg U (1998) Adrenoceptors. The IUPHAR Compendium of Receptor Characterization and Classification

Cavalli A, Lattion AL, Hummler E, Nenniger M, Pedrazzini T, Aubert JF, Michel MC, Yang M, Lembo G, Vecchione C, Mostardini M, Schmidt A, Beermann F, Cotecchia S (1997) Decreased blood pressure response in mice deficient of the α_{1b}-adrenergic receptor. Proc Natl Acad Sci USA 94:11589–11594

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Couldwell C, Jackson A, O'Brien H, Chess-Williams R (1993) Characterization of the α_1-adrenoceptors of rat prostate gland. J Pharm Pharmacol 45:922–924

Eltze M, Boer R (1992) The adrenoceptor agonist, SDZ NVI 085, discriminates between α_{1A}-and α_{1B}-adrenoceptor-subtypes in vas deferens, kidney and aorta of the rat. Eur J Pharmacol 224:125–136

Endoh M, Takanashi M, Norota I (1992) Role of alpha$_{1A}$ adrenoceptor subtype in production of the positive inotropic effect mediated via myocardial alpha$_1$ adrenoceptors in the rabbit papillary muscle: influence of selective alpha$_{1A}$ subtype antagonists WB 4101 and 5-methylurapidil. Naunyn-Schmiedeberg's Arch Pharmacol 345:578–585

García-Sáinz JA (1993) α_1-adrenergic action: receptor subtypes, signal transduction and regulation. Cell Signal 5:539–547

García-Sáinz JA, Romero-Avila MT, Hernandez RA, Macias-Silva M, Olivares-Reyes A, González-Espinosa C (1992) Species heterogeneity of hepatic α_1-adrenoceptors: α_{1A}-, α_{1B}- and α_{1C}-subtypes. Biochem Biophys Res Commun 186:760–767

Gleason MM, Hieble JP (1992) The α_2-adrenoreceptors of the human retinoblasoma cell line (Y79) may represent an additional example of the α_{2C}-adrenoceptor. Br. J Pharmacol 107:222–225

Graham RM, Perez DM, Hawa J (1996) Alpha1 adrenergic receptor subtypes. Molecular structure, function and signaling. Circ Res 78:737–749

Greengrass P, Bremner R (1979) Binding characteristics of ^{3}H-prazosin to rat brain α-adrenergic receptors. Eur J Pharmacol 55:323–326

Guicheney P, Meyer P (1981) Binding of [^{3}H]-prazosin and [^{3}H]-dihydroergocryptine to rat cardiac α-adrenoceptors. Br J Pharmac 73:33–39

Hawrylyshyn KA, Michelotti GA, Cogé F, Guénin SP, Schwinn DA (2004) Update on human α_1-adrenoceptor subtype signaling and genomic organization. Trends Pharmacol Sci 25 :449–455

Hieble JP, Ruffolo RR Jr (1997) Recent advances in the identification of α_1- and α_2-adrenoceptor subtypes. Therapeutic implications. Expert Opin Invest Drugs 6:367–387

Hieble JP, Bylund DB, Clarke DE, Eikenburg DC, Langer SZ, Lefkowitz RJ, Minneman KP, Ruffolo RR Jr (1995) International Union of Pharmacology: X. Recommendation for nomenclature of α_1-adrenoceptors. Consensus update. Pharmacol Rev 47:267–270

Hoffman BB, de Lean A, Wood CL, Schocken DD, Lefkowitz RJ (1979) Alpha-adrenergic receptor subtypes: Quantitative assessment by ligand binding. Life Sci 24:1739–1746

Kenny BB, Chalmers DH, Philpott PC, Naylor AM (1995) Characterization of an α_{1D}-adrenoceptor mediating the contractile response of rat aorta to adrenaline. Br J Pharmacol 115:981–986

Miach PJ, Dausse JP, Cardot A, Meyer P (1980) ^{3}H-prazosin binds specifically to 'α_1'-adrenoceptors in rat brain. Naunyn-Schmiedeberg's Arch Pharmacol 312:23–26

Michel AD, et al (1989) Identification of a single α_{1A}-adrenoceptor corresponding to the α_{1A}-subtype in rat submaxillary gland. Br J Pharmacol 98:833–889

Minneman KP, Esbenshade TA (1994) α_1-adrenergic receptor subtypes. Ann Rev Pharmacol Toxicol 34:117–133

Ohmura T, Oshita M, Kigoshi S, Muramatsu I (1992) Identification of α_1-adrenoceptor subtypes in the rat vas deferens: binding and functional studies. Br J Pharmacol 107:697–704

Oshita M, Kigoshi S, Muramatsu I (1993) Pharmacological characterization of two distinct α_1-adrenoceptor subtypes in rabbit thoracic aorta. Br J Pharmacol 108:1071–1076

Regan JW, Cotecchia S (1992) The α-adrenergic receptors: new subtypes, pharmacology, and coupling mechanisms. In: Brann MR (ed) Molecular Biology of G-Protein-coupled receptors. Birkhäuser, Boston Basel Berlin, pp 76–112

Ruffolo RR, Stadel JM, Hieble JP (1994) α-adrenoceptors: recent developments. Med Res Rev 14:279–270

Satoh M, Kojima C, Takayanagi I (1992) Characterization of α_1-adrenoceptor subtypes labeled by [^{3}H]prazosin in single cells prepared from rabbit thoracic aorta. Eur J Pharmacol 221:35–41

Schwinn DA, Lomasney JW (1992) Pharmacologic characterization of cloned α_1-adrenoceptor subtypes: selective antagonists suggest the existence of a fourth subtype. Eur J Pharmacol – Mol Pharmacol Sect 227:433–436

Stam WB, Van der Graaf PH, Saxena PR (1998) Functional characterization of the pharmacological profile of the putative α_{1B}-adrenoceptor antagonist, (+)-cyclazosin. Eur J Pharmacol 361:79–83

Timmermans PBMWM, Karamat Ali F, Kwa HY, Schoop AMC, Slothorst-Grisdijk FP, van Zwieten PA (1981) Identical antagonist selectivity of central and peripheral alpha$_1$ adrenoceptors. Mol Pharmacol 20:295–301

Vargas HM, Cunningham D, Zhou L, Hartman HB, Gorman AJ (1993) Cardiovascular effects of chloroethylclonidine, a irreversible α_{1B}-adrenoceptor antagonist, in the unanesthetized rat: a pharmacological analysis *in vivo* and *in vitro*. J Pharm Exp Ther 266:864–871

Veenstra DMJ, van Buuren KJH, Nijkamp FP (1992) Determination of α_1-adrenoceptor subtype selectivity by [^{3}H]-prazosin displacement studies in guinea-pig cerebral cortex and rat spleen membranes. Br J Pharmacol 107:202–206

Waitling KJ (ed) (2006) The Sigma-RBI handbook of receptor classification and signal transduction, 5th edn. Sigma-Aldrich, St Louis, Mo., pp 88–89

A.1.1.2 α_2-Adrenoreceptor Binding

PURPOSE AND RATIONALE

α_2-adrenoceptors are widely distributed and are activated by norepinephrine released from sympathetic nerve terminals or by epinephrine released from the adrenal medulla or from some neurons in the CNS. The most extensively characterized action is the prejunctionally mediated inhibition of the release of neurotransmitters from many peripheral and central neurons. α_2-adrenoceptors are also present at postjunctional sites, where they mediate actions such as smooth muscle contraction, platelet aggregation and inhibition of insulin secretion. Activation of postsynaptic α_2-adrenoceptors in the brainstem results in an inhibition of sympathetic outflow in the periphery.

Clonidine is a centrally-acting antihypertensive agent, which lowers blood pressure mostly through reducing sympathetic tone by acting at the nucleus tractus solitarius in the brain stem (Kobinger and Walland 1967). Clonidine can, however, act at both peripheral and central α_2-receptors. Peripherally administered clonidine causes a brief increase in blood pressure followed by a prolonged decrease (Rand and Wilson 1968). Functional studies (^{3}H-NE release) indicate a presynaptic mechanism for clonidine (Langer 1977,

1981; Starke 1977). However, lesioning studies fail to confirm a presynaptic location for clonidine receptors in either the CNS or periphery (U'Prichard et al. 1979; Bylund and Martinez 1981; U'Prichard et al. 1980). No change in clonidine receptor sites was seen after 6-hydroxydopamine lesions in cerebral cortex. This may be due to the fact that α_2-receptors are both pre- and postsynaptic (Hieble et al. 1988).

Alpha-adrenergic agonists most potently displace ^{3}H-clonidine. Ergot compounds, dopamine agonists and mianserin are also fairly potent (U'Prichard et al. 1977). A survey on functions mediated by alpha-2 adrenergic receptors was given by Ruffolo et al. (1988) and on the role of neurotransmitters in the central regulation of the cardiovascular system by McCall (1990). Although clonidine relieves the autonomic symptoms of morphine withdrawal (Gold et al. 1978), there is no evidence for a direct α_2/opiate-receptor interaction.

The purpose of this assay is to assess the interaction of hypotensive agents with central α_2-receptors and determine possible clonidine-like mechanisms of action. Clonidine binding may also be relevant to the activity of other classes of drugs such as antidepressants that interact with α_2-receptors.

PROCEDURE

Reagents

1. Tris buffer pH 7.7
   a) 57.2 g Tris HCl q.s. to 1 liter
      (0.5 M Tris buffer, pH 7.7)
      16.2 g Tris base
   b) make a 1:10 dilution in distilled H_2O
      (0.05 M Tris buffer, pH 7.7)
2. Tris buffer containing physiological ions
   a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
$CaCl_2$	0.222 g
$MgCl_2$	0.204 g

   q.s to 100 ml in 0.5 M Tris buffer
   b) Dilute 1:10 in distilled H_2O.
      This yields 0.05 M Tris HCl, pH 7.7; containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM)
3. [4-^{3}H]-Clonidine hydrochloride (20–30 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: ^{3}H-Clonidine is made up to a concentration of 120 nM and 50 µl are added to each tube (yielding a final concentration of 3 nM in the 2 ml volume assay).
4. Clonidine-HCl is obtained from Boehringer Ingelheim.
   A stock solution of 0.1 mM clonidine is made up to determine non-specific binding. This yields a final concentration of 1 µM in the assay (20 µl to 2 ml).
5. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, so that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay and higher or lower concentrations can be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation and the cortical tissue is rapidly dissected. The tissue is homogenized in 50 volumes of 0.05 M Tris buffer pH 7.7 (buffer 1b) with the Brinkman Polytron, and centrifuged at 40,000 *g* for 15 min. The supernatant is discarded and the final pellet rehomogenized in 50 volumes of buffer 2b. This tissue suspension is then stored on ice. The final tissue concentration is 10 mg/ml. Specific binding is 1% of the total added ligand and 80% of total bound ligand.

Assay

100 µl	0.5 M Tris – physiological salts pH 7.7 (buffer 2a)
830 µl	H_2O
20 µl	Vehicle (for total binding) or 0.1 mM clonidine (for nonspecific binding) or appropriate drug concentration.
50 µl	^{3}H-clonidine stock solution
1000 µl	tissue suspension.

Tissue homogenates are incubated for 20 min at 25°C with 3 nM ^{3}H-clonidine and varying drug concentrations, and immediately filtered under reduced pressure on Whatman GF-B filters. The filters are washed with 3 five ml volumes of 0.05 M Tris buffer pH 7.7, and transferred to scintillation vials. Specific clonidine binding is defined as the difference between total bound radioactivity and that bound in the presence of 1 µM clonidine.

EVALUATION

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

MODIFICATIONS OF THE METHOD

Perry and U'Prichard (1981) described [^{3}H]rauwolscine (α-yohimbine) as a specific radioligand for brain α_2-adrenergic receptors.

Goldberg and Robertson (1983) reviewed yohimbine as a pharmacological probe for the study of the α_2-adrenoreceptor.

Pimoule et al. (1983) characterized [^{3}H]RX 781094 [(imidazolinyl-2)-2benzodioxane-1,4] as a specific α_2-adrenoceptor antagonist radioligand.

Murphy and Bylund (1988) characterized alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line.

Binding of the radioligand [^{3}H]-rauwolscine to the α_{2A}-adrenoceptor subtype can be measured in membranes prepared from rabbit spleens (Michel et al. 1989).

Binding of the radioligand [^{3}H]-yohimbine to the α_{2B}-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat kidney cortices (Connaughton and Docherty 1989).

SUBTYPES OF THE α_2-ADRENOCEPTOR

Using ^{3}H-rauwolscine as ligand Broadhurst et al. (1988) studied the existence of two alpha$_2$-adrenoreceptor subtypes.

Bylund et al. (1988) used [^{3}H]-yohimbine and [^{3}H]-rauwolscine to study alpha-2A and alpha-2B adrenergic subtypes in tissues and cell lines containing only one subtype.

Brown et al. (1990) found that [^{3}H]-yohimbine labels at α_{2A}- and α_{2B}-adrenoceptors whereas [^{3}H]-idazoxan labels the α_{2A}-adrenoceptor and, in addition, an imidazoline binding site.

Several subtypes of the α_2-adrenoceptor have been identified by pharmacological means (α_{2A}-, α_{2B}-, α_{2C}-, and α_{2D}-adrenoceptors; Ruffulo 1990; Uhlén and Wikberg 1990; Gleason and Hieble 1992; Satoh and Takayanagi 1992; Takano et al. 1992; Ruffolo et al. 1993) or by recombinant technology as α_{2a}-, α_{2b}-, α_{2c}-adrenoceptors (Bylund et al. 1994; Hieble et al. 1995; Hieble and Ruffolo 1996; Alexander et al. 2001; Waitling 2006).

Gleason and Hieble (1992) reported that the α_2-adrenoreceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C}-adrenoceptor.

Marjamäki et al. (1993) recommended the use of recombinant human α_2-adrenoceptors to characterize subtype selectivity of antagonist binding.

All three α_2-adrenoceptor types serve as autoreceptors in postganglionic sympathetic neurons (Trendelenburg et al. 2003).

Uhlén et al. (1994) found that the α_2-adrenergic radioligand [^{3}H]-MK912 is α_{2C}-selective among human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors.

Uhlén et al. (1998) tested the binding of the radioligand [^{3}H]RS79948–197 to human, guinea pig and pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors and compared the values with MK912, RX821002, rauwolscine and yohimbine. [^{3}H]RS79948–197 was non-selective for the α_2-adrenoceptor subtypes, showing high affinity for all three.

REFERENCES AND FURTHER READING

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Boyajian CL, Leslie FM (1987) Pharmacological evidence for alpha-2 adrenoceptor heterogeneity: Differential binding properties of [^{3}H]rauwolscine and [^{3}H]idazoxan in rat brain. J Pharmacol Exp Ther 241:1092–1098

Brasch H (1991) No influence of prejunctional α_2-adrenoceptors on the effects of nicotine and tyramine in guinea-pig atria. J Auton Pharmacol 11:37–44

Broadhurst AM, Alexander BS, Wood MD (1988) Heterogeneous ^{3}H-rauwolscine binding sites in rat cortex: two alpha$_2$-adrenoreceptor subtypes or an additional non-adrenergic interaction? Life Sci 43:83–92

Brown CM, MacKinnon AC, McGrath JC, Spedding M, Kilpatrick AT (1990) α_2-adrenoceptors subtypes and imidazoline-like binding sites in the rat brain. Br J Pharmacol 99:803–809

Bylund DB (1978) Subtypes of α_2-adrenoceptors: pharmacological and molecular biological evidence converge. Trends Pharmacol Sci 9:356–361

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR, Trendelenburg U (1994) IV. International union of pharmacology nomenclature of adrenoceptors. Pharmacol Rev 46:121–136

Bylund DB, Martinez JR (1981) Postsynaptic localization of α_2-adrenergic receptors in rat submandibular gland. J Neurosci 1:1003–1007

Bylund DB, Ray-Prenger C, Murphy TJ (1988) Alpha-2A and alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. J Pharmacol Exp Ther 245:600–607

Connaughton S, Docherty R (1989) Functional evidence for heterogeneity of peripheral prejunctional α_2-adrenoceptors. Br J Pharmacol 101:285–290

Gleason MM, Hieble JP (1992) The α_2-adrenoceptors of the human retinoblastoma cell line (Y79) may represent an additional example for the α_{2C}-adrenoceptor. Br J Pharmacol 107:222–225

Gold MS, Redmond DE, Kleber HD (1978) Clonidine blocks acute opiate withdrawal symptoms. Lancet 2:599–602

Goldberg MR, Robertson D (1983) Yohimbine: a pharmacological probe for the study of the α_2-adrenoreceptor. Pharmacol Rev 35:143–180

Hieble JP, Sulpicio AC, Nichols AJ, Willette RN, Ruffulo RR (1988) Pharmacologic characterization of SK&F

104078, a novel alpha-2 adrenoceptor antagonist which discriminates between pre- and postjunctional alpha-2 adrenoceptors. J Pharm Exp Ther 247:645–652
Hieble JP, Ruffolo RR Jr, Sulpicio AC (1995) Functional subclassification of α_2-adrenoreceptors. Pharmacol Commun 6:91–97
Hieble JP, Ruffolo RR Jr, Sulpicio AC, Naselsky DP, Conway TM, Ellis C, Swift AM, Ganguli S, Bergsma DJ (1996) Subclassification and nomenclature of α_1- and α_2-adrenoceptors. In: Jucker E (ed) Progress in Drug Research. Birkhäuser Verlag, pp 81–130
Kobinger W, Walland A (1967) Investigations into the mechanism of the hypotensive effect of 2-(2,6-dichlorphenylamino)-2-imidazoline-HCl. Eur J Pharmacol 2:155–162
Langer SZ (1977) Presynaptic receptors and their role in the regulation of transmitter release. Br J Pharmacol 60:481–497
Langer SZ (1981) Presynaptic regulation of the release of catecholamines. Pharmacol Rev 32:337–362
Marjamäki A, Luomala K, Ala-Uotila S, Scheinin M (1993) Use of recombinant human α_2-adrenoceptors to characterize subtype selectivity of antagonist binding. Eur J Pharmacol, Mol Pharmacol Sect 246:219–226
McCall RB (1990) Role of neurotransmitters in the central regulation of the cardiovascular system. Progr Drug Res 35:25–84
Michel AD, Loury DN, Withing RL (1989) Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}- and α_{2B}-adrenoceptor subtypes. Br J Pharmacol 98:890–897
Murphy TJ, Bylund DB (1988) Characterization of alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line. J Pharmacol Exp Ther 244:571–578
Perry BD, U'Prichard DC (1981) [^{3}H]rauwolscine (α-yohimbine): a specific radioligand for brain α_2-adrenergic receptors. Eur J Pharmacol 76:461–464
Pimoule C, Scatton B, Langer SZ (1983) [^{3}H]RX 781094: a new antagonist ligand labels α_2-adrenoceptors in the rat brain cortex. Eur J Pharmacol 95:79–85
Rand MJ, Wilson J (1968) Mechanisms of the pressor and depressor actions of St 155 (2-(2,6-dichlorophenylamino-2-imidazoline hydrochloride) (Catapres). Eur J Pharmacol 3:27–33
Ruffulo RR (1990) α_2-adrenoceptor agonists and antagonists. Neurotransmissions 6 (2):1–5
Ruffulo RR, Nichols AJ, Hieble JP (1988) Functions mediated by alpha-2 adrenergic receptors. Humana Press, Clifton, pp 187–280
Ruffolo RR, Nichols AJ, Stadel JM, Hieble JP (1993) Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. Annu Rev Pharmacol Toxicol 33:243–279
Satoh M, Takayanagi I (1992) Identification and characterization of the α_{2D}-adrenoceptor subtype in single cells prepared from guinea pig tracheal smooth muscle. Japan J Pharmacol 60:393–395
Starke K (1977) Regulation of noradrenaline release by presynaptic receptor systems. Rev Physiol Biochem Pharmacol 77:1–124
Summers RJ, Barnett DB, Nahorski SR (1983) Characteristics of adrenoceptors in homogenates of human cerebral cortex labelled with (^{3}H)-rauwolscine. Life Sci 33:1105–1112
Takano Y, Takano M, Yaksh TL (1992) The effect of intrathecally administered imiloxan and WB4101: possible role of α_2-adrenoceptor subtypes in the spinal cord. Eur J Pharmacol 219:465–468
Trendelenburg AU, Philipp M, Meyer A, Klebroff W, Hein L, Starke K (2003) All three alpha2-adrenoceptor types serve as autoreceptors in postganglionic sympathetic neurons. Naunyn-Schmiedebergs Arch Pharmacol 368:504–512
U'Prichard DC, Greenberg DA, Snyder SH (1977) Binding characteristics of a radiolabeled agonist and antagonist at central nervous system alpha noradrenergic receptors. Mol Pharmacol 13:454–473
U'Prichard DC, Bechtel WD, Rouot B, Snyder SH (1979) Multiple apparent alpha-noradrenergic receptor binding sites in rat brain: Effect of 6-hydroxydopamine. Mol Pharmacol 15:47–60
U'Prichard DC, Reisine TD, Mason ST, Fibiger MC, Yamamura HI (1980) Modulation of rat brain α- and β-adrenergic receptor populations by lesion of the dorsal noradrenergic bundle. Brain Res 187:143–154
Uhlén S, Wikberg JES (1990) Spinal cord α_2-adrenoceptors are of the α_{2A}-subtype: comparison with α_{2A}- and α_{2B}-adrenoceptors in rat spleen, cerebral cortex and kidney using [^{3}H]-RX821002 ligand binding. Pharmacol Toxicol 69:341–350
Uhlén S, Porter AC, Neubig RR (1994) The novel α_2-adrenergic radioligand [^{3}H]-MK912 is α_{2C}-selective among human α_{2A}-, α_{2B}- and -α_{2C} adrenoceptors. J Pharmacol Exp Ther 271:1558–1565
Uhlén S, Dambrova M, Näsman J, Schiöth HB, Gu Y, Wikberg-Mattson A, Wikberg JE (1998) [^{3}H]RS79948–197 binding to human, guinea pig and pig α_{2A}-, α_{2B}- and -α_{2C} adrenoceptors. Comparison with MK912, RX821002, rauwolscine and yohimbine. Eur J Pharmacol 343:93–101
Waitling KJ (2006) The Sigma RBI handbook of receptor classification and signal transduction, 5th edn. Sigma-Aldrich, St Louis, Mo., pp 90–91

A.1.1.3 Electrically Stimulated Release of [^{3}H]Norepinephrine from Brain Slices

PURPOSE AND RATIONALE

The existence of presynaptic receptors which regulate the evoked release of neurotransmitters has been functionally demonstrated in both peripheral and central nervous system (Langer 1981; Starke 1981; Raiteri et al. 1984; Miller 1998). Presynaptic adrenergic α_2-receptors regulate the evoked release of norepinephrine, comprising a short negative feedback loop. Alpha-2 agonists, such as clonidine and guanabenz, inhibit evoked release and alpha-2 antagonists, such as yohimbine and idazoxan, enhance evoked release.

The assay is used as a biochemical screen for agents which enhance or inhibit release of [^{3}H]norepinephrine (^{3}H-NE) and is particularly useful for testing receptor function of α_2-adrenergic agonists and antagonists.

The procedures used emphasize delicate care of slices. By treating slices with great care, one is able to incubate at low tracer concentrations of ^{3}H-NE (25 nM), thus minimizing nonspecific labeling of releasable pools other than those in noradrenergic nerve terminals. It also permits the use of low (and more physiological) stimulation parameters, which allow the

neurons to recover easily between stimulations and do not flood the synaptic cleft with released NE, which would compete with any applied drug thus decreasing sensitivity.

PROCEDURE

This assay is based on the method described by Zahniser et al. (1986).

A Reagents

1. Krebs-Henseleit bicarbonate buffer, pH 7.4 (KHBB):

NaCl	118.4 mM
KCl	4.7 mM
$MgSO_4 \times 7\ H_2O$	1.2 mM
KH_2PO_4	2.2 mM
$NaHCO_3$	24.9 mM
$CaCl_2$	1.3 mM
dextrose (added prior to use)	11.1 mM

   The buffer is aerated for 60 min with 95% O_2, 5% CO_2 on ice and pH is checked.
2. Levo-[Ring-2,5,6-^{3}H]-norepinephrine (specific activity 40–50 Ci/mmol) is obtained from New England Nuclear.
   The final desired concentration of ^{3}H-NE is 25 nM. 0.125 nmol is added to 5 ml KHBB.
3. Test compounds
   For most assays, a 1 mM stock solution of the test compound is made up in a suitable solvent and diluted such that the final concentration in the assay is 1 µM. Higher or lower concentrations may be used depending on the potency of the drug.

B Instrumentation

Neurotransmitter release apparatus consisting of:

- oscilloscope B8K, Precision Model 1420, dual-trace microscope (Dynascan Corp.)
- constant current unit, Grass model CCU1 (Grass Instr. Co.)
- stimulator, model S44, solid state square wave stimulator (Grass Instr. Co.)
- pump, Watson-Marlow, model 502 SHR, standard drive module; model 501 M multichannel pumphead (Bacon Technical Instr.)
- circulator, Haake D8 immersion circulator (Haake Buchler Instr. Inc.)
- fraction collector, Isco Retriever IV fraction collector (Isco Inc.)

C Tissue Preparation

Male Wistar rats (100–150 g) are decapitated, cortical tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue chopper. The slices are made individually and removed from the razor blade by twirling an artist's paint brush underneath the slice. Care should be taken not to compress the slice or impale it on the bristles. The slices are placed in cold, oxygenated buffer (10–20 ml) and incubated at 35°C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 5 ml of cold oxygenated buffer is added, and enough [^{3}H]NE to bring the final concentration to 25 nM. This is then incubated and shaken for 30 min at 35°C under oxygen. After this step, the buffer is decanted and the "loaded" slices are rapidly placed on the nylon mesh in the stimulation chambers using a cut-off pipetman tip.

D Assay

To establish a stable baseline, control buffer is pumped through the chamber for 1 h at a flow rate of 0.7 ml/min before the first stimulation. One hour is allowed to pass before the second stimulation. When drugs are used, each concentration is prepared in a separate flask in control buffer and allowed to equilibrate with the tissue slice 20 min before the second stimulation. The experiment is stopped 40 min after the second stimulation.

Stimulation parameters are set at 5 Hz (2 ms duration) for 60 s, with 1 ms delay and voltage setting of 440 SIU (250 Ω).

After the experiment is completed, the chambers are washed with distilled water for at least 20 min, then 200 ml of 20% methanol in distilled water, then distilled water again for at least 20 min.

EVALUATION

After conversion of dpm, percent fractional release is calculated for each fraction, using the spreadsheet program.

Percent fractional release is defined as the amount of radiolabeled compound released divided by the amount present in the tissue at that moment in time. "Spontaneous release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) are the summed differences between the percent fractional release during stimulation and the appropriate SP value.

The effects of drugs can be reported as S_2/S_1 ratios. To normalize the data, drug effects can be estimated by first calculating S_2/S_1 values for control and drug-treated slices and then expressing the S_2/S_1 value for the drug-treated slices as a percentage of the

S_2/S_1 value for the control slices for each experiment. Each condition should be tested in slices from each animal.

REFERENCES AND FURTHER READING

Langer SZ (1981) Presynaptic regulation of the release of catecholamines. Pharmacol Rev 32:337–362

Miller RJ (1998) Presynaptic receptors. Ann Rev Pharmacol Toxicol 38:201–227

Raiteri M, et al (1984) In: Handbook of Neurochemistry, Vol. 6, Plenum Publishing Corporation, New York, pp 431–462,

Starke K (1981) Presynaptic receptors. Ann. Rev. Pharmacol. Toxicol. 21:7–30

Zahniser NR, et al (1986) In: Chemical and Functional Assays of Receptor Binding, 1986, Short Course 1, Syllabus, published by Society for Neuroscience, Washington D.C., pp 73–81

A.1.1.4 Imidazoline Receptor Binding

PURPOSE AND RATIONALE

Imidazoline receptors constitute a family of nonadrenergic high-affinity binding sites for clonidine, idazoxan, and allied drugs. Drugs selectively binding to imidazoline receptors are expected to have less side effects than clonidine (Ernsberger et al. 1992, 1997; Molderings et al. 1992; Limon et al. 1992). One major subclass, the I_1 receptors, being mainly distributed in the brain and brain stem, partly mediates the central hypotensive action of clonidine-like drugs. The I_2 receptors, an other subclass, are mitochondrial, not G protein coupled, and have diversified functions. They may be involved in neuroprotection for cerebral ischemia. Two binding sites of [^{3}H]p-aminoclonidine, α_2-adrenoceptors and imidazoline binding sites, could be separated (Ernsberger et al. 1987; Bricca et al. 1988; Kamisaki et al. 1990). At least 3 subtypes of imidazoline/guanidinium-receptive sites have been found by photoaffinity labeling (Lanier et al. 1993).

Several endogenous ligands for imidazoline receptors, collectively termed clonidine displacing substances (CDSs), have been detected in tissues and serum (Reis et al. 1995; Chan et al. 1997).

An endogenous substance with clonidine-like properties originally isolated from brain which binds selectively to imidazoline receptors was described by Atlas and Burstein (1984), Ernsberger et al. (1988), Atlas (1991), Meeley et al. (1992), Dontenwill et al. (1992, Ragunathan and Reis 1996). The endogenous substance agmatine, a decarboxylated arginine, may be the physiological agonist at imidazoline receptors acting as neurotransmitter (Li et al. 1994; Gonzales et al. 1996; Head et al. 1997; Herman 1997; Reis and Regunathan 2000). Further candidates of endogenous ligands are discussed (Reis and Ragunathan 1998).

A critical review on imidazoline binding sites is given by Eglen et al. (1998).

PROCEDURE

Tissue Preparation

Whole bovine brains and adrenal glands are obtained from a local slaughterhouse. The lateral medulla oblongata is isolated by a sagittal section through the lateral margin of the pyramids and then bisected. The ventral half is defined as the ventrolateral medulla.

Fresh bovine adrenal glands are perfused retrogradely through the adrenal vein twice with 25 ml ice-cold Krebs-Henseleit bicarbonate buffer. The glands are perfused again with 25 ml ice-cold Krebs-Henseleit buffer containing 0.025% collagenase (type I, Sigma Chemical), incubated at room temperature for 1 h, then perfused with 25 ml fresh buffer containing collagenase and incubated for 30 min at 35°C. The digested glands are split, and the medulla is removed from the cortex. Adrenal medullae are minced and incubated while being stirred for 30 min at 37°C. The digest is filtered and centrifuged at 200 *g* for 30 min at 20°C. The cell pellet is resuspended in 30 ml Krebs' solution without collagenase, recentrifuged, flash-frozen, and stored at –70°C.

Membrane Preparation

Fresh bovine ventrolateral medulla and collagenase-digested rat renal medulla are homogenized with a Polytron (Tekmar Tissumizer; setting 80 for 15 s twice) in 20 vol of ice-chilled HEPES-buffered isotonic sucrose (pH 7.4) containing the protease inhibitors 1,10-phenanthroline (100 µM) and phenylmethylsulfonyl fluoride (50 µM). Bovine adrenomedullary chromaffin cells are homogenized in 15 ml HEPES-buffered isotonic sucrose by 10 strokes in a glass/glass handhold homogenizer. The homogenates are centrifuged at 1000 *g* for 5 min at 4°C to remove nuclei and debris. The pellets (P1) are resuspended in 20 ml of homogenization buffer and centrifuged again at 1000 *g* for 5 min. The supernatants are centrifuged at 48,000 *g* for 18 min at 4°C, and the resulting pellet (P2) is resuspended in 10–25 vol 50 mM Tris-HCl buffer (pH 7.7) containing 5 mM EDTA. After recentrifugation at 48,000 *g* for 18 min, the resulting membrane pellet is resuspended in Tris-HCl containing 25 mM NaCl, preincubated for 30 min at 25°C, chilled on ice, centrifuged again, resuspended a final time in Tris-HCl alone, centrifuged, flash-frozen, and stored at –70°C.

Binding Assays

For determination of specific binding to I_1-imidazoline sites and α_2-adrenergic receptors radioligand binding assays are performed with [^{3}H]clonidine, [^{3}H]*p*-iodoclonidine, or [^{3}H]moxonidine. Membranes are slowly thawed and resuspended in Tris-HCl or Tris-HEPES buffer (pH 7.7, 25°C). Assays are conducted in a total volume of 250 µl in polypropylene 96 well plates (Beckman Macrowell). Each well contains 125 µl membrane suspension, 25 µl radioligand, and 100 µl drug or vehicle. Incubations are initiated by the addition of membrane suspension and carried out for 40 min at 25°C. Nonspecific binding is defined in the presence of either piperoxan or phentolamine (0.1 mM), which are imidazoline-adrenergic agents. Specific α_2-adrenergic binding is defined by epinephrine (0.1 mM). In experiments with catecholamines, all samples contain ascorbic acid in a final concentration of 0.001%. Incubations are terminated by vacuum filtration over Reeves-Angel or Whatman GF/C fiberglass filters using a cell harvester (Brandel). The filters are washed four times with 5 ml ice-cold Tris-HCl, placed in scintillation vials, covered with 4 ml scintillation cocktail and counted at 50% efficiency. Protein is assayed by a modified Lowry et al. method (Peterson 1977) using a deoxycholate-trichloroacetic acid protein precipitation technique which provides a rapid quantitative recovery of soluble and membrane proteins from interfering substances even in very dilute solutions. Sodium dodecyl sulfate is added to alleviate possible nonionic and cationic detergent and lipid interferences, and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins.

EVALUATION

Data are obtained as disintegrations per min and transferred to the Equilibrium Binding Data Analysis program (McPherson 1985). Then, several experiments are analyzed simultaneously with the LIGAND program for non-linear curve fitting (Munson and Rodbard 1980). IC_{50} values are estimated from inhibition curves by non-linear curve fitting (Mutolsky and Ransnas 1987). Protein assay data are also analyzed by non-linear curve fitting (McPherson 1985).

MODIFICATIONS OF THE METHOD

Tesson et al. (1991) defined the subcellular localization of imidazoline-guanidinium-receptive sites by performing binding studies with the radioligand [^{3}H]idazoxan.

Lanier et al. (1993) visualized multiple imidazoline/guanidinium-receptive sites with the photoaffinity adduct 2-[3-azido-4-[^{125}I]iodo-phenoxy]methyl imidazoline.

Molderings et al. (1991) characterized imidazoline receptors involved in the modulation of noradrenaline release in the rabbit pulmonary artery pre-incubated with [^{3}H]noradrenaline.

Molderings and Göthert (1995) determined electrically or K^+-evoked tritium overflow from superfused rabbit aortic strips pre-incubated with [^{3}H]noradrenaline in order to characterize presynaptic imidazoline receptors which mediate noradrenaline release and compared them with I_1- and I_2-imidazoline radioligand binding sites.

Ernsberger et al. (1995) described optimization of radioligand binding assays for I_1 imidazoline sites.

Munk et al. (1996) reported the synthesis and pharmacological evaluation of a potent imidazoline-1 receptor specific agent.

Piletz et al. (1996) compared the affinities of several ligands for [^{125}I]p-iodoclonidine binding at human platelet I_1 imidazole binding sites.

Several selective ligands for imidazoline I_2 receptors have been identified, such as:

- LSL 60101 (Alemany et al. 1995; Menargues et al. 1995),
- RS-45041–190 (MacKinnon et al. 1995; Brown et al. 1995),
- RX801077 (= 2-BFI = 2-(2-benzofuranoyl)-2-imidazoline and analogues (Jordan et al. 1996; Lione et al. 1996; Alemany et al. 1997; Hosseini et al. 1997; Wiest and Steinberg 1997; Hudson et al. 1997).

REFERENCES AND FURTHER READING

Alemany R, Olmos G, Escriba PV, Menargues A, Obach R, Garcia-Sevilla JA (1995) LSL, 60101, a selective ligand for imidazoline I_2 receptors, on glial fibrillary acidic protein concentration. Eur J Pharmacol 280:205–210

Alemany R, Olmos G, Garcia-Sevilla JA (1997) Labeling of I2B-imidazoline receptors by [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) in rat brain and liver. Characterization, regulation and relation to monoamine oxidase enzymes. Naunyn-Schmiedeberg's Arch Pharmacol 356:39–47

Atlas D (1991) Clonidine-displacing substance (CDS) and its putative imidazoline receptor. New leads for further divergence of α_2-adrenergic receptor activity. Biochem Pharmacol 41:1541–1549

Atlas D, Burstein Y (1984) Isolation and partial purification of a clonidine-displacing endogenous brain substance. Eur J Biochem 144:287–293

Bricca G, Dontenwill M, Molines A, Feldman J, Belcourt A, Bousquet P (1989) The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. Eur J Pharmacol 162:1–9

Brown CM, MacKinnon AC, Redfern WS, William A, Linton C, Stewart M, Clague RU, Clark R, Spedding M (1995) RS-45041–190: A selective, high affinity ligand for I_2 imidazoline receptors. Br J Pharmacol 116:1737–1744

Bousquet P (1995) Imidazoline receptors: From basic concepts to recent developments. J Cardiovasc Pharmacol 26/Suppl 2:S1–S6

Bousquet P (1998) Imidazoline receptors: how many, where and why? Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1, R 195

Chan SLF, Atlas D, James RFL, Morgan NG (1997) The effect of the putative endogenous imidazoline receptor ligand, clonidine-displacing substance, on insulin secretion from rat and human islets of Langerhans. Br J Pharmacol 120:926–932

Dontenwill M, Molines A, Verdun A, Bricca G, Belcourt A, Bousquet P (1992) A circulating imidazoline-like substance cross-reacts with anti-clonidine antibodies: high levels in hypertensive patients. Fundam Clin Pharmacol 6, Suppl 6:49s

Eglen RM, Hudson AL, Kendall DA, Nutt DJ, Morgan NG, Wilson VC, Dillon MP (1998) 'Seeing through a glass darkly': casting light on imidazoline 'I' sites. Trends Pharmacol Sci 19:381–390

Ernsberger P, Meeley MP, Mann JJ, Reis DJ (1987) Clonidine binds to imidazoline binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. Eur J Pharmacol 134:1–13

Ernsberger P, Meeley MP, Reis DJ (1988) An endogenous substance with clonidine-like properties: selective binding to imidazole sites in the ventrolateral medulla. Brain Res 441:309–318

Ernsberger P, Giuliano R, Willette RN, Reis DJ (1990) Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. J Pharm Exp Ther 253:408–418

Ernsberger PR, Westbrooks KL, Christen MO, Schäfer SG (1992) A second generation of centrally acting antihypertensive agents act on putative I_1-imidazoline receptors. J Cardiovasc Pharmacol 20, Suppl 4:S1–S10

Ernsberger P, Piletz JE, Graff LM, Graves ME (1995) Optimization of radioligand binding assays for I_1 imidazoline sites. Ann New York Acad Sci 763:163–168

Ernsberger P, Friedman JE, Koletsky RJ (1997) The I_1-imidazoline receptor: From binding site to therapeutic target in cardiovascular disease. J Hypertens/Suppl 15:S9–S23

Gonzalez C, Regunathan S, Reis DJ, Estrada C (1996) Agmatine, an endogenous modulator of noradrenergic transmission in the rat tail artery. Br J Pharmacol 119:677–684

Hamilton CA (1995) Imidazoline receptors, subclassification, and drug-induced regulation. Ann New Acad Sci 763:57–65

Head GA, Chan CKS, Godwin SJ (1997) Central cardiovascular actions of agmatine, a putative clonidine-displacing substance, in conscious rabbits. Neurochem Int 30:37–45

Herman ZS (1997) Agmatine – a novel endogenous ligand of imidazoline receptors. Pol J Pharmacol 49:85–88

Hieble JP, Ruffolo RR (1992) Imidazoline receptors: historical perspective. Fundam Clin Pharmacol 6 (Suppl 1):7s–13s

Hieble JP, Ruffolo RR (1995) Possible structural and functional relationships between imidazoline receptors and α_2-adrenoceptors. Ann New Acad Sci 763:8–21

Hosseini AR, King PR, Louis WJ, Gundlach AL (1997) [^{3}H]2-(2-Benzofuranyl)-2-imidazoline, a highly selective radioligand for imidazoline I_2 receptor binding sites. Naunyn Schmiedeberg's Arch Pharmacol 355:131–138

Hudson AL, Chapleo CB, Lewis JW, Husbands S, Grivas K, Mallard NJ, Nutt DJ (1997) Identification of ligands selective for central I_2 imidazoline binding sites. Neurochem Int 30:47–53

Jordan S, Jackson HC, Nutt DJ, Handley SL (1996) Discrimination stimulus produced by the imidazoline I_2 site ligand, 2-BFI. J Psychopharmacol 10:273–278

Kamisaki Y, Ishikawa T, Takao Y, Omodani H, Kuno N, Itoh T (1990) Binding of [^{3}H]*p*-aminoclonidine to two sites, α_2-adrenoceptors and imidazoline binding sites: distribution of imidazoline binding sites in rat brain. Brain Res 514:15–21

Lanier SM, Ivkovic B, Singh I, Neumeyer JL, Bakthavachalam V (1993) Visualization of multiple imidazoline/guanidinium-receptive sites. J Biol Chem 268:16047–16051

Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ (1994) Agmatine: an endogenous clonidine-displacing substance in the brain. Science 263:966–969

Limon I, Coupry I, Tesson F, Lachaud-Pettiti V, Parini A (1992) Renal imidazoline-guanidinium receptive site: a potential target for antihypertensive drugs. J Cardiovasc Pharmacol 20, Suppl 4:S21–S23

Lione LA, Nutt DJ, Hudson AL (1996) [^{3}H]2-(2-benzofuranyl)-2-imidazoline: A new selective high affinity radioligand for the study of rabbit brain imidazoline I_2 receptors. Eur J Pharmacol 304:221–229

MacKinnon AC, Stewart M, Olverman HJ, Spedding M, Brown CM (1993) [^{3}H]p-aminoclonidine and [^{3}H]idazoxan label different populations of imidazoline sites on rat kidney. Eur J Pharmacol 232:79–87

MacKinnon AC, Redfern WS, Brown CM (1995) [^{3}H]-RS-45041–190: A selective high affinity ligand for I_2 imidazoline receptors. Br J Pharmacol 116:1729–1736

Mc Pherson GA (1985) Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. J Pharmacol Meth 14:213–218

Meeley MP, Hensley ML, Ernsberger P, Felsen D, Reis DJ (1992) Evidence for a bioactive clonidine-displacing substance in peripheral tissues and serum. Biochem Pharmacol 44:733–740

Menargues A, Cedo M, Artiga O, Obach R, Garcia-Sevilla JA (1995) Effects of the I_2 imidazoline receptor ligand LSL 60101 on various models of anorexia in rats. Ann New York Acad Sci 763:494–496

Molderings GJ, Göthert M (1995) Inhibitory presynaptic imidazoline receptors on sympathetic nerves in the rabbit aorta differ from I_1- and I_2-imidazoline binding sites. Naunyn-Schmiedeberg's Arch Pharmacol 351:507–516

Molderings GJ, Hentrich F, Göthert M (1991) Pharmacological characterization of the imidazoline receptor which mediates inhibition of noradrenaline release in the rabbit pulmonary artery. Naunyn-Schmiedeberg's Arch Pharmacol 344:630–638

Molderings GJ, Michel MC, Göthert M, Christen O, Schäfer SG (1992) Imidazolrezeptoren: Angriffsort einer neuen Generation von antihypertensiven Arzneimitteln. Dtsch Med Wschr 117:67–71

Munk SA, Lai RK, Burke JE, Arasasingham PN, Kharlamb AB, Manlapaz CA, Padillo EU, Wijono MK, Hasson DW, Wheeler LA, Garst ME (1996) Synthesis and pharmacological evaluation of 2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane: a potent imidazoline-1 receptor specific agent. J Med Chem 39:1193–1195

Munson PJ, Rodbard D (1980) LIGAND, a versatile computerized approach for characterization of ligand binding systems. Anal Biochem 107:220–239

Musgrave IF, Krautwurst D, Schulz G (1996) Imidazoline binding sites and signal transduction pathways. Clin Exp Pharmacol Physiol 23:990–994
Mutolsky HJ, Ransnas LA (1987) Fitting curves for data using non-linear regression: a practical and non mathematical review. FASEB J 1:365–374
Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
Piletz JE, Zhu H, Chikkala DN (1996) Comparison of ligand binding affinities at human I_1 imidazole binding sites and the high affinity state of α_2 adrenoceptor subtypes. J Pharmacol Exper Ther 279:694–702
Regunathan S, Reis DJ (1996) Imidazoline receptors and their endogenous ligands. Annu Rev Pharmacol Toxicol 36:511–544
Regunathan S, Evinger MJ, Meeley MP, Reis DJ (1991) Effects of clonidine and other imidazole-receptor binding agents on second messenger systems and calcium influx in bovine adrenal chromaffin cells. Biochem Pharmacol 42:2011–2018
Reis DJ, Ragunathan S (1998) Endogenous ligands of imidazoline receptors. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1, R 195
Reis DJ, Regunanthan S (2000) Is agmatine a novel neurotransmitter in brain? Trends Pharmacol Sci 21:187–193
Reis DJ, Li G, Regunathan S (1995) Endogenous ligands of imidazoline receptors: Classic and immunoreactive Clonidine-displacing substance and agmatine. Ann New York Acad Sci 763:295–313
Tesson F, Prip-Buus C, Lemoine A, Pegorier JP, Parini A (1991) Subcellular distribution of imidazoline-guanidinium-receptive sites in human and rabbit liver. J Biol Chem 266:155–160
Van Zwieten (1997) Central imidazoline (I_1) receptors as targets of centrally acting antihypertensives: Moxonidine and rilmenidine. J Hypertens 15:117–125
Wang H, Regunathan S, Ruggiero DA, Milner TA, Meeley MP, Reis DJ (1993) Biochemical and immunohistochemical characterization of imidazoline receptor protein. Am J Hypertens 6:77A
Wiest SA, Steinberg MI (1997) Binding of [^{3}H]2-(2-benzofuranyl)-2-imidazoline (BFI) to human brain: Potentiation by tranylcypromine. Life Sci 60:605–615
Yu A, Frishman WH (1996) Imidazoline receptor agonist drugs: A new approach to the treatment of systemic hypertension. J Clin Pharmacol 36:98–111

A.1.1.5 β-Adrenoreceptor Binding

PURPOSE AND RATIONALE

β-adrenoceptors are widely distributed, found at both central and peripheral sites, and are activated either via norepinephrine released from sympathetic nerve terminals or via epinephrine released from the adrenal medulla. Important physiological consequences of β-adrenoceptor activation include stimulation of cardiac rate and force, relaxation of vascular, urogenital and bronchial smooth muscle, stimulation of renin secretion from the juxtaglomerular apparatus, stimulation of insulin and glucagon secretion from the endocrine pancreas, stimulation of glycogenolysis in liver and skeletal muscle and stimulation of lipolysis in the adipocyte.

Three β-adrenoceptor proteins have been cloned, and the characteristics of these recombinant receptors correspond with those of the three well characterized β-adrenoceptors on native tissue, designated as β_1, β_2 and β_3. The possible roles of β_3-adrenoceptors in the cardiovascular system were discussed by Gauthier et al. (2000). An additional β-adrenoceptor modulatingcardiac contractility has been designated as the β_4-adrenoceptor (Kaumann et al. 1998).

While it was initially thought that cardiac stimulation involved primarily the β_1-adrenoceptor, it now appears that all of the receptor subtypes may be involved. Bronchodilation appears to be mediated by the β_2-adrenoceptor. The β_3-adrenoceptor is responsible for lipolysis in white adipose tissue and thermogenesis in the brown adipose tissue found in rodents. Renin release appears to be mediated by the β_1-adrenoceptor (Waitling 2006).

The β-adrenoceptor population of plasma membranes from bovine heart ventricles consists of 75–80% β_1- and 20–25% β_2-adrenoceptors. The use of this tissue allows a parallel investigation of the binding characteristics of drugs at both the β_1- and β_2-adrenoceptors. Both, the β_1- and β_2-adrenoceptors coexist in rat ventricular myocytes, but stimulation of these receptor subtypes elicits qualitatively different cell responses at the levels of ionic channels, the myofilaments, and sarcoplasmic reticulum (Xiao and Lakatta 1993).

A constant concentration of the radioligand ^{3}H-dihydroalprenolol (^{3}H-DHA) (4–6 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine heart ventricles. If the test drug exhibits any affinity to β-adrenoceptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more effective is the test drug.

PROCEDURE

Materials and Solutions

preparation buffer:

Tris-HCl	5 mM
$MgCl_2 \times 6\,H_2O$	1 mM
D(+)-sucrose	250 mM
pH 7.4	

310 mOsm sodium phosphate buffer:

pH 7.4	rinse buffer:
Tris-HCl	50 mM
$MgCl_2 \times 6\,H_2O$	10 mM
pH 7.4	

incubation buffer:

Tris-HCl	50 mM
$MgCl_2 \times 6\,H_2O$	10 mM
ascorbic acid	1.6 mM
catechol	0.3 mM
pH 7.4	

radioligand:

(–)^{3}H-dihydroalprenolol × HCl

(^{3}H-DHA) specific activity 1.48–2.59 TBq/mmol (40–70 Ci/mmol) (NEN)

for inhibition of ^{3}H-dihydroalprenolol binding in non-specific binding experiments:

(–)isoprenaline(+)bitartrate salt (Sigma)

Bovine hearts are obtained freshly from the local slaughter house. The lower part of the left ventricle from 5 hearts is separated and kept in ice-cold preparation buffer. In the laboratory, approx. 60 g wet weight from the five ventricle pieces are minced with a scalpel into 2–3 mm pieces.

Membrane Preparation

Ventricles are homogenized by Ultra-Turrax (1 g tissue/10 ml buffer), the homogenate is filtered through gauze and centrifuged at 500 *g* (4°C) for 10 min. The pellets are discarded, the supernatant is collected, and centrifuged at 40,000 *g* for 20 min. The resulting pellets are resuspended in approx. 300 ml 310 mOsm sodium phosphate buffer, homogenized by Ultra-Turrax, and centrifuged as before. The final pellets are dissolved (by Ultra-Turrax) in sodium phosphate buffer corresponding to 1 g ventricle wet weight/2 ml buffer. The membrane suspension is immediately stored in aliquots of 5–20 ml at –77°C. Protein concentration of the membrane suspension is determined according to the method of Lowry et al. with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 40,000 *g* (4°C) for 20 min. The pellets are resuspended in a volume of ice-cold rinse buffer, yielding a membrane suspension with a protein content of approx. 2.0 mg/ml. After homogenizing by Ultra-Turrax, the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

All incubation samples are performed in triplicate.

The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

total binding:

- 50 µl ^{3}H-DHA
  (12 concentrations, 3×10^{-10}–4×10^{-8} M)
- 50 µl incubation buffer

non-specific binding:

- 50 µl ^{3}H-DHA
  (4 concentrations, 3×10^{-10}–4×10^{-8} M)
- 50 µl (–)isoprenaline (10^{-5} M)

Competition Experiments

- 50 µl ^{3}H-DHA
  (1 constant concentration, 4–6 × 10^{-9} M)
- 50 µl incubation buffer without or with non-labeled test drug
  (15 concentrations 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (approx. 2 mg protein/ml). The samples are incubated for 60 min in a shaking water bath at 25°C. The reaction is stopped by rapid vacuum filtration of the total incubation volume over glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free activity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding–non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-DHA versus non-labeled drug by a computer-sup-

ported analysis of the binding data.

$$K_i = \frac{K_D\ ^3H \times IC_{50}}{K_D\ ^3H + [^3H]}$$

IC_{50} = concentration of the test drug, which competes with 50% of specifically bound ^{3}H-DHA in the competition experiment

$[^3H]$ = concentration of ^{3}H-DHA in the competition experiment.

$K_D\ ^3H$ = dissociation constant of ^{3}H-DHA, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data

propranolol hydrochloride $K_i = 6–8 \times 10^{-9}$ mol/l

MODIFICATIONS OF THE METHOD

Abrahamsson et al. (1988) performed a receptor binding study on the β_1- and β_2-adrenoceptor affinity of atenolol and metoprolol in tissues from the rat, the guinea pig and man with various radioligands, such as [^{125}I](±)hydroxybenzylpindolol, [^{125}I](–)pindolol, [^{3}H](–)dihydroalprenolol, and [^{3}H](–)CGP 12177.

Fleisher and Pinnas (1985) used specific binding of (–)[^{3}H]dihydroalprenolol to rat lung membranes for *in vitro* studies on the relative potency of bronchodilator agents.

REFERENCES AND FURTHER READING

Abrahamsson T, Ek B, Nerme V (1988) The β_1- and β_2-adrenoceptor affinity of atenolol and metoprolol: a receptor-binding study performed with different ligands in tissues from the rat, the guinea pig and man

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Fleisher JH, Pinnas JL (1985) *In vitro* studies on the relative potency of bronchodilator agents. Lung 163:161–171

Gauthier C, Langin D, Balligand JL (2000) β_3-adrenoceptors in the cardiovascular system. Trends Pharmacol Sci 21:426–431

Hedberg A, Minneman KP, Molinoff PB (1980) Differential distribution of beta-1 and beta-2 adrenergic receptors in cat and guinea-pig heart. J Pharmacol Exp Ther 212:503–508

Kaumann AJ, Preitner F, Sarsero D (1998), Molenaar P, Revelli JP, Giacobino JP (–)CGP 12177 causes cardiostimulation and binds to cardiac putative β_4-adrenoceptors in both wild-type and β_3-adrenoceptor knockout mice. Mol Pharmacol 53:670–675

Minneman KP, Hegstrand LR, Molinoff PB (1979) The pharmacological specificity of beta-1 and beta-2 adrenergic receptors in rat heart and lung *in vitro*. Mol Pharmacol 16:21–33

Waitling KJ (2006) The Sigma RBI handbook of receptor classification and signal transduction, 5th edn. Sigma-Aldrich, St Louis, Mo., pp 92–93

Wiemer G, Wellstein A, Palm D, Hattingberg HM v, Brockmeier D (1982) Properties of agonist binding at the β-adrenoceptor of the rat reticulocyte. Naunyn-Schmiedeberg's Arch Pharmacol. 321:11–19

Xiao RP, Lakatta EG (1993) β_1-adrenoceptor stimulation and β_2-adrenoceptor stimulation differ in their effect on contraction, cytosolic Ca^{2+}, and Ca^{2+} current in single rat ventricular cells. Circ Res 73:286–300

A.1.1.6 β_1-Adrenoreceptor Binding

PURPOSE AND RATIONALE

β-adrenergic receptors were differentiated from α-receptors (Ahlquist 1948) and subsequently divided into 2 distinct subtypes, β_1 and β_2 (Lands et al. 1967) based on differing pharmacology in different tissues. β-receptors have been labelled in a number of tissues including heart, lung, erythrocytes and brain using the β-agonists [^{3}H]-epinephrine (U'Prichard et al. 1978), or [^{3}H]-hydroxybenzylisoproterenol (Lefkowitz and Williams 1977) or the β-receptor antagonists [^{3}H]-alprenolol (Mukherjee et al. 1975), [^{3}H]-dihydroalprenolol (DHA) (U'Prichard et al. 1978; Bylund and Snyder 1976) and (^{125}I)-iodohydroxypindolol (Weiland et al. 1980). DHA is a potent β-antagonist (Mukherjee et al. 1975), which labels both β_1 and β_2 adrenergic receptors. The binding characteristics of this ligand in brain were described by Bylund and Snyder (1976), who showed that antagonists competed potently and agonists less potently although stereospecificity was maintained. The pharmacology of binding was consistent with β_1-receptor occupancy. Lesioning studies (Wolfe et al. 1982), combined with non-linear regression analysis of data have shown that while β-receptors in rat cerebellum are primarily of the β_2 subtype, the β_1 occurring in rat cerebral cortex are physiologically more significant. The assay can be used to evaluate the direct interaction of drugs with β-receptors labelled by [^{3}H]-dihydroalprenolol.

PROCEDURE

Reagents

Tris buffer, pH 8.0

1. a) 44.4 g Tris HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base
   b) Dilute 1:10 in distilled water. (0.05 M Tris, pH 8.0)

2. (–)-[propyl-1,2,3-^{3}H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled H_2O and 50 µl is added to each tube (this yields a final concentration of 1 nM in the 1 ml assay).
3. (±)-propranolol HCl is obtained from Ayerst.
   A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution is added to 3 tubes to determine nonspecific binding (yields a final concentration of 1 µM in a 1 ml assay).
4. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound.

Tissue Preparation

Male rats are decapitated and the brains rapidly removed. The cerebral cortices are dissected free, weighed and homogenized in 50 ml of ice-cold 0.05 Tris buffer, pH 8.0. This homogenate is centrifuged at 40,000 *g*, the supernatant decanted and the pellet resuspended and recentrifuged at 40,000 *g*. The final pellet is resuspended in the initial volume of fresh 0.05 Tris buffer, pH 8.0. This tissue suspension is then stored on ice. The final tissue concentration in the assay is 10 mg/ml. Specific binding is about 3% of the total added ligand and 80% of the total bound ligand.

Assay

380 µl	H_2O
50 µl	0.5 Tris buffer, pH 8.0
20 µl	Vehicle (for total binding) or 50 µM (±) propranolol (for nonspecific binding) or appropriate drug concentration
50 µl	^{3}H-DHA stock solution
500 µl	tissue suspension.

The tissue homogenates are incubated for 15 min at 25°C with 1 nM ^{3}H-DHA and varying drug concentrations. With each binding assay, triplicate samples are incubated with 1 µM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 A as a useful tool for quantitating β_1 and β_2 adrenoceptors.

REFERENCES AND FURTHER READING

Ahlquist RP (1948) Study of adrenotropic receptors. Am J Physiol 153:586–600

Bylund DB, Snyder SH (1976) Beta adrenergic receptor binding in membrane preparations from mammalian brain. Mol Pharmacol 12:568–580

Dooley DJ, Bittiger H, Reymann NC (1986) CGP 20712 A: A useful tool for quantitating β_1 and β_2 adrenoceptors. Eur J Pharmacol 130:137–139

Haeusler G (1990) Pharmacology of β-blockers: classical aspects and recent developments. J Cardiovasc Pharmacol 16 (Suppl 5):S1–S9

Lands AM, Arnold A, McAuliff JP, Luduena FP, Brown TG (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature 214:597–598

Lefkowitz RJ, Williams LT (1977) Catecholamine binding to the β-adrenergic receptor. Proc Nat Acad Sci 74:515–519

Mukherjee C, Caron MG, Coverstone M, Lefkowitz RJ (1975) Identification of adenylate cyclase-coupled β-adrenergic receptors in frog erythrocytes with (–)-[^{3}H]-alprenolol. J Biol Chem 250:4869–4876

U'Prichard DC, Bylund DB, Snyder SH (1978) (±)-[^{3}H]Epinephrine and (–)-[^{3}H] dihydroalprenolol binding to β_1- and β_2-noradrenergic receptors in brain, heart and lung membranes. J Biol Chem 253:5090–5102

Weiland GA, Minneman KD, Molinoff PB (1980) Thermodynamics of agonist and antagonist interaction with mammalian β-adrenergic receptors. Mol Pharmacol 18:341–347

Wolfe BB, Minneman KP, Molinoff PB (1982) Selective increases in the density of cerebellar β_1-adrenergic receptors. Brain Res 234:474–479

A.1.1.7 β_2-Adrenoreceptor Binding

PURPOSE AND RATIONALE

Lands et al. (1967) classified β-receptors into β_1 and β_2 subtypes according to differences in the action of various catecholamines. Synthesis of more selective β-antagonists has helped to confirm the existence of receptor subtypes. Based on catecholamine pharmacology and differences in the tissue distribution, it has been suggested that the β_1-receptor serves as the receptor for norepinephrine acting as a neurotransmitter and the β_2-receptor serves as a receptor for epinephrine acting as a hormone. (Nahorski 1981;

Ariens and Simonis 1983; Lefkowitz et al. 1983; Minneman 1983). Since [^{3}H]-dihydroalprenolol is a nonspecific ligand, it is necessary to select a tissue which is enriched in β_2-receptors in order to convey specificity to this assay. Tissues with predominantly β_2-receptors include lung (U'Prichard et al. 1978; Ariens and Simonis 1983; Lefkowitz et al. 1983), cerebellum (Lefkowitz et al. 1983; Minneman et al. 1983), rat and frog erythrocytes (Mukherjee et al. 1975; Lefkowitz et al. 1983) and ciliary process (Nathanson 1985) whereas, forebrain, heart and avian erythrocytes are relatively enriched in the β_1-subtype (Lefkowitz et al. 1983). Due to poor binding characteristics in cerebellum, rat lung is chosen as the tissue for β_2-adrenergic receptors.

A compound with β_2-selectivity would be less likely to produce cardiac effects but more likely to produce bronchiolar constriction. The test is used to determine the affinity of compounds for the β_2-adrenergic receptor subtype. A measure of receptor subtype selectivity can be determined when data are compared with those obtained in the β_1-adrenergic assay in rat cerebral cortex.

The present nomenclature of β_1, β_2, and β_3 receptors was reviewed by Alexander et al. (2001).

PROCEDURE

Reagents

1. Tris buffers, pH 8.0
   a) 44.4 g Tris HCl q.s. to 1 liter (0.5 M Tris, pH 8.0) 26.5 g Tris base
   b) Dilute 1:10 in distilled water (0.05 M Tris, pH 8.0)
2. (–)-[propyl-1,2,3-^{3}H] Dihydroalprenolol hydrochloride (45–52 Ci/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: A stock solution of 20 nM ^{3}H-DHA is made up in distilled water and 50 µl is added to each tube (this yields a final concentration of 1 nM in the assay)
3. (±)-propranolol HCl is obtained from Ayerst.
   A 1 mM propranolol stock solution is made up in distilled water and further diluted 1:20 in distilled water to give 50 µM propranolol solution. Twenty µl of dilute stock solution are added to 3 tubes to determine nonspecific binding (yielding a final concentration of 1 µM in a 1 ml assay).
4. Test compounds:
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the compound to be tested.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation and the lungs removed, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 8.0 using a Tekmar homogenizer. The homogenate is passed through a cheese cloth and centrifuged at 40,000 *g* for 15 min. The final membrane pellet is resuspended in the original volume of Tris buffer, pH 8.0, and used in the assay.

Assay

380 µl	H_2O
50 µl	0.5 Tris buffer, pH 8.0
20 µl	Vehicle (for total binding) or 50 µM (±)-propranolol (for nonspecific binding) or appropriate drug concentration
50 µl	^{3}H-DHA stock solution
500 µl	tissue suspension.

The tissue homogenates are incubated for 15 min at 25°C with 1 nM ^{3}H-DHA and varying drug concentrations. In each binding assay, triplicate samples are incubated with 1 µM (±)-propranolol under identical conditions to determine nonspecific binding. The assay is stopped by vacuum filtration through Whatman GF/B filters which are washed 3 times with 5 ml of ice-cold 0.05 Tris buffer, pH 8.0. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

EVALUATION

The percent inhibition of each drug concentration is the mean of triplicate determinations. IC_{50} values are obtained by computer-derived log-probit analysis.

MODIFICATIONS OF THE METHOD

Dooley et al. (1986) recommended CGP 20712 as a useful tool for quantitating β_1- and β_2-adrenoceptors.

McCrea and Hill (1993) described salmeterol as a long-acting β-adrenoceptor agonist mediating cyclic AMP accumulation in the B50 neuroblastoma cell line.

Sarsero et al. (1998) recommended (–)[^{3}H]-CGP 12177A as radioligand for the putative β_4-adrenoceptor.

McConnell et al. (1991, 1992; Owicki and Parce 1992) used a special apparatus, the 'cytosensor microphysiometer' which measures the rate of proton excretion from cultured cells. Chinese hamster ovary cells

were transfected with human β_2-adrenergic receptors. The β_2-adrenergic receptor activates adenylate cyclase resulting in an increase in the cyclic AMP concentration within the cell which can be measured as acidification. Addition of 10 µM isoproterenol, 500 µM 8-bromo cyclic AMP, or 10 µg/ml forskolin induced a reversible acidification.

Hoffmann et al. (2004) compared human β-adrenergic receptor subtypes using characterization of stably transfected receptors in CHO cells.

PROCEDURE

cDNA of human β-adrenergic receptors cDNAs coding for human β-adrenergic receptors in pcDNA3 expression vectors were verified by sequencing and comparison with the respective GeneBank entries. The translated amino acid sequences corresponded to the published sequences for the β_1-adrenergic receptor (Frielle et al. 1987), β_2-adrenergic receptor (Schofield et al. 1987), and β_3-adrenergic receptor (Emorine et Y al. 1989). With respect to polymorphisms, the β-adrenergic receptors used in this study corresponded to the following variants: β_1-receptor 49-Ser, 389-Gly; β_2-receptor 16-Arg, 27-Gln, 164-Thr; β_3-receptor 64-Trp. All of the variants correspond to the sequences originally termed wild-type.

Stable Transfection of Cells

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, Md., USA) were transfected with plasmid DNA for stable expression using the calcium phosphate precipitation method (Chen and Okayama 1987) as described for the rat A_1 adenosine receptor (Freund et al. 1994). Positive clones were selected with 600 µg/ml of the neomycin analog G-418, and single clonal lines were isolated by limiting dilution. Expression of the receptor was verified by radioligand binding.

Cell culture and membrane preparation Chinese hamster ovary cells stably transfected with human β-adrenergic receptor subtypes were grown adherently and maintained in Dulbecco's Modified Eagle's Medium with nutrient mixture F12 (DMEM/F12), containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and geneticin (G-418, 0.2 mg/ml) at 37°C in 5% CO_2/95% air. Cells were split 2 or 3 times weekly at a ratio of between 1:5 and 1:15. In order to harvest cells the culture medium was removed, cells were washed twice with PBS and membranes were prepared or cells were frozen on the dishes for later preparation of membranes. Crude membrane fractions were prepared from fresh (measurement of adenylyl cyclase) or frozen cells (radioligand binding). The resulting membrane pellets were resuspended in 50 mM Tris/HCl buffer pH 7.4 to give a final protein concentration of 1–2 mg/ml.

Radioligand Binding Studies and Adenylyl Cyclase Activity

The radioligand binding experiments were performed with membranes prepared as described above. Assays were done in a volume of 200 µl in 50 mM Tris/HCl, pH 7.4 (assay buffer) in the presence of 100 µM GTP to ensure monophasic binding curves for agonists. For saturation binding experiments at human β_1- and β_2-receptors up to 400 pM ^{125}I-CYP and for β_3-receptors up to 1,500 pM ^{125}I-CYP were used. Non-specific binding was determined in the presence of 10 µM alprenolol. For competition binding, 50 pM ^{125}I-CYP in the case of β_1- and β_2-receptors, or 80 pM ^{125}I-CYP for β_3-receptors were used. For most of the competition binding experiments membranes with intermediate receptor expression (β_1: 367 ± 75 fmol/mg protein, β_2: 282 ± 19 fmol/mg protein, β_3: 377 ± 82 fmol/mg protein) were used. For selected compounds it was demonstrated that higher receptor expression did not affect K_i values (data not shown). Membranes were incubated for 90 min at 30°C, filtered through Whatman GF/C filters, and washed 3 times with ice-cold assay buffer. Samples were counted in a γ-counter (Wallac 1480 wizard 3). K_D-values for ^{125}I-CYP were calculated by non-linear curve fitting with the program SCTFIT. Ligand IC_{50} values were calculated using Origin 6.1 (OriginLab Corporation, Northampton, Mass., USA) and were transformed to K_i values according to Cheng and Prusoff (1973).

Adenylyl cyclase activity in cell membranes was determined according to Jakobs et al. (1976). Membrane protein (50 µg) was added to an incubation mixture with final concentrations of 50 mM Tris/HCl pH 7.4, 100 µM cAMP, 0.2% BSA, 10 µM GTP, 100 µM ATP, 1 mM $MgCl_2$, 100 µM IBMX, 15 mM phosphocreatine, and 300 U/ml of creatine kinase. Membranes were incubated with about 200,000 cpm of [α-^{32}P]-ATP for 20 min in the incubation mixture as described (Klotz et al. 1985). Accumulation of [α-^{32}P]-cAMP was linear over at least 20 min under all conditions. The reaction was stopped by addition of 400 µl of 125 mM ZnAc-solution and 500 µl of 144 mM Na_2CO_3. Samples were centrifuged for 5 min at 14,000 rpm in a laboratory microcentrifuge. Then, 800 µl of the resulting supernatant wase finally applied to alumina WN-6 (Sigma) columns that were eluted twice with 2 ml of 100 mM Tris/HCl pH 7.4.

The eluates were counted in a β-counter (Beckmann LS 1801).

Niclauss et al. (2006) compared the ability of three radioligands, [^{125}I]-cyanopindolol, [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol, to label the three human β-adrenoceptor subtypes. Saturation and competition binding experiments were performed using membrane preparations from Chinese hamster ovary cells stably transfected with the three subtypes. While [^{3}H]-CGP 12,177 had very similar affinity for β_1- and β_2-adrenoceptors (about 40 pM), [^{125}I]-cyanopindolol and [^{3}H]-dihydroalprenolol had four- to sixfold higher affinity for β_2- as compared to β_1-adrenoceptors (10 vs. 45 and 187 vs. 1,021 pM, respectively). The affinity of [^{125}I]-cyanopindolol at β_3-adrenoceptors was considerably lower (440 pM) than at the other two subtypes. The β_3-adrenoceptor affinity of [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol was so low that it could not be estimated within the tested range of radioligand concentrations (up to 4,000 pM and 30,000 pM for [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol, respectively). All three radioligands were ill-suited to labeling β_3-adrenoceptors, particularly in preparations co-expressing multiple subtypes. In the absence of alternatives, [^{125}I]-cyanopindolol appears the least unsuitable for labeling β_3-adrenoceptors. At present, there is still a need for high-affinity radioligands that are selective for β_3-adrenoceptors.

REFERENCES AND FURTHER READING

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Ariens EJ, Simonis AM (1983) Physiological and pharmacological aspects of adrenergic receptor classification. Biochem Pharmacol 32:1539–1545

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Collis MG (1983) Evidence for an A_1 adenosine receptor in the guinea pig atrium. Br J Pharmac78:207–212

Deighton NM, Motomura S, Bals S, Zerkowski HR, Brodde OE (1992) Characterization of the beta adrenoceptor subtype(s) mediating the positive inotropic effects of epinine, dopamine, dobutamine, denopamine and xamoterol in isolated human right atrium. J Pharm Exp Ther 262:532–538

Dooley DJ, Bittiger H, Reymann NC (1986) CGP 20712: A useful tool for quantitating β_1- and β_2-adrenoceptors. Eur J Pharmacol 130:137–139

Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD (1989) Molecular characterization of the human beta 3-adrenergic receptor. Science 245:1118–1121

Freund S, Ungerer M, Lohse MJ (1994) A_1 adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and phospholipase C. Naunyn-Schmiedebergs Arch Pharmacol 350:49–56

Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK (1987) Cloning of the cDNA for the human β_1-adrenergic receptor. Proc Natl Acad Sci USA 84:7290–7294

Hoffmann C, Leitz MR, Ober-dorf-Maass S, Lohse MJ, Klotz KN (2004) Comparative pharmacology of human β-adrenergic receptor subtypes – characterization of stably transfected receptors in CHO cells. Naunyn Schmiedbergs Arch Pharmacol 369:151–159

Jakobs KH, Saur W, Schultz G (1976) Reduction of adenylyl cyclase activity in lysates of human platelets by the alpha-adrenergic component of epinephrine. J Cyclic Nucleotide Res 2:381–392

Klotz K-N, Cristalli G, Grifantini M, Vittori S, Lohse MJ (1985) Photoaffinity labeling of A_1-adenosine receptors. J Biol Chem 260:14659–14664

Klotz K-N, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes – characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedebergs Arch Pharmacol 357:1–9

Lands AM, Arnold A, McAuliffe JP, Luduena FP, Brown TG (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature (London) 214:597–598

Lefkowitz RJ, Stadel JM, Caron MG (1983) Adenylate cyclase coupled beta-adrenergic receptors: Structure and mechanisms of activation and desensitization. Ann Rev Biochem 52:159–186

McConnell HM, Rice P, Wada GH, Owicki JC, Parce JW (1991) The microphysiometer biosensor. Curr Opin Struct Biol 1:647–652

McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG, Pitchford S (1992) The Cytosensor Microphysiometer: biological applications of silicon technology. Science 257:1906–1912

McCrea KE, Hill SJ (1993) Salmeterol, a long acting β-adrenoceptor agonist mediating cyclic AMP accumulation in a neuronal cell line. Br J Pharmacol 110:619–626

Minneman KP, Wolfe BB, Pittman RN, Molinoff PB (1983) β-adrenergic receptor subtypes in rat brain. In: Segawa T (ed) Molecular Pharmacology of Neurotransmitter Receptors. Raven Press, New York

Mukherjee C, Caron MG, Coverstone M, Lefkowitz RJ (1975) Identification of adenylate cyclase-coupled β-adrenergic receptors in frog erythrocytes with (–)-^{3}H-Alprenolol. J Biol Chem 250:4869–4876

Nahorski SR (1981) Identification and significance of beta-adrenoceptor subtypes. TIPS, April 1981:95–98

Nathanson JA (1985) Differential inhibition of beta adrenergic receptors in human and rat ciliary process and heart. J Pharmacol Exp Ther 232:119–126

Niclauss N, Michel-Reher MB, Alewijnse AE, Michel MC (2006) Comparison of three radioligands for the human b-adrenoceptor types. Naunyn-Schmiedebergs Arch Pharmacol 374:99–105

Owicki JC, Parce JW (1992) Biosensors based on the energy metabolism of living cells: The physical chemistry and cell biology of extracellular acidification. Biosensors Bioelectronics 7:255–272

Sarsero D, Molenaar P, Kaumann AJ (1998) Validity of (–)-[^{3}H]-CGP 12177A as radioligand for the putative β_4-adrenoceptor in rat striatum. Br J Pharmacol 123:371–380

Schofield PR, Rhee LM, Peralta EG (1987) Primary structure of the human beta-adrenergic receptor gene. Nucleic Acids Res 15:3636

U'Prichard DC, Bylund DB, Snyder SH (1978) (±)-^{3}H-Epinephrine and (–)-^{3}H-dihydroalprenolol binding to β_1 and β_2 noradrenergic receptors in brain, heart and lung membranes. J Biol Chem 253:5090–5102

A.1.1.8 Adenosine A_1 Receptor Binding

GENERAL CONSIDERATIONS

Adenosine receptors belong to the class of purinoceptors (Burnstock 1972, 1981; Olsson and Pearson 1990). Purinoceptors are divided into two general types on the basis of recognized natural ligands:

P_1 receptors recognize adenosine and AMP and P_2 receptors recognize ATP and AMP. Fredholm et al. (1994), Abbracchio and Burnstock (1994, Jacobson et al. 2000) proposed a nomenclature system which is now widely accepted: two families of P2 purinoceptors, P2X ionotropic ligand-gated ion channel receptors (North 2002) and P2Y metabotropic G-protein-coupled receptors (Costanzi et al. 2004). The nomenclature of seven subtypes of P2X receptors and six subtypes of P2Y receptors has been agreed by the NC-IUPHAR Subcommittee (Burnstock 2001; Alexander et al. 2001; Waitling 2006).

The effects of adenosine are mediated effects through cAMP (Sattin and Rall 1970; VanCalker et al. 1978). It was discovered that adenosine could either inhibit or stimulate the formation of cAMP. The discovery of dual effects on adenylate cyclase led to the proposal of two distinct adenosine receptors referred to as the A_1 and A_2 receptors. The A_1 subtype of adenosine receptor mediates the inhibition of adenylate cyclase; whereas, the A_2 subtype mediates stimulation of adenylate cyclase. The methylxanthines are relatively nonselective inhibitors of adenosine receptor subtypes and their pharmacological properties are thought to be mostly due to antagonism of these receptors.

Comparison of adenosine receptors with other G-protein linked receptors indicates that they comprise a family of G protein coupled receptors that can be grouped by subtypes or by species. Thus, in addition to A_1 and A_2, several authors described A_{1a}, A_{1b}, A_{2a}, A_{2b}, A_3, and A_4 receptors with species dependent differences (Jacobson et al. 1992 1996; Zhou et al. 1992; Linden et al. 1993; Salvatore et al. 1993; Linden et al. 1994; Fredholm et al. 1994; Alexander et al. 2001). The nomenclature and classification of adenosine receptors were published by Fredholm et al. (2001) and Waitling (2006). Klotz (2000) reviewed adenosine receptors and their ligands. The four subtypes of adenosine receptors referred to as A_1, A_{2A}, A_{2B}, and A_3 are members of the superfamily of G-protein-coupled receptors. The most recently discovered member of the adenosine receptor family, the A3 receptor, has a unique pharmacological profile (Salvatore et al. 1993; Avila et al. 2002).

PURPOSE AND RATIONALE

The purpose of this assay is to measure the affinity of test compounds for adenosine (A_1) receptors. Evidence for an A_1 adenosine receptor in the guinea pig atrium was given by Collis (1983). Adenosine plays a physiological role in many systems, including platelet aggregation, lipolysis, steroidogenesis and smooth muscle tone (Daly 1982).The vasodilatory and cardiac depressant effects of adenosine are well known. In addition to cardiovascular effects, adenosine has marked effects in the CNS including depression of electrophysiological activity (Siggins and Schubert 1981), anticonvulsant activity, analgesic properties (Ahlijanian and Takemori 1985) and inhibition of neurotransmitter release (Harms et al. 1979).

The agonist, [^{3}H]cyclohexyladenosine (CHA), has affinity for the A_1 receptor in the nanomolar concentration range and has proven to be a suitable ligand for A_1 receptor assays (Bruns et al. 1980; Bruns et al. 1986). Selective A_1 (Schingnitz et al. 1991) and A_2 antagonists (Shimada et al. 1992; Jacobson et al. 1993) have been described. Adenosine and its nucleotides have not only a cardiovascular but predominantly a cerebral activity (Phillis and Wu 1981; Daly 1982; Fredholm et al. 1982).

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
   b) 0.05 M Tris buffer, pH 7.7
2. Adenosine deaminase is obtained from Sigma Chemical Co.
   Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7 for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.
3. Cyclohexyladenosine, N^6-[Adenine-2,8-^{3}H] (specific activity 34 mCi/mmol) is obtained from New England Nuclear.
   For IC_{50} determinations: [^{3}H]CHA is made up to a concentration of 40 nM and 50 µl are added to each tube. This yields a final concentration of 1 nM in the assay.
4. Theophylline is obtained from Regis Chemical Co.
   A 100 mM stock solution is made up in deionized water. 20 µl are added to each of 3 tubes for the de-

termination of nonspecific binding, yielding a 1 mM final concentration in the assay.

5. Test compounds
   For most assays, a 1 mM stock solution is prepared in DMSO and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Whole brains minus cerebellum are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 0.05 M Tris buffer containing 0.1 U/ml of adenosine deaminase.

Assay

1000 µl	tissue suspension
930 µl	H_2O
20 µl	vehicle
	or theophylline
	or appropriate concentration of test compound
50 µl	^{3}H-CHA

The tubes are incubated for 2 hours at 25°C. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml liquiscintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 1 mM theophylline. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors (Bruns et al. 1986) precludes the simple calculation of K_i values by the Cheng–Prusoff equation.

MODIFICATIONS OF THE METHOD

Stiles et al. (1985) used ^{125}I-labeled N^6-2-(4-aminophenyl)ethyladenosine as a selective ligand to probe the structure of A_1 receptors.

Lohse et al. (1987) described 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as a high affinity antagonist radioligand for A_1 adenosine receptors.

Klotz et al. (1989) described 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) as a high affinity agonist radioligand for A_1 adenosine receptors.

Von Lubitz et al. (1995) studied the therapeutic implications of chronic NMDA receptor stimulation on adenosine A_1 receptors.

The partial agonism of theophylline-7-riboside on the adenosine A_1 receptor has been reported by Ijzerman et al. (1994).

Libert et al. (1992) reported the cloning and functional characterization of a human A_1 adenosine receptor.

REFERENCES AND FURTHER READING

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475

Ahlijanian MK, Takemori AE (1985) Effects of (–)-N^6-R-phenylisopropyladenosine (PIA) and caffeine on nociception and morphine-induced analgesia, tolerance and dependence in mice. Eur J Pharmacol 112:171–179

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Avila MY, Stone RA, Civan MM (2002) Knockout of A_3 adenosine receptors reduces mouse intraocular pressure. Invest Ophthalmol Vis Sci 43:3021–3026

Bruns RF, Daly JW, Snyder SH (1980) Adenosine receptors in brain membranes: Binding of N^6-cyclohexyl [^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]phenylxanthine. Proc Natl Acad Sci 77:5547–5551

Bruns RF, Lu GH, Pugsley TA (1986) Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol Pharmacol 29:331–346

Burnstock G (1972) Purinergic nerves. Pharmacol Rev 24:509–581

Burnstock G (ed) (1981) Purinergic receptors. Receptors and recognition. Chapman & Hall, London, Ser. B, Vol. 12

Burnstock G (2001) Purine-mediated signalling in pain and visceral perception. Trends Pharmacol Sci 22:182–188

Costanzi S, Mamedova L, Gao ZG, Jacobson KA (2004) Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis and homology modelling. J Med Chem 47:5393–5404

Daly JW (1982) Adenosine receptors: Targets for future drugs. J Med Chem 25:197–207

Fredholm BB, Jonzon B, Lindgren E, Lindström K (1982) Adenosine receptors mediating cyclic AMP production in the rat hippocampus. J Neurochem 39:165–175

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. Pharmacol Rev 46:143–156

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527–552

Hamilton HW, Taylor MD, Steffen RP, Haleen SJ, Bruns RF (1987) Correlation of adenosine receptor affinities and cardiovascular activity. Life Sci 41:2295–2302

Harms HH, Wardeh G, Mulder AH (1979) Effects of adenosine on depolarization-induced release of various radiolabelled

neurotransmitters from slices of rat corpus striatum. Neuropharmacol 18:577–580
Ijzerman AP, van der Wenden EM, von Frijtag Drabbe Künzel JK, Mathôt RAA, Danhof M, Borea PA, Varani K (1994) Partial agonism of theophylline-7-riboside on adenosine receptors. Naunyn Schmiedeberg's Arch Pharmacol 350:638–645
Jacobson KA (1996) Specific ligands for the adenosine receptor family. Neurotransmissions 12:1–6
Jacobson KA, van Galen PJ, Williams M (1992a) Adenosine receptors: Pharmacology, structure-activity relationships, and therapeutic potential. J Med Chem 35:407–422
Jacobson KA, van Galen PJM, Williams M (1992b) Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. J Med Chem 35:409–422
Jacobson KA, Gallo-Rodriguez C, Melman N, Fischer B, Maillard M, van Bergen A, van Galen P, Karton Y (1993) Structure-activity relationships of 8-styrylxanthines as A_2-selective antagonists. J Med Chem 36:1333–1342
Jacobson KA, King BF, Burnstock GF (2000) Pharmacological characterization of P2 (nucleotide) receptors. Celltransmissions 16:3–16
Klotz KN, Lohse MJ, Schwabe U, Cristalli G, Vittori S, Grifantini M (1989) 2-Chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) – a high affinity agonist radioligand for A_1 adenosine receptors. Naunyn-Schmiedeberg's Arch Pharmacol 340:679–683
Klotz KN (2000) Adenosine receptors and their ligands. Naunyn-Schmiedebergs Arch Pharmacol 362:382–391
Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, Vassart G, Ensinger JA, Mendia KD (1992) Cloning and functional characterization of a human A_1 adenosine receptor. Biochem Biophys Res Commun 187:919–926
Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA (1993) Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. Mol Pharmacol 44:524–532
Linden J, Jacobson ME, Hutchins C, Williams M (1994) Adenosine receptors. In: Peroutka SJ (ed) Handbook of Receptors and Channels. G Protein Coupled Receptors. CRC Press, Boca Raton, Vol 1, pp 29–44
Lohse MJ, Klotz KN, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA (1987) 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a high affinity antagonist radioligand for A_1 adenosine receptors. Naunyn-Schmiedeberg's Arch Pharmacol 336:204–210
Murphy KMM, Snyder SH (1982) Heterogeneity of adenosine A_1 receptor binding in brain tissue. Mol Pharmacol 22:250–257
North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067
Olsson RA, Pearson JC (1990) Cardiovascular purinoceptors. Pharmacol Rev 70:761–845
Phillis JW, Wu PH (1981) The role of adenosine and its nucleotides in central synaptic transmission. Prog Neurobiol 16:178–239
Salvatore CA, Jacobson ME, Taylor HE, Linden J (1993) Molecular cloning and characterization of the human A_3 adenosine receptor. Proc Natl Acad Sci 90:10365–10369
Sattin A, Rall TW (1970) The effect of adenosine and adenine nucleotides on the adenosine 3′, 5′-phosphate content of guinea pig cerebral cortical slices. Mol Pharmacol 6:13–17
Schingnitz G, Küfner-Mühl U, Ensinger H, Lehr E, Kühn FJ (1991) Selective A_1-antagonists for treatment of cognitive deficits. Nucleosides and Nucleotides 10:1067–1076
Schwabe U, Trost T (1980) Characterization of adenosine receptors in rat brain by (–)[^{3}H]N^6-phenylisopropyladenosine. Naunyn Schmiedeberg's Arch Pharmacol 313:179–187
Shimada J, Suzuki F, Nonaka H, Ishii A, Ichikawa S (1992) (*E*)-1,3-Dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthines: Potent and selective A_2 antagonists. J Med Chem 35:2342–2345
Siggins GR, Schubert P (1981) Adenosine depression of hippocampal neurons *in vitro*: An intracellular study of dose-dependent actions on synaptic and membrane potentials. Neurosci Letters 23:55–60
Stiles GL, Daly DT, Olsson RA (1985) The A_1 receptor. Identification of the binding subunit by photoaffinity crosslinking. J Biol Chem 260:10806–10811
VanCalker D, Müller M, Hamprecht B (1978) Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. Nature 276:839–841
Von Lubitz DKJE, Kim J, Beenhakker M, Carter MF, Lin RCS, Meshulam Y, Daly JW, Shi D, Zhou LM, Jacobson KA (1995) Chronic NMDA receptor stimulation: therapeutic implications of its effect on adenosine A_1 receptors. Eur J Pharmacol 283:185–192
Waitling KJ (2006) The Sigma RBI handbook of receptor classification and signal transduction, 5th edn. Sigma-Aldrich, St Louis, Mo., pp 86–87, 130–137
Zhou QY, Li C, Olah ME Johnson RA, Stiles GL (1992) Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. Proc Natl Acad Sci 89:7432–7436

A.1.1.9 Adenosine A_2 Receptor Binding

PURPOSE AND RATIONALE

The A_2 receptor is a low-affinity binding site for adenosine (Daly et al. 1981). Activation of the A_2 receptor subtype by agonists mediates an increase in adenylate cyclase activity, while the A_1 receptor has the opposite effect. Although many of the physiological effects of adenosine seem to correlate with activity at the A_1 receptor, the effect on coronary blood flow correlates with activation of A_2 receptors (Hamilton et al. 1987).

This assay uses ^{3}H-NECA (5′-N-ethylcarboxamido[8-^{3}H]adenosine) to label A_2 receptors in rat striatum by the method described by Bruns et al. (1986). Comparison of data from this assay and the A_1 receptor assay provides a measure of selectivity for these two receptors.

PROCEDURE

Reagents

1. a) 0.5 M Tris buffer, pH 7.7
   b) 0.05 M Tris buffer, pH 7.7
   c) 0.05 M Tris buffer, pH 7.7, containing 12 mM $CaCl_2$ (final assay concentration: 10 mM)
2. Adenosine deaminase is obtained from Sigma Chemical Co.

Adenosine deaminase is added to 0.05 M Tris-HCl buffer, pH 7.7, containing 12 mM $CaCl_2$ for final resuspension of the membrane pellet, such that the concentration in the assay is 0.1 U/ml of tissue.

3. 5′-N-Ethylcarboxamido[8-^{3}H]adenosine (specific activity 23–40 mCi/mmol) is obtained from Amersham.
   For IC_{50} determinations: ^{3}H-NECA is made up to a concentration of 80 nM and 50 µl is added to each tube. This yields a final concentration of 4 nM in the assay.
4. Cyclopentyladenosine (CPA) is obtained from Research Biochemicals Inc.
   A 5 mM stock solution is made up in DMSO. 20 µl are added to each of 3 tubes for the determination of nonspecific binding, yielding a 100 µM final concentration in the assay.
   Since [^{3}H]NECA is not a specific ligand for A_2 receptors, CPA is added to all other tubes to mask the A_1 receptors at a final concentration of 50 nM.
5. Test compounds
   For most assays, a 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 2×10^{-5} to 2×10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are sacrificed by decapitation. Striata are removed, weighed and homogenized in 10 volumes of ice-cold 0.05 M Tris buffer, pH 7.7. The homogenate is centrifuged at 48,000 *g* for 10 min, the supernatant decanted, the pellet resuspended in the same volume of buffer and centrifuged again as before. The final pellet is resuspended in 100 volumes of 0.05 M Tris buffer containing 10 mM $CaCl_2$ and 0.1 U/ml of adenosine deaminase.

Assay

830 µl	tissue suspension
100 µl	CPA
20 µl	vehicle or CPA or appropriate concentration of test compound
50 µl	^{3}H-NECA

The tubes are incubated at 25°C for 2 hours. The assay is stopped by vacuum filtration through Whatman GF/B filters which are then washed 3 times with 5 ml of 0.05 M Tris buffer. The filters are then placed into scintillation vials with 10 ml Liquiscint scintillation cocktail, left to soak overnight and counted.

EVALUATION

Specific binding is defined as the difference between total binding and binding in the presence of 100 µM CPA. IC_{50} values are calculated from the percent specific binding at each drug concentration.

The complexity of interaction of adenosine ligands with receptors precludes the simple calculation of K_i values by the Cheng–Prusoff equation.

MODIFICATIONS OF THE METHOD

Jarvis et al. (1989) reported on [^{3}H]CGS 21 680, a selective A_2 adenosine receptor agonist which directly labels A_2 receptors in rat brain. [^{3}H]CGS 21 680 binding was greatest in striatal membranes with negligible specific binding obtained in rat cortical membranes.

Gurden et al. (1993) described the functional characterization of three adenosine receptor types.

Hutchinson et al. (1990) described 2-(arylalkylamino)adenosin-5′-uronamides as a new class of highly selective adenosine A_2 receptor ligands.

A_{2A} Adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells have been characterized with radioligand binding and by activation of adenylate cyclase (Hide et al. 1992).

Nonaka et al. (1994) reported on KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxantine), a potent and selective adenosine A_2 receptor antagonist.

The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist has been reported by Poucher et al. (1955).

Monopoli et al. (1994) described the pharmacology of the selective $A_{2\alpha}$ adenosine receptor agonist 2-hexynyl-5′-N-ethylcarboxamidoadenosine.

Jacobson et al. (1993) described structure-activity relationships of 8-styrylxanthines as A_2-selective adenosine antagonists.

Varani et al. (1996) reported pharmacological and biochemical characterization of purified A_{2a} adenosine receptors in human platelet membranes by [^{3}H]-CGS 21680 binding.

Van der Ploeg et al. (1996) characterized adenosine A_2 receptors in human T-cell leukemia Jurkat cells and rat phaeochromocytoma PC12 cells using adenosine receptor agonists.

REFERENCES AND FURTHER READING

Bruns RF, Lu GH, Pugsley TA (1986) Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol Pharmacol 29:331–346

Daly JW, Bruns RF, Snyder SH (1981) Adenosine receptors in the central nervous system: relationship to the central actions of methylxanthines. Life Sci 28:2083–2097

Gurden MF, Coates J, Ellis F, Evans B, Foster M, Hornby E, Kennedy I, Martin DP, Strong P, Vardey CJ, Wheeldon A (1993) Functional characterization of three adenosine receptor types. Br J Pharmacol 109:693–698

Hamilton HW, Taylor MD, Steffen RP, Haleen SJ, Bruns RF (1987) Correlation of adenosine receptor affinities and cardiovascular activity. Life Sci 41:2295–2302

Hide I, Padgett WL, Jacobson KA, Daly JW (1992) A_{2A} Adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: Characterization with radioligand binding and by activation of adenylate cyclase. Mol Pharmacol 41:352–359

Hutchinson AJ, Williams M, de Jesus R, Yokoyama R, Oei HH, Ghai GR, Webb RL, Zoganas HC, Stone GA, Jarvis MF (1990) 2-(Arylalkylamino)adenosin-5′-uronamides: a new class of highly selective adenosine A_2 receptor ligands. J Med Chem 33:1919–1924

Jacobson KA, Gallo-Rodriguez C, Melman N, Fischer B, Maillard M, van Bergen A, van Galen PJM, Karton Y (1993) Structure-activity relationships of 8-styrylxanthines as A_2-selective adenosine antagonists. J Med Chem 36:1333–1342

Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA, Williams M (1989) [^{3}H]CGS 21 680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. J Pharm Exp Ther 251:888–893

Monopoli A, Conti A, Zocchi C, Casati C, Volpini R, Cristalli G, Ongini E (1994) Pharmacology of the new selective $A_{2\alpha}$ adenosine receptor agonist 2-hexynyl-5′-N-ethylcarboxamidoadenosine. Arzneim Forsch/Drug Res 44:1296–1304

Nonaka H, Ichimura M, Takeda M, Nonaka Y, Shimada J, Suzuki F, Yamaguchi K, Kase H (1994) KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxantine), a potent and selective adenosine A_2 receptor antagonist. Eur J Pharmacol Mol Pharmacol Sect 267:335–341

Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PWR, Jones G, Collis MG (1955) The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist. Br J Pharmacol 115:1096–1102

Parkinson FE, Fredholm BB (1991) Effects of propentofylline on adenosine A_1 and A_2 receptors and nitrobenzylthioinosine-sensitive nucleoside transporters: quantitative autoradiographic analysis. Eur J Pharmacol 202:361–366

Van der Ploeg I, Ahlberg, Parkinson FE, Olsson RA, Fredholm BB (1996) Functional characterization of adenosine A_2 receptors in Jurkat cells and PC12 cells using adenosine receptor agonists. Naunyn Schmiedebergs Arch Pharmacol 353:250–260

Varani K, Gessi S, Dalpiaz A, Borea PA (1996) Pharmacological and biochemical characterization of purified A_{2a} adenosine receptors in human platelet membranes by [^{3}H]-CGS 21680 binding. Br J Pharmacol 117:1693–1701

A.1.1.10 Adenosine A_3 Receptor Binding

PURPOSE AND RATIONALE

The A_3 adenosine receptor has been cloned and characterized by Zhou et al. (1992). A possible role in reproduction has been discussed. The role of central A_3 adenosine receptors may be the mediation of behavioral depressant effects (Jacobson et al. 1993). The design of selective ligands of A_3 adenosine receptors and the therapeutic concepts including effects on locomotor activity, cardiovascular effects, effects in cerebral ischemia (von Lubitz et al. 1994), in cardiac preconditioning and as antagonists in inflammation and asthma has been discussed by Jacobson et al. (1995). Von Lubitz et al. (1995) noticed some anticonvulsive activity of the adenosine A_3 receptor selective agonist IB-MECA (N^6-(3-iodobenzyl) adenosine-5′-N-methyl-carboxamide). Stimulation of the A_3 adenosine receptor facilitates release of allergic mediators in mast cells (Ramkumar et al. 1993) inducing hypotension in the rat (Hannon et al. 1995). A binding site model and structure-activity relationships for the rat A_3 adenosine receptor are described by van Galen et al. (1994).

PROCEDURE

Cell culture and membrane preparation

Chinese hamster ovary (CHO) cells stably expressing the rat A_3 adenosine receptor are grown in F-12 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively) at 37° in a 5% CO_2 atmosphere. When cells reach confluency, they are washed twice with 10 ml of ice-cold lysis buffer (10 mM EDTA, pH 7.4). After addition of 5 ml of lysis buffer, cells are mechanically scraped and homogenized in an ice-cold Dounce homogenizer. The suspension is centrifuged at 43,000 *g* for 10 min. The pellet is suspended in the minimum volume of ice-cold 50 mM Tris/10 mM $MgCl_2$/1 mM EDTA (pH 8.26 at 5°C) buffer required for the binding assay and homogenized in a Dounce homogenizer. Aminodeaminase (ADA, Boehringer Mannheim) is added to a final concentration of 3 units/ml and the suspension is incubated at 37°C for 15 min; the membrane suspension is subsequently kept on ice until use.

Radioligand binding assay

Binding of [^{125}I]APNEA (N^6-2-(4-aminophenyl)-ethyladenosine) to CHO cells stably transfected with the rat A_3 adenosine receptor clone is performed according to Stiles et al. (1985). Assays are performed in 50/10/1 buffer in glass tubes and contain 100 µl of the membrane suspension, 50 µl of inhibitor. Incubations are carried out in duplicate for 1 h at 37°C and are terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester. Tubes are washed three times with 3 ml of buffer. Radioactivity is determined in a Beckman γ -counter. Non-specific binding is determined in the presence of 40 µM R-PIA = N^6-[(R)]-1-methyl-2-phenylethyl]adenosine.

EVALUATION

K_i values are calculated according to Cheng and Prusoff (1973), assuming a K_d for [^{125}I]APNEA of 17 nM.

MODIFICATIONS OF THE METHOD

^{125}I-4-aminobenzyl-5′-N-methylcarboxamidoadenosine has been recommended as a high affinity radioligand for the rat A_3 adenosine receptor (Olah et al. 1994).

Molecular cloning and functional expression of a sheep A_3 adenosine receptor has been reported by Linden et al. (1993).

G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain was reported by Abbracchio et al. (1995).

Molecular cloning and characterization of the human A_3 adenosine receptor was reported by Salvatore et al. (1993).

The differential interaction of the rat A_3 adenosine receptor with multiple G-proteins has been described by Palmer et al. (1995).

Baraldi and Borea (2000) described new potent and selective human adenosine A_3 receptor antagonists using radioligand binding studies to the human A_3 receptor.

REFERENCES AND FURTHER READING

Abbracchio MP, Brambilla R, Ceruti S, Kim HO, von Lubitz DKJE, Jacobson KA, Cattabeni F (1995) G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain. Mol Pharmacol 48:1038–1045

Baraldi PG, Borea PA (2000) New potent and selective human adenosine A_3 receptor antagonists. Trends Pharmacol Sci 21:456–459

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of the inhibitor which causes 50% inhibition (IC_{50}) of an enzyme reaction. Biochem Pharmacol 22:3099–3108

Hannon JP, Pfannkuche HJ, Fozard JR (1995) A role for mast cells in adenosine A_3 receptor-mediated hypotension in the rat. Br J Pharmacol 115:945–952

Jacobson KA, Nikodijevic O, Shi D, Gallo-Rodriguez C, Olah ME, Stiles GR, Daly JW (1993) A role of central A_3 adenosine receptors. Mediation of behavioral depressant effects. FEBS Lett 336:57–60

Jacobson KA, Kim HO, Siddiqi SM, Olah ME, Stiles GL, von Lubitz DKJE (1995) A_3 adenosine receptors: design of selective ligands of and therapeutic concepts. Drugs Future 20:689–699

Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA (1993) Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. Mol Pharmacol 44:524–552

Olah ME, Gallo-Rodriguez C, Jacobson KA, Stiles GL (1994) ^{125}I-4-aminobenzyl-5′-N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. Mol Pharmacol 45:978–982

Palmer TM, Gettys TW, Stiles GL (1995) Differential interaction with and regulation of multiple G-proteins by the rat A_3 adenosine receptor. J Biol Chem 270:16895–16902

Ramkumar V, Stiles GL, Beaven M, Ali H (1993) The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J Biol Chem 268:16887–16890

Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG (1993) Molecular cloning and characterization of the human A_3 adenosine receptor. Proc Natl Acad Sci USA 90:10365–10369

Stiles GL, Daly DT, Olsson RA (1985) The adenosine A_1 receptor. Identification of the binding subunit by photoaffinity cross-linking. J Biol Chem 260:10806–10811

van Galen PJM, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, Ijzerman AP, Stiles GL, Jacobson KA (1994) A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. Mol Pharmacol 45:1101–1111

Von Lubitz DKJE, Lin RCS, Popik P, Carter MF, Jacobson KA (1994) Adenosine A_3 receptor stimulation and cerebral ischemia. Eur J Pharmacol 263:59–67

Von Lubitz DKJE, Carter MF, Deutsch SI, Lin RCS, Mastropaolo J, Meshulam Y, Jacobson KA (1995) The effects of adenosine A_3 receptor stimulation on seizures in mice. Eur J Pharmacol 275:23–29

Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL (1992) Molecular cloning and characterization of the adenosine receptor: The A_3 adenosine receptor. Proc Natl Acad Sci USA 89:7432–7336

A.1.1.11 Inhibition of Adenosine Uptake in Human Erythrocytes

PURPOSE AND RATIONALE

Adenosine regulates multiple physiological functions in animals and humans. It plays a potent neuromodulatory role mainly by inhibiting the presynaptic transmitter release, e. g. of glutamate and aspartate. It is released by synaptic stimulation and during hypoxia in the central and peripheral nervous system. Adenosine plays a neuroprotective role in hypoxia and ischemia since it reduces the excessive stimulation of the NMDA receptors. The use of adenosine uptake inhibitors has been proposed as a new therapeutic strategy for hypoxic/ischemic disease. Due to its vasodilatory action adenosine plays a key role in the regulation of coronary and cerebral blood flow. The rapid cellular uptake of adenosine by erythrocytes is a reason for the short duration of action of adenosine.

Human erythrocytes are used as a cellular model to detect adenosine uptake inhibitors. Erythrocytes are treated with test compound and thereafter incubated with ^{3}H-adenosine. The uptake of ^{3}H-adenosine is evaluated in relation to the untreated control group.

Dipyridamole is a potent inhibitor of adenosine uptake (IC_{50} of 3×10^{-7} M).

Standard compounds:

- theophylline
- dipyridamole (Persantin)
- propentofylline (HWA 285)

PROCEDURE

Materials and solutions

isotonic glycyl-glycine buffer, pH 7.4

KCl	5.0 mM
NaCl	119.5 mM
$MgCl_2$	2.0 mM
glycyl-glycine	50 mM
Na_2HPO_4	2.0 mM
2-[^{3}H]-adenosine (specific activity 0.2 μCi/μmol)	5 μM

Buffer-washed fresh human erythrocytes are depleted of ATP by incubation in an isotonic glycyl-glycine buffer at 37°C. Aliquots of the erythrocyte suspensions are incubated for 2 min in fresh glycyl-glycine buffer solution containing additional 10 mM glucose and test- or standard compound. In screening assays, test compounds are added at a concentration of 5×10^{-4} M. Drugs showing an effect in this assays, are further tested at a concentration range of 10^{-5}–5×10^{-4} M to determine IC_{50} values (triplicate samples for each concentration).

The suspension is then incubated with 5 μM radioactively labelled 2-[^{3}H]-adenosine for 30 s. The adenosine uptake is stopped by adding cold buffer (4°C) containing 5 μM adenosine, 10 μM glucose and 7.4 μM dipyridamole. After centrifugation, the tritium radioactivity is determined in the supernatant.

EVALUATION

The percent change of ^{3}H-adenosine uptake relative to the vehicle control group is determined. The ^{3}H-adenosine uptake of the control group is taken as 100%; subsequent results are expressed as percentages of this.

IC_{50} values are determined by plotting the percent inhibition against test compound concentration; IC_{50} is defined as the dose of drug leading to a 50% inhibition of adenosine uptake.

Statistical evaluation is performed by means of the Student's *t*-test.

Standard data:

- IC_{50} of dipyridamole 3×10^{-7} M

MODIFICATIONS OF THE METHOD

Marangos et al. (1982), Verma and Marangos (1985) recommended [^{3}H]nitrobenzylthioinosine binding as a probe for the study of adenosine uptake sites in brain of various species. The highest density of binding sites were found in the caudate and hypothalamus of human and rat brain.

REFERENCES AND FURTHER READING

Bowmer CJ, Yates MS (1989) Therapeutic potential for new selective adenosine receptor ligands and metabolism inhibitors. Trends Pharmacol Sci 10:339–341

Geiger JD, Fyda DM (1991) Adenosine transport in nervous system tissues. In: Stone TW (ed) Adenosine in the Nervous System. Academic Press, London, San Diego, New York, pp 1–23

Marangos PJ, Patel J, Clark-Rosenberg R, Martino AM (1982) [^{3}H]Nitrobenzylthioinosine binding as a probe for the study of adenosine uptake sites in brain. J Neurochem 39:184–191

Porsche E (1982) Effects of methylxanthine derivates on the adenosine uptake in human erythrocytes. IRCS Med Sci 10:389

Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB (1992) Adenosine and brain ischemia. Cerebrovasc Brain Metab Rev 4:346–369

Verma A, Marangos PJ (1985) Nitrobenzylthioinosine binding in brain: an interspecies study. Life Sci 36:283–290

Winn HR, Rubio GR, Berne RM (1981) The role of adenosine in the regulation of cerebral blood flow. J Cerebr Blood Flow Metab 1:239–244

A.1.1.12 Inhibition of Vasopeptidases

GENERAL CONSIDERATIONS

Vasopeptidase inhibitors (VPIs) inhibit both angiotensin converting enzyme (ACE) and neprilysin (NEP) and can thus reduce the activity of the renin-angiotensin system and potentiate the vasodilatory, natriuretic and antiproliferative effects of bradykinin and natriuretic peptides (Burnett 1999; Bralet and Schwartz 2001). Combined inhibition of neutral endopeptidase 24.11 (NEP) and ACE is a candidate therapy for hypertension and cardiac failure (Duncan et al. 1999). Heath et al. (1995) described the quantification of a dual ACE-I-converting enzyme-neutral endopeptidase inhibitor and the active thiol metabolite in dog plasma by high-performance liquid chromatography with ultraviolet absorption detection. Dumoulin et al. (1995) studied the metabolism of bradykinin by the rat coronary vascular bed and found that combined treatment with the ACE inhibitor enalaprilate and the NEP inhibitor retrothiorphan reduced bradykinin degradation to lower values than enalaprilate alone.

Hubner et al. (2001) reported *in-vitro* and *in-vivo* inhibition of rat neutral endopeptidase and ACE with the vasopeptidase inhibitor gemopatrilat. Dumoulin et

al. (2001) compared the effects of a vasopeptidase inhibitor with those of neutral endopeptidase and ACE inhibitors on bradykinin metabolism in the rat coronary bed.

Crackower et al. (2002) described type-2 ACE (ACE2) as an essential regulator of heart function. In three different rat models of hypertension, ACE2 messenger RNA and protein expression were markedly reduced. Targeted disruption of ACE2 in mice resulted in a severe cardiac contractility defect.

REFERENCES AND FURTHER READING

Bralet J, Schwartz J-C (2001) Vasopeptidase inhibitors: an emerging class of cardiovascular drugs. Trends Pharmacol Sci 22:106–109

Burnett JC (1999) Vasopeptidase inhibition: a new concept in blood pressure management. J Hypertens 17 [Suppl 1]:S37–S43

Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, Penninger JM (2002) Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature 417:822–828

Dumoulin MJ, Adam A, Blais C Jr, Lamontagne D (1998) Metabolism of bradykinin by the rat coronary vascular bed. Cardiovasc Res 38:229–236

Dumoulin MJ, Adam A, Rouleau JL, Lamontagne D (2001) Comparison of a vasopeptidase inhibitor with neutral endopeptidase and angiotensin-converting enzyme inhibitors on bradykinin metabolism in the rat coronary bed. J Cardiovasc Pharmacol 37:359–366

Duncan AM, James GM, Anastasopoulos F, Kladis A, Briscoe TA, Campbell DJ (1999) Interaction between neutral endopeptidase and angiotensin converting enzyme in rats with myocardial infarction: effects on cardiac hypertrophy and angiotensin and bradykinin peptide levels. J Pharmacol Exp Ther 289:295–303

Heath TG, Massad DD, Carroll JI, Mathews BS, Chang J, Scott DO, Kuo BS, Toren PC (1995) Quantification of a dual angiotensin I-converting enzyme-neutral endopeptidase inhibitor and the active thiol metabolite in dog plasma by high-performance liquid chromatography with ultraviolet absorption detection. J Chromatogr B670:91–101

Hubner RA, Kubota E, Casley DJ, Johnston CI, Burrell LM (2001) In-vitro and in vivo inhibition of rat neutral endopeptidase and angiotensin converting enzyme with the vasopeptidase inhibitor gemopatrilat. J Hypertens 19:941–946

A.1.1.12.1 Inhibition of the Angiotensin-Converting Enzyme in Vitro

PURPOSE AND RATIONALE

An *in vitro* system can be used to screen potential angiotensin-converting enzyme inhibitors. Fluorescence generated by an artificial substrate in presence or absence of the inhibitor is measured to detect inhibitory activity.

PROCEDURE

Reagents

1. 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
2. 10 mM potassium phosphate buffer, pH 8.3
3. Substrate: O-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (molecular weight 482) (Bachem Gentec. Inc., Torrance, California, USA)
   a) stock solution: 10 mg substrate in 10 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl
   b) working solution: 2 ml stock solution is added to 18 ml 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl; final concentration in the assay is 170.2 µM.
4. Test compounds
   Compounds are made up to a concentration of 1 mM in 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl or 10% methanol in Tris/NaCl if insoluble in aqueous buffer alone. This will give a final concentration in the assay of 0.1 mM. If inhibition is seen, further dilution in Tris/NaCl should be made.

Enzyme preparation

Lung tissue from 10 rats is diced and homogenized in a blender with 3 pulses of 15 s each. The homogenate is centrifuged at 5000 *g* for 10 min. The pellet is discarded, the supernatant is dialyzed against three 1 liter changes of 10 mM potassium phosphate buffer, pH 8.3 overnight in the cold and then centrifuged at 40,000 *g* for 20 min. The pellet is discarded, 390 mg $(NH_4)_2SO_4$ is added for each ml of supernatant. This will give 60% saturation. The solution is stirred on ice for 15 min. The pellet formed is dissolved in 15 ml potassium phosphate buffer, pH 8.3 and dialyzed against the same buffer overnight in the cold with three 1 liter changes. Some protein will precipitate during dialysis. The suspension is centrifuged at 40,000 *g* for 20 min and the supernatant is discarded. The final solubilized enzyme preparation can be aliquoted and stored at –20°C at least 6 months.

Enzyme inhibition studies

1. Enzyme activity is measured with a Perkin Elmer LS-5 Fluorescence Spectrophotometer or equivalent at an excitation wavelength of 357 nm and an emission wavelength of 424 nm.
2. Enzyme assay
   50 µl vehicle or inhibitor solution and 40 µl enzyme are preincubated for 5 min, then 410 µl substrate working solution is added.

Samples are mixed by drawing fluid back up into the pipette and by pipetting into the cuvette. For the initial

control run of the day, the auto zero is pushed immediately after placing the sample in the cuvette.

EVALUATION

The individual fluorescence slope is measured and % inhibition is calculated as follows:

$$\%\,\text{inhibition} = \%\,\text{inhibition} = \left(100 - \frac{\text{slope in presence of inhibitor}}{\text{control slope}}\right) \times 100$$

Inhibitor concentrations on either side of the IC_{50} should be tested to generate a dose-response curve. The IC_{50} is calculated using Litchfield-Wilcoxon log-probit analysis.

Standard data:

- IC_{50} values for inhibition of angiotensin I-converting enzyme
- Compound IC_{50} [M]
- Captopril 6.9×10^{-9}

MODIFICATIONS OF THE METHOD

Other assays use the cleavage of hippuric acid from tripeptides (Hip-Gly-Gly or Hip-His-Leu) whereby hippuric acid is either tritium labelled or determined spectrophotometrically (Cushman and Cheung 1969, 1971; Friedland and Silverstein 1976; Santos et al. 1985; Hecker et al. 1994).

Bünning (1984) studied the binding and inhibition kinetics of ramipril and ramiprilate (Hoe 498 diacid) with highly purified angiotensin converting enzyme using furanacryloyl-Phe-Gly-Gly as substrate.

The importance of tissue converting enzyme inhibition in addition to inhibition in plasma has been verified in several studies (Unger et al. 1984, 1985; Linz and Schölkens 1987).

Eriksson et al. (2002), Oudit et al. (2003), and Danilczyk et al. (2003, 2004) discussed the role of the homolog of angiotensin-converting enzyme ACE2 in cardiovascular physiology. ACE2 appears to negatively regulate the renin-angiotensin system and cleaves Ang I and Ang II into the inactive Ang 1–9 and Ang 1–7. ACE2 differs in its specificity and physiological role from ACE.

REFERENCES AND FURTHER READING

Bünning P (1984) Inhibition of angiotensin converting enzyme by 2-[N-[(S)-1-carboxy-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (Hoe 489 diacid). Arzneim Forsch Drug Res 34:1406–1410

Cushman DW, Cheung HS (1969) A simple substrate for assay of dog lung angiotensin converting enzyme. Fed Proc 28:799

Cushman DW, Cheung HS (1971) Spectrophotometric assay and properties of the angiotensin converting enzyme of rabbit lung. Biochem Pharmacol 20:1637–1648

Danilczyk U, Eriksson U, Crackower MA, Penninger JM (2003) A story of two ACEs. J Mol Med 61:227–234

Danilczyk U, Eriksson U, Oudit GY, Penninger JM (2004) Physiological roles of the angiotensin-converting enzyme 2. Cell Mol Life Sci 61:2714–2719

Dzau VJ, Pratt RE (1986) Renin-angiotensin system: Biology, physiology, and pharmacology. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan MD (eds) The Heart and Cardiovascular System. Vol. 2, Chapter 69, pp 1631–1662. Raven Press New York

Eriksson U, Danilczyk U, Penninger JM (2002) Just the beginning: novel functions for angiotensin-converting enzymes. Curr Biol 12:R745–R7542

Friedland J, Silverstein E (1976) A sensitive fluorimetric assay for serum angiotensin converting enzyme. Am J Clin Path 66:416–424

Hayakari M, Kondo Y, Izumi H (1978) A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. Analyt Biochem 84:361–369

Hecker M, Pörtsi I, Bara AT, Busse R (1994) Potentiation by ACE inhibitors of the dilator response to bradykinin in the coronary microcirculation: interaction at the receptor level. Br J Pharmacol 111:238–244

Linz W, Schölkens BA (1987) Influence of local converting enzyme inhibition on angiotensin and bradykinin effects in ischemic hearts. J Cardiovasc Pharmacol 10 (Suppl 7):S75–S82

Oudit GY, Crackower MA, Backx PH, Penninger JM (2003) The role of ACE2 in cardiovascular physiology. Trends Cardiovasc Med 13:93–1101

Pre J, Bladier D (1983) A rapid and sensitive spectrophotometric method for routine determination of serum angiotensin I converting enzyme activity. IRCS Medical Sci 11:220–221

Santos RAS, Krieger EM, Greene LJ (1985) An improved fluorimetric assay of rat serum and plasma converting enzyme. Hypertension 7:244–252

Unger T, Fleck T, Ganten D, Rettig F (1984) 2-[N-[(S)-1-Ethoxycarbonyl-3-phenylpropyl-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (Hoe 498): antihypertensive action and persistent inhibition of tissue converting enzyme activity in spontaneously hypertensive rats. Arzneim Forsch/Drug Res 34:1426–1430

Unger T, Ganten D, Lang RE, Schölkens BA (1985) Persistent tissue converting enzyme inhibition following chronic treatment with Hoe 498 and MK 421 in spontaneously hypertensive rats. J Cardiovasc Pharmacol 7:36–41

A.1.1.12.2 Inhibition of Neutral Endopeptidase (Neprilysin)

PURPOSE AND RATIONALE

Neutral endopeptidase cleaves various peptides, such as enkephalins, kinins, chemotactic peptide, atrial natriuretic factor, and substance P. Reviews on neutral endopeptidase 24.11 (enkephalinase) were given by Erdös and Skidgel (1989) and by Roques et al. (1993). Structural requirements were investigated by Santos et al. (2002).

Several enzymatic assays have been developed for measuring neutral endopeptidase (NEP) activity, such as radiolabeled methods (Vogel and Altstein 1977; Llorens et al. 1982) and colorimetric assays (Almenoff et al. 1981; Almenoff and Orlowsky 1984); fluorometric assay (Florentin et al. 1984; Goudreau et al. 1994). Burell et al. (1997) and Hubner et al. (2001) used the selective NEP inhibitor radioligand ^{125}I-labelled RB104.

Cavalho et al. (1995, 1996) described a highly selective assay for neutral endopeptidase based on the cleavage of a fluorogenic substrate related to Leu-enkephalin.

PROCEDURE

A recombinant soluble form of NEP (rNEP) was expressed using a baculovirus/insect-cell system and purified by immunoaffinity.

The substrate (10 nmol) was incubated with rNEP (100 ng) in a final volume of 100 µl of 50 mM Tris-HCl buffer, pH 7.4, at 37°C for 30 min. For the inhibition assays, the enzyme was preincubated with 1 µM thiorphan or 1 µM captopril for 20 min before its incubation with the substrate. The reaction was stopped by heating for 5 min at 100°C. After centrifugation at 10,000 *g* for 10 min, the supernatant fraction was injected into an HPLC column and eluted with a 20%–40% gradient of acetonitrile containing 0.05% trifluoroacetic acid over a period of 30 min, at a flow rate of 1 ml/min. The substrate and products, detected by both UV absorbance (220 nm) and fluorescence (λ_{em} = 420 nm, λ_{ex} = 320 nm) with the detectors arranged in series, were collected to identify the cleavage site by amino acid analysis.

EVALUATION

Kinetic parameters for the NEP-catalyzed hydrolysis were determined from the double-reciprocal Lineweaver-Burkq plots.

MODIFICATIONS OF THE METHOD

Sulpizio et al. (2004) described the determination of **NEP activity in tissues** after *in vivo* treatment of rats with ACE inhibitors. After sacrifice of the animals, approximately 250 mg of kidney tissue was homogenized in 6 volumes of 0.1 M KH_2PO_4, pH 8.3, 0.3 M NaCl, and 1 µM $ZnSO_4$, using a Teflon-glass motor-driven pestle. NEP activity was measured by adding 35 µl of homogenate to wells containing 5 µl buffer or 10 phosphoramidon. Next, 10 µl of 2.5 mM *N*-dansyl-D-ala-gly-p-nitro-phe-gly substrate (Florentin et al. 1984) was added to each sample to yield a 0.5 mM final concentration and incubated for 4 min at 37°C. Subsequently, 100 µl of 10% TCA was added and plates were centrifuged to pellet precipitated proteins. Then 50 µl of supernatant was added to 100 µl of 100% ethanol and 50 µl of 1 N NaOH in a black fluorometric plate. After 10 min, plates were read at 590 nm emission, 320 nm excitation in a fluorometer.

Zhang et al. (1994) described an ELISA for the neuropeptide endopeptidase 3.4.24.11 in human serum and leukocytes.

Gros et al. (1989) studied the protection of atrial natriuretic factor against degradation and the diuretic and natriuretic responses after *in vivo* inhibition of enkephalinase (EC 3.4.24.11) by acetorphan. Increased tissue neutral endopeptidase 24.11 activity in spontaneously hypertensive hamsters was reported by Vishwanata et al. (1998). Graf et al. (1998) studied regulation of neutral endopeptidase 24.11 in human vascular smooth muscle cells by glucocorticoids and protein kinase C.

Pham et al. (1992) described the effects of a selective endopeptidase inhibitor on renal function and blood pressure in conscious normotensive Wistar and hypertensive DOCA-salt rats.

NEP is involved in organ systems other than the cardiovascular system, for example the brain and lung.

Ratti et al. (2001) studied the correlation between neutral endopeptidase (NEP) in serum and the degree of bronchial hyperreactivity.

Shirotani et al. (2001) found that neprilysin degrades both amyloid β peptides 1–40 and 1–42 very rapidly and efficiently. Newell et al. (2003) found that thiorphan-induced neprilysin inhibition raises amyloid β levels in rabbit cortex and cerebrospinal fluid.

Facchinetti et al. (2003) described the ontogeny, regional and cellular distribution of metalloprotease neprilysin 2 (**NEP2**) in the rat in comparison with neprilysin and endothelin-converting enzyme-1.

REFERENCES AND FURTHER READING

Almenoff J, Orlowsky M (1984) Biochemical and immunological properties of a membrane-bound brain metalloendopeptidase: comparison with thermolysin-like kidney neutral metalloendopeptidase. J Neurochem 42:151–157

Almenoff J, Wilk S, Orlowsky M (1981) Membrane bound pituitary metalloendopeptidase: apparent identity to enkephalinase. Biochem Biophys Res Commun 102:206–214

Burrell LM, Farina M, Risvanis J, Woollard D, Casely D, Johnston CI (1997) Inhibition of neutral endopeptidase, the degradative enzyme for natriuretic peptides, in rat kidney after oral SCH 42495. Clin Sci (Lond) 93:43–50

Cavalho KM, Boilleau G, Franca MS, Medeiros MA, Camargo AC, Juliano L (1995) A new fluorometric assay for neutral endopeptidase (EC 3.4.24.11). Braz J Med Biol Res 28:1055–1059

Cavalho KM, Boileau G, Camargo ACM, Juliano L (1996) A highly selective assay for neutral endopeptidase based on the cleavage of a fluorogenic substrate related to Leu-enkephalin. Anal Biochem 237:167–173

Erdös EG, Skidgel RA (1989) Neutral endopeptidase 24.11 (enkephalinase) and related regulatory peptide hormones. FASB J 3:145–151

Facchinetti P, Rose C, Schwartz JC, Ouimet T (2003) Ontogeny, regional and cellular distribution of the novel metalloprotease neprilysin 2 in the rat: a comparison with neprilysin and endothelin-converting enzyme-1. Neuroscience 118:627–639

Florentin D, Sassi A, Roques BP (1984) A highly sensitive fluorometric assay for "enkephalinase", a neutral metalloendopeptidase that releases tyrosine-glycine-glycine from enkephalins. Anal Biochem 141:62–69

Goudreau N, Guis C, Soleilhac JM, Roques BP (1994) Dns-gly-(*p*-NO_2)phe-βala, a specific fluorogenic substrate for neutral endopeptidase 24.11. Anal Biochem 219:87–95

Graf K, Schäper C, Gräge M, Fleck E, Kunkel G (1998) Glucocorticoids and protein kinase C regulate neutral endopeptidase 24.11 in human vascular smooth muscle cells. Basic Res Cardiol 93:11–17

Gros C, Souque A, Schwarzt JC, Duchier J, Cournot A, Baumer O, Lecomte JM (1989) Protection of atrial natriuretic factor against degradation: diuretic and natriuretic responses after *in vivo* inhibition of enkephalinase (EC 3.4.24.11) by acetorphan. Proc Natl Acad Sci USA 86:7580–7584

Hubner RA, Kubota E, Casley DJ, Johnston CI, Burrell LM (2001) In-vitro and in vivo inhibition of rat neutral endopeptidase and angiotensin converting enzyme with the vasopetidase inhibitor gemopatrilat. J Hypertens 19:941–946

Llorens C, Malfroy B, Schwartz JC, Gacel G, Roques BP, Morgat JL, Javoy-Agid F, Agid Y (1982) Enkephalin dipeptidyl carboxypeptidase (enkephalinase) activity: selective radioimmunoassay, properties and regional distribution in human brain. J Neurochem 39:1081–1089

Newell AJ, Sue LI, Scott S, Rauschkolb PK, Walker DG, Potter PE, Beach TG (2003) Thiorphan-induced neprilysin inhibition raises amyloid β levels in rabbit cortex and cerebrospinal fluid. Neurosci Lett 350:178–180

Pham I, el Amrani AI, Fournie-Zaluski MC, Corvol P, Roques B, Michel JB (1992) Effects of the selective endopeptidase inhibitor, retrothiophan, on renal function and blood pressure in conscious normotensive Wistar and hypertensive DOCA-salt rats. J Cardiovasc Pharmacol 20:847–857

Ratti H, Zhang M, Kunkel G (2001) Correlation between neutral endopeptidase (NEP) 3.4.24.11 in serum and the degree of bronchial hyperreactivity. Regul Pept 97:181–186

Roques BP, Noble F, Dauge V, Fournie-Zaluski MC, Beaumont A (1993) Neutral endopeptidase 24:11: structure, inhibition, and experimental and clinical pharmacology. Pharamcol Rev 45:87–146

Santos AN, Wulfänger J, Helbing G, Blosz T, Langner J, Riemann D (2002) Two C-terminal cysteines are necessary for proper folding of the peptidase neprilysin/CD10. Biochem Biophys Res Commun 295:423–427

Shirotani K, Tsubuki S, Iwata N, Takaki Y, Harigaya W, Maruyama K, Kiryu-Seo S, Kiyama H, Iwata H, Tomita T, Iwatsubo T, Saido TC (2001) Neprilysin degrades both amyloid β peptides 1–40 and 1–42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases. J Biol Chem 276:21895–21901

Sulpizio AC, Pullen MA, Edward RM, Brooks DP (2004) The effect of acute angiotensin-converting enzyme and neutral endopeptidase 24.11 inhibition on plasma extravasation in the rat. J Pharmacol Exp Ther 309:1141–1147

Vishwanata JK, Davis RC, Blumberg S, Gao XP, Rubinstein I (1998) Increased tissue neutral endopeptidase 24.11 activity in spontaneously hypertensive hamsters. Am J Hypertens 11:585–590

Vogel Z, Altstein M (1977) The adsorption of enkephalin to porous polystyrene beads: a simple assay for enkephalin hydrolysis: FEBS Lett 80:332–335

Zhang M, Niehus J, Schnellbacher T, Müller S, Graf K, Schultz KD, Baumgarten CR, Lucas C, Kunkel G (1994) ELISA for the neuropeptide endopeptidase 3.4.24.11 in human serum and leukocytes. Peptides 15:843–848

A.1.1.13 Quantitative Autoradiographic Localization of Angiotensin-Converting Enzyme

PURPOSE AND RATIONALE

Cardiac angiotensin converting enzyme can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40°C. Frozen section (20 µm) are cut in a cryostat at –20°C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4°C and then stored at –80°C.

Quantitative autoradiography

Radioligand: MK351A is a tyrosyl derivative of lisinopril, a potent competitive inhibitor of ACE. MK351A is iodinated by the chloramine T method and separated free from ^{125}I by SP Sephadex C25 column chromatography.

^{125}I-MK351A binding: The sections are preincubated in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 2% bovine serum albumin for 15 min at 20°C. The sections are then incubated with 11.1 KBq/ml ^{125}I-MK351A in the same buffer for 60 min at 20°C. Nonspecific binding is determined in the presence of 10^{-6} mol/L MK351A or lisinopril. Binding isotherms are determined using a set of serial sections incubated with 10^{-12}–10^{-6} mol/L lisinopril for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h

at room temperature. After exposure, the sections are fixed in formaldehyde and stained with haematoxylin and eosin. The optical density of the X-ray films is quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

REFERENCES AND FURTHER READING

Kohzuki M, Johnston CI, Chai SY, Jackson B, Perich R, Paxton D, Mendelson FAO (1991) Measurement of angiotensin converting enzyme induction and inhibition using quantitative *in vitro* autoradiography: tissue selective induction after chronic lisinopril treatment. J Hypertens 9:579–587

Kohzuki M, Kanazawa M, Yoshida K, Kamimoto M, Wu XM, Jiang ZL, Yasujima M Abe K, Johnston CI, Sato T (1996) Cardiac angiotensin converting enzyme and endothelin receptor in rats with chronic myocardial infarction. Jpn Circ J 60:972–980

Mendelsohn FAO, Chai S, Dunbar SY et al (1984) *In vitro* autoradiographic localization of angiotensin-converting enzyme in rat brain using ^{125}I-labelled MK351A. J Hypertens 2, Suppl 3:41–44

Munson PJ, Rodbard D (1980) LIGAND, a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239

A.1.1.14 Angiotensin Antagonism

The renin-angiotensin-aldosterone hormonal axis is the major long-term control for regulation of both arterial blood pressure and sodium balance. It supports normotension or hypertension via angiotensin vasoconstriction and angiotensin plus aldosterone-induced renal sodium retention (Laragh 1993; Unger and Schölkens 2004).

Volpe et al. (1995) Wagner et al. (1996) showed that regulation of aldosterone biosynthesis by adrenal renin is mediated through AT_1 receptors in renin transgenic rats.

Easthope and Jarvis (2002) reviewed pharmacological, pharmacokinetic and clinical data on the angiotensin II antagonist candesartan cilexetil.

REFERENCES AND FURTHER READING

Easthope SE, Jarvis B (2002) Candesartan Cilexetil. An update of its use in essential hypertension. Drugs 62:1253–1287

Laragh JH (1993) The renin system and new understanding of the complications of hypertension and their treatment. Arzneim Forsch/Drug Res 43:247–254

Unger T, Schölkens BA (eds) (2004) Angiotensin. Handbook of experimental pharmacology, Vol 1 (548 pp) and Vol II (603 pp). Springer, Berlin Heidelberg New York, p 163Volpe M, Rubattu S, Gigante B, Ganten D, Porcellini A, Russo R, Romano M, Enea E, Lee MA, Trimarco B (1995) Regulation of aldosterone biosynthesis by adrenal renin is mediated through AT_1 receptors in renin transgenic rats. Circ Res 77:73–79

Wagner J, Thile F, Ganten D (1996) The renin-angiotensin system in transgenic rats. Pediatr Nephrol 10:108–112

A.1.1.14.1 Angiotensin II Receptor Binding

PURPOSE AND RATIONALE

Angiotensin II receptor subtypes, AT_1 and AT_2, have been identified by structurally dissimilar antagonists, by different distribution in organs of various species and with specific radioligands (Chiu et al. 1989, 1990, 1992, 1993; Chang and Lotti 1991; Gibson et al. 1991; Chansel et al. 1992; Steckelings et al. 1992; Aiyar et al. 1993; Barnes et al. 1993; Bossé et al. 1993; Bottari et al. 1993; Dzau et al. 1993; Feuillan et al. 1993; van Meel et al. 1993; Alexander et al. 2001). These two types of receptors have been cloned (Sasaki et al. 1991; Murphy et al. 1991; Mukoyama et al. 1993; Kambayashi et al. 1993). Two other mammalian receptors named AT_3 and AT_4 have been described (de Gasparo et al. 1998).

The functional correlates of angiotensin II receptors have been discussed by Timmermans et al. (1992, 1993; Bernstein and Berk 1993). Most effects of angiotensin are mediated via the AT_1 receptors, but a possible role of angiotensin II subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts has been suggested (Wiemer et al. 1993a, b). Clearance studies in dogs indicated that the angiotensin type 2 receptor may be related to water handling in the kidney (Keiser et al. 1992).

Evidence for AT_1 receptor subtypes (AT_{1A} and AT_{1B}) has been reported (Iwai and Inagami 1992; Kakar et al. 1992; Balmforth et al. 1994; Matsubara et al. 1994; Bauer and Reams 1995; de Gasparo et al. 1998).

Chai et al. (2004) described the properties of the angiotensin IV/AT_4 receptor.

The assay described below is used to determine the affinity of test compounds to the angiotensin II receptor by measuring their inhibitory activity on the bind-

ing of ^{3}H-angiotensin II to a plasma membrane preparation from rat or bovine adrenal cortex.

PROCEDURE

Fresh bovine adrenal glands are obtained from the local slaughter house. For rat adrenal glands, male Sprague-Dawley rats weighing 250–300 g are sacrificed. The adrenals are separated from fat tissue and the medullae removed. The cortices are minced and homogenized in 5 mM Tris buffer containing 1 mM $MgCl_2$ and 250 mM sucrose, pH 7.4, using a chilled Potter homogenizer. The homogenate is centrifuged at 3000 *g* and 4°C for 10 min. The supernatant is recentrifuged at 39,000 *g* and 4°C for 10 min. The pellets are resuspended in 75 mM Tris buffer containing 25 mM $MgCl_2$, pH 7.4, and recentrifuged twice at 39,000 *g* and 4°C for 10 min. After the last centrifugation, the pellets are suspended in 75 mM Tris buffer containing 25 mM $MgCl_2$ and 250 mM sucrose, pH 7.4. Samples of 0.5 ml are frozen in liquid nitrogen and stored at –70°C.

In the competition experiment, 50 µl ^{3}H-angiotensin II (one constant concentration of $0.5–1 \times 10^{-9}$ M), and 50 µl test compound (6 concentrations, $10^{-5}–10^{-10}$ M) and 100 µl membrane suspension from rat or bovine adrenal cortex (approx. 250 mg wet weight/ml) per sample are incubated in a bath shaker at 25°C for 60 min. The incubation buffer contains 50 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$ and 0.2% bovine serum albumin, pH 7.4.

Saturation experiments are performed with 12 concentrations of ^{3}H-angiotensin II ($15–0.007 \times 10^{-9}$ M). Total binding is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled angiotensin II (10^{-6} M).

The reaction is stopped by rapid vacuum filtration through glass fiber filters. Thereby the membrane-bound radioactivity is separated from the free one. The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.

EVALUATION OF RESULTS

The following parameters are calculated:

- total binding of ^{3}H-angiotensin II
- non-specific binding: binding of ^{3}H-angiotensin II in the presence of mepyramine or doxepine
- specific binding = total binding–non-specific binding
- % inhibition of ^{3}H-angiotensin II binding: 100–specific binding as percentage of control value

The dissociation constant (K_i) and the IC_{50} value of the test drug are determined from the competition experiment of ^{3}H-angiotensin II versus non-labeled drug by a computer-supported analysis of the binding data (McPherson 1985).

MODIFICATIONS OF THE METHOD

Olins et al. (1993) performed competition studies in rat uterine smooth muscle membranes and rat adrenal cortex membranes using [^{125}I] labeled angiotensin II.

Membranes from cultured rat aortic smooth muscle cells and from human myometrium were used for binding studies with [^{125}I] labeled angiotensin II by Criscone et al. (1993).

Wiener et al. (1993) used membrane preparations from rat lung and adrenal medulla for binding studies with [^{125}I] labeled angiotensin II.

Bradbury et al. (1993) used a guinea pig adrenal membrane preparation to study nonpeptide angiotensin II receptor antagonists.

Cazaubon et al. (1993) prepared purified plasma membranes from rat livers for [^{125}I] AII binding assays.

Noda et al. (1993) described the inhibition of rabbit aorta angiotensin II (AII) receptor by a non-peptide AII antagonist.

Kushida et al. (1995) tested AT II receptor binding in particulate fractions of rat mesenteric artery and rat adrenal cortex and medulla with ^{125}I-AT II.

Chang et al. (1995) used rabbit aorta, rat adrenal and human AT_1 receptors in CHO cells and AT_2 receptors from rat adrenal and brain to characterize a non-peptide angiotensin antagonist.

Aiyar et al. (1995) tested inhibition of [^{125}I]-angiotensin II or [^{125}I]angiotensin II (Sar^1,Ile^8) binding in various membrane and cell preparations, such as rat mesenteric artery, rat adrenal cortex, rat aortic smooth muscle cell, human liver, recombinant human AT_1 receptor, bovine cerebellum, and bovine ovary.

Caussade et al. (1995) tested [^{125}I]Sar^1,Ile^8-angiotensin II binding to rat adrenal membranes and rat aortic smooth muscle cells.

Using [^{125}I]Sar^1,Ile^8-angiotensin II as radioligand, de Gasparo and Whitebread (1995) compared the affinity constants of valsartan and losartan in liver and adrenal of rat and marmoset, human adrenal and in rat aortic smooth muscle cells.

Webb et al. (1993) transfected the vascular angiotensin II receptor cDNA (AT_{1A}) into Chinese hamster ovary cells to generate the stable cell line CHO-AT_{1A} and recommended these cells as a useful model

to study AT_{1A} receptor domains, which are critical to signaling pathways.

Kiyama et al. (1995) used COS cells transfected with a cDNA encoding a human AT_1 angiotensin II receptor to evaluate nonpeptide angiotensin II receptor antagonists.

Mizuno et al. (1995) used bovine adrenal cortical membranes, Nozawa et al. (1997) membrane fractions from rat aorta, bovine cerebellum and human myocardium and [^{125}I]angiotensin II as radioligand.

Renzetti et al. (1995a, b) used membranes from rat adrenal cortex and bovine cerebellum for binding assays with [^{3}H]angiotensin II as radioligand.

Inter-species differences in angiotensin AT_1 receptors were investigated by Kawano et al. (1998).

The angiotensin II receptor subtype having a high affinity for lorsatan has been designated angiotensin AT_1 receptor and the receptor having a high affinity for PD123177 (1-(3-methyl-4-aminophenyl) methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazo[3,5-c]pyridine-6-carboxylic acid) as angiotensin AT_2 receptor (Bumpus et al. 1991; Nozawa et al. 1994; Chang et al. 1995).

In order to determine affinity for the angiotensin AT_1 subtype in a radioligand binding assay with ^{125}I-sarcosine^1, isoleucine^8 angiotensin II, Chang and Lotti (1991), Chang et al. 1995), Wong et al. (1995) incubated membranes of tissues with both AT_1 and AT_2 receptors in the presence of 1 μM PD121981 (which occupied all the AT_2 binding sites) and for the angiotensin AT_2 subtype in the presence of 1 μM lorsatan (which occupied all the AT_1 binding sites).

Hilditch et al. (1995) used membranes from rat livers and [^{3}H]-AT II for the determination of binding affinity at AT_1 receptors, or membranes from bovine cerebellum and [^{125}I]-Tyr^4-AT II for AT_2 receptors.

Lu et al. (1995) studied the influence of freezing on the binding of ^{125}I-sarcosine^1, isoleucine^8 angiotensin II to angiotensin II receptor subtypes in the rat. The results suggested that studies of AII receptor subtypes that involve freezing of the tissue underestimate the density and affinity of the AT_1 receptor subtype.

REFERENCES AND FURTHER READING

Aiyar N, Griffin E, Shu A, Heys R, Bergsma DJ, Weinstock J, Edwards R (1993) Characterization of [^{3}H]SK&F 108655 as a radioligand for angiotensin type-1 receptor. J Recept Res 13:849–861

Aiyar N, Baker E, Vickery-Clark L, Ohlstein EH, Gellai M, Fredrickson TA, Brooks DP, Weinstock J, Weidley EF, Edwards RM (1995) Pharmacology of a potent long-acting imidazole-5-acrylic acid angiotensin AT_1 receptor antagonist. Eur J Pharmacol 283:63–72

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Balmforth AJ, Bryson SE, Aylett AJ, Warburton B, Ball SG, Pun KT, Middlemiss D, Drew GM (1994) Comparative pharmacology of recombinant AT_{1A}, AT_{1B} and human AT_1 receptors expressed by transfected COS-M6 cells. Br J Pharmacol 112:277–281

Barnes JM, Steward LJ, Barber PC, Barner NM (1993) Identification and characterization of angiotensin II receptor subtypes in human brain. Eur J Pharmacol 230:251–258

Bauer JH, Reams GP (1995) The angiotensin II Type 1 receptor antagonists. A new class of antihypertensive drugs

Bernstein KE, Berk BC (1993) The biology of angiotensin II receptors. Am J Kidney Dis 22:745–754

Bossé R, Servant G, Zhou LM, Boulay G, Guillemette G, Escher E (1993) Sar^1-p-Benzoylphenylalanine-angiotensin, a new photoaffinity probe for selective labeling of the type 2 angiotensin receptor. Regul Peptides 44:215.223

Bottari SP, de Gasparo M, Steckelings UM, Levens NR (1993) Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications. Front Neuroendocrin 14:123–171

Bradbury RH, Allott CP, Dennis M, Girdwood JA, Kenny PW, Major JS, Oldham AA, Ratcliffe AH, Rivett JE, Roberts DA, Robins PJ (1993) New nonpeptide angiotensin II receptor antagonists. 3. Synthesis, biological properties, and structure-activity relationships of 2-alkyl-4-(biphenylmethoxy)pyridine derivatives. J Med Chem 36:1245–1254

Bürgisser E, Raine AEG, Erne P, Kamber B, Bühler FR (1985) Human cardiac plasma concentrations of atrial natriuretic peptide quantified by radioreceptor assay. Biochem Biophys Res Comm 133:1201–1209

Caussade F, Virone-Oddos A, Delchambre C, Cazes M, Versigny A, Cloarec A (1995) *In vitro* pharmacological characterization of UP 269–6, a novel nonpeptide angiotensin II receptor antagonist. Fundam Clin Pharmacol 9:119–128

Cazaubon C, Gougat J, Bousquet F, Guiraudou P, Gayraud R, Lacour C, Roccon A, Galindo G, Barthelmy G, Gautret B, Bernhart C, Perreaut P, Breliere JC, le Fur G, Nisato D (1993) Pharmacological characterization of SR 47436, a new non-peptide AT_1 subtype angiotensin II receptor antagonist. J Pharmacol Exp Ther 265:826–834

Chai S, Fernando R, Peck G, Ye S, Mendelson F, Jenkins T, Albiston A (2004) What's new in the renin-angiotensin system? The angiotensin IV/AT_4 receptor. Cell Mol Life Sci 61:2728–2737

Chang RSL, Lotti VJ (1991) Angiotensin receptor subtypes in rat, rabbit and monkey tissues: relative distribution and species dependency. Life Sci 49:1485–1490

Chang RSL, Lotti VJ, Chen TB, O'Malley SS, Bedensky RJ, Kling PJ, Kivlighn SD, Siegl PKS, Ondeyka D, Greenlee WJ, Mantlo NB (1995) *In vitro* pharmacology of an angiotensin AT_1 receptor antagonist with balanced affinity for AT_2 receptors. Eur J Pharmacol 294:429–437

Chansel D, Czekalski S, Pham P, Ardaillou R (1992) Characterization of angiotensin II receptor subtypes in human glomeruli and mesangial cells. Am J Physiol 262:F432–F441

Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, Timmermans PBMWM (1989) Identification of angiotensin II receptor subtypes. Biochem Biophys Res Commun 165:196–203

Chiu AT, McCall DE, Ardecky RJ, Duncia JV, Nguyen TT, Timmermans PBMWM (1990) Angiotensin II receptor

subtypes and their selective nonpeptide ligands. Receptor 1:33–40

Chiu AT, Carini DJ, Duncia JV, Leung KH, McCall DE, Price WA, Wong PC, Smith RD, Wexler RR, Timmermans PBMWM (1991) DuP 532: A second generation of nonpeptide angiotensin II receptor antagonists. Biochem Biophys Res Comm 177:209–217

Chiu AT, McCall DE, Roscoe WA (1992) [^{125}I]EXP985: a highly potent and specific nonpeptide radioligand for the AT_1 angiotensin receptor. Biochem Biophys Res Commun 188:1030–1039

Chiu AT, Leung KH, Smith RD, Timmermans PBMWM (1993) Defining angiotensin receptor subtypes. In: Raizada MK, Phillips MI, Sumners C (eds) Cellular and Molecular Biology of the Renin-Angiotensin System. CRC Press, Boca Raton, pp 245–271

Criscione L, de Gasparo M, Bühlmayer P, Whitebread S, Ramjoué HPR, Wood J (1993) Pharmacological profile of valsartan: a potent, orally active, nonpeptide antagonist of angiotensin II AT_1-receptor subtype. Br J Pharmacol 110:761–771

Cox HM, Munday KA, Poat JA (1984) Inactivation of [^{125}I]angiotensin II binding sites in rat renal cortex epithelial membranes by dithiothreitol. Biochem Pharmacol 33:4057–4062

De Gasparo M, Whitebread S (1995) Binding of valsartan to mammalian angiotensin AT_1 receptors. Regul Peptides 59:303–311

De Gasparo M, Catt KJ, Inagami T (1998) Angiotensin receptors. In: Girdlestone D (ed) The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR Media, London, pp 80–86

Dzau VJ, Sasamura H, Hein L (1993) Heterogeneity of angiotensin synthetic pathways and receptor subtypes: Physiological and pharmacological implications. J Hypertens 11:S13–S18

Entzeroth M, Hadamovsky S (1991) Angiotensin II receptors in the rat lung are of the AII-1 subtype. Eur J Pharmacol Mol Pharmacol Sec 206:237–241

Feuillan PP, Millan MA, Aguilera G (1993) Angiotensin II binding sites in the rat fetus: characterization of receptor subtypes and interaction with guanyl nucleotides. Regul Peptides 44:159–169

Gibson RE, Thorpe HH, Cartwright ME, Frank JD, Schorn TW, Bunting PB, Siegl PKS (1991) Angiotensin II receptor subtypes in renal cortex of rats and monkeys. Am J Physiol 261:F512

Hilditch A, Hunt AAE, Travers A, Polley J, Drew GM, Middlemiss D, Judd DB, Ross BC, Robertson MJ (1995) Pharmacological effects of GR138950, a novel angiotensin AT_1 receptor antagonist. J Pharm Exp Ther 272:750–757

Iwai N, Inagami T (1992) Identification of two subtypes in the rat type1 angiotensin II receptor. FEBS Lett 298:257–260

Kakar SS, Sellers JC, Devor DC, Musgrove LC, Neill JD (1992) Angiotensin II type-1 receptor subtype cDNAs: Differential tissue expression and hormonal regulation. Biochem Biophys Res Commun 183:1090–1096

Kambayashi Y, Bardhan S, Takahashi K, Tsuzuki S, Inui H, Hamakubo T, Inagami T (1993) Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. J Biol Chem 268:24543–24546

Kawano KI, Fujishima K, Nagura J, Yasoda S, Shinki, Hachisu M, Konno F (1998) Nonpeptide angiotensin receptor antagonist recognizes inter-species differences in angiotensin AT_1 receptors. Eur J Pharmacol 357:33–39

Keiser JA, Bjork FA, Hodges JC, Taylor DG (1992) Renal hemodynamic and excretory responses to PD123319 and losartan, nonapeptide AT_1 and AT_2 subtype-specific angiotensin II ligands. J Pharm Exp Ther 262:1154–1160

Kiyama R, Hayashi K, Hara M, Fujimoto M, Kawabata T, Kawakami M, Nakajima S, Fujishita T (1995) Synthesis and evaluation of novel pyrazolo[1,5-a]pyrimidine derivatives as non-peptide angiotensin II receptor antagonists. Chem Pharm Bull 43:960–965

Kushida H, Nomura S, Morita O, Harasawa Y, Suzuki M, Nakano M, Ozawa K, Kunihara M (1995) Pharmacological characterization of the nonpeptide angiotensin II receptor antagonist, U-97018. J Pharmacol Exp Ther 274:1042–1053

Lu XY, Zhang W, Grove KL, Speth RC (1995) Influence of freezing on the binding of ^{125}I-sarcosine^{1}, isoleucine^{8} angiotensin II to angiotensin II receptor subtypes in the rat. J Pharmacol Toxicol Meth 33:83–90

McPherson GA (1985) Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J Pharmacol Meth 14:213–228

Matsubara H, Kanasaki M, Murasawa S, Tsukaguchi Y, Nio Y, Inada M (1994) Differential gene expression and regulation of angiotensin receptor subtypes in rat cardiac fibroblasts an cardiomyocytes in culture. J Clin Invest 93:1592–1601

Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanagisawa H, Koike H (1995) Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. Eur J Pharmacol 285:181–188

Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, Dzau VJ (1993) Expression cloning of type-2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J Biol Chem 268:24539–24542

Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE (1991) Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. Nature 351:233–236

Noda M, Shibouta Y, Inada Y, Ojima M, Wada T, Sanada T, Kubo K, Kohara Y, Naka T, Nishikawa K (1993) Inhibition of rabbit aorta angiotensin II (AII) receptor by CV-11974, a new nonpeptide AII antagonist. Biochem Pharmacol 46:311–318

Nozawa Y, Haruno A, Oda N, Yamasaki Y, Matsuura N, Yamada S, Inabe K, Kimura R, Suzuki H, Hoshino T (1994) Angiotensin II receptor subtypes in bovine and human ventricular myocardium. J Pharmacol Exp Ther 270:566–571

Nozawa Y, Haruno A, Oda N, Yamasaki Y, Matsuura N, Miyake H, Yamada S, Kimura R (1997) Pharmacological profile of TH-142177, a novel orally active AT_1-receptor antagonist. Fundam Clin Pharmacol 11:395–401

Olins GM, Corpus VM, Chen ST, McMahon EG, Paloma MA, McGraw DE, Smits GJ, Null CL, Brown MA, Bittner SE, Koepke JB, Blehm DJ, Schuh JR, Baierl CS, Schmidt RE, Cook CS, Reitz DB, Penick MA, Manning RE, Blaine EH (1993) Pharmacology of SC-52458, an orally active, nonpeptide angiotensin AT_1 receptor antagonist. J Cardiovasc Pharmacol 22:617–625

Renzetti AR, Criscuoli M, Salimbeni A, Subissi A (1995a) Molecular pharmacology of LR-B/081, e new non-peptide angiotensin AT_1 receptor antagonist. Eur J Pharmacol Mol Pharmacol Sect 290:151–156

Renzetti AR, Cucchi P, Guelfi M, Cirillo R, Salimbeni A, Subissi A, Giachetti A (1995b) Pharmacology of LR-B/057, a novel active AT_1 receptor antagonist. J Cardiovasc Pharmacol 25:354–360

Robertson MJ, Barnes JC, Drew GM, Clark KL, Marshall FH, Michel A, Middlemiss D, Ross BC, Scopes D, Dowle MD (1992) Pharmacological profile of GR117289 *in vitro*:

a novel, potent and specific non-peptide angiotensin AT_1 receptor antagonist. Br J Pharmacol 107:1173–1180
Sasaki K, Yamono Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T (1991) Cloning and expression of a complementary DANN encoding a bovine adrenal angiotensin II type-1 receptor. Nature 351:230–233
Steckelings UM, Bottari SP, Unger T (1992) Angiotensin receptor subtypes in the brain. TIPS 13:365–368
Timmermans PBMWM, Benfield P, Chiu AT, Herblin WF, Wong PC, Smith RD (1992) Angiotensin II receptors and functional correlates. Am J Hypertens 5:221S–235S
Timmermans PBMWM, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee PJ, Wexler RR, Saye JAM, Smith RD (1993) Angiotensin receptors and angiotensin receptor antagonists. Pharmacol Rev 45:205–251
van Meel JCA, Entzeroth M, Hauel N, Narr B, Ries U, Wienen W (1993) Angiotensin II receptor antagonists. Arzneim Forsch/Drug Res 43:242–246
Webb ML, Monshizadegan H, Dickinson KE, Serafino R, Moreland S, Michel I, Seiler SM, Murphy TJ (1993) Binding and signal transduction of the vascular angiotensin II (AT_{1A}) receptor cDNA stably expressed in Chinese hamster cells. Regul Pept 44:131–139
Wiemer G, Schölkens BA, Busse R, Wagner A, Heitsch H, Linz W (1993b) The functional role of angiotensin II-subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts. Pharm Pharmacol Lett 3:24–27
Wiemer G, Schölkens BA, Wagner A, Heitsch H, Linz W (1993a) The possible role of angiotensin II subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts. J Hypertens 11, Suppl 5:S234–S235
Wienen W, Hauel N, van Meel JCA, Narr B, Ries U, Entzeroth M (1993) Pharmacological characterization of the novel nonpeptide angiotensin II receptor antagonist, BIBR 277. Br J Pharmacol 110:245–252
Wong PC, Quan ML, Saye JAM, Bernard R, Crain EJ Jr, NcCall DE, Watson CA, Zaspel AM, Smith RD, Wexler RR, Timmermans PBMWM, Chiu AT (1995) Pharmacology of XR510, a potent orally active nonpeptide angiotensin II AT_1 receptor antagonist with high affinity to the AT_2 receptor subtype. J Cardiovasc Pharmacol 26:354–362

A.1.1.14.2 AT_2 Receptor Binding

PURPOSE AND RATIONALE

In addition to the AT_1 receptor, the AT_2 receptor has attracted increasing interest (Gallinat et al. 2000; Nouet and Nahmias 2000). Steckelings et al. (2005) reviewed the knowledge of AT2 receptor distribution, signaling and function with an emphasis on growth/anti-growth, differentiation, and the regeneration of neuronal tissue.

Wan et al. (2004a, 2004b) described a porcine myometrial membrane AT_2 receptor assay.

PROCEDURE

Myometrial membranes are prepared from porcine uteri. Nielsen et al. (1997) found that in myometrium from non-pregnant sows, the Ang II receptors were almost exclusively AT_2 receptors. A presumable interference by binding to AT_1 receptors was blocked by addition of 1 µM losartan. Binding of [^{125}I]Ang II to membranes was conducted in a final volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, homogenate corresponding to 10 mg of the original tissue weight, [^{125}I]Ang II (80,000–85,000 cpm, 0.03 nM), and variable concentration of test substance. Samples were incubated at 25°C for 1.5 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets, which had been pre-soaked overnight with 0.3% polyethylamine, using a Brandel cell harvester. The filters were washed with 3 × 3 ml of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a γ-counter. All determinations were performed in triplicate.

EVALUATION

The characteristics of the Ang-II-binding AT_2 receptor were determined by using six different concentrations (0.03–5 nmol/l) of the labeled [^{125}I]-Ang II. Non-specific binding was determined in the presence of 1 µM Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [^{125}I]-Ang II. IC_{50} was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK).

MODIFICATIONS OF THE METHOD

Whitebread et al. (1991) described the radioligand CGP 42112A as a high-affinity and highly selective ligand for the characterization of angiotensin AT_2 receptors. Heemskerk and Saavedra (1995) performed quantitative autoradiography of Ang II AT_2 receptors with [^{125}I]CGP 42112.

Heerding et al. (1997) performed mutational analysis of the Ang II type 2 receptor in order to study the contribution of conserved extracellular amino acids.

Hoe et al. (2003) reported the molecular cloning, characterization, and distribution of the gerbil Ang II AT_2 receptor.

Utsunomiya et al. (2005) described Ang II AT_2 receptor localization in cardiovascular tissues by its antibody developed in AT_2 gene-deleted mice.

REFERENCES AND FURTHER READING

Gallinat S, Busche S, Raizada MK, Summers C (2000) The angiotensin II type 2 receptor: an enigma with multiple variation. Am J Physiol 278:E357–E374
Heemskerk FJM, Saavedra JM (1995) Quantitative autoradiography of angiotensin II AT_2 receptors with [^{125}I]CGP 42112. Brain Res 677:29–38
Heerding JN, Yee DK, Jacobs SL, Fluharty SJ (1997) Mutational analysis of the angiotensin II type 2 receptor: contri-

bution of conserved extracellular amino acids. Regul Pept 72:97–103
Hoe KL, Armando I, Baiardi G, Sreenath T, Kulkarni A, Martínez A, Saavedra JM (2003) Molecular cloning, characterization, and distribution of the gerbil angiotensin II AT_2 receptor. Am J Physiol 285:R1373–R1383
Nielsen AH, Schauser K, Winther H, Dantzer V, Poulsen K (1997) Angiotensin II receptors and renin in the porcine uterus: myometrial AT2 and endometrial AT1 receptors are down-regulated during gestation. Clin Exp Pharmacol Physiol 24:309–314
Nouet S, Nahmias C (2000) Signal transduction from the angiotensin II AT_2 receptor. Trends Endocrinol Metab 11:1–6
Steckelings UM, Kaschira E, Unger T (2005) The AT2 receptor – A matter of love and hate. Peptides 26:1401–1409
Utsunomiya H, Nakamura M, Kakudo K, Inagami T, Tamura M (2005) Angiotensin II AT_2 receptor localization in cardiovascular tissues by its antibody developed in AT_2 gene-deleted mice. Regul Pept 126:155–161
Wan Y, Wallinder C, Johansson B, Holm M, Mahalingam AK, Wu X, Botros M, Karlén A, Pettersson A, Nyberg F, Fändriks L, Hallberg A, Alterman M (2004a) First reported nonpeptide AT_1 receptor agonist (L-162,313) acts as an AT_2 receptor agonist in vivo. J Med Chem 47:1536–1540
Wan Y, Wallinder C, Plouffe B, Beaudry H, Mahalingam AK, Wu X, Johansson B, Holm M, Botros M, Karlén A, Pettersson A, Nyberg F, Fändriks L, Gallo-Payer N, Hallberg A, Alterman M (2004b) Design, synthesis and biological evaluation of the first selective nonpeptide AT_2 receptor agonist. J Med Chem 47:5995–6008
Whitebread SE, Taylor V, Bottari SP, Kamber B, de Gasparo M (1991) Radioligand CGP 42112A: a novel high affinity and highly selective ligand for the characterization of angiotensin AT_a receptors. Biochem Biophys Res Commun 181:1365–1371

A.1.1.14.3 Angiotensin II Induced Contraction in Isolated Rabbit Aorta

PURPOSE AND RATIONALE

The isolated rabbit aorta has been used to evaluate angiotensin II agonists (Liu 1993) and angiotensin II antagonists (Chang et al. 1992, 1994; Noda et al. 1993; Aiyar et al. 1995; Cirillo et al. 1995; Kushida et al. 1995; Mochizuki et al. 1995; Renzetti et al. 1995; Wong et al. 1995; Hong et al. 1998; Kawano et al. 1998).

PROCEDURE

New Zealand White male rabbits weighing 2–3 kg are sacrificed and exsanguinated. The thoracic aorta is removed and cleaned from adherent fat and connective tissue. The vascular endothelium is removed by gently rubbing the intimal surface of the vessel. Spiral aortic strips (2–3 mm wide and 30 mm long) are prepared and mounted in 5 ml organ baths containing Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 4.7 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, glucose 10 mM, pH 7.4). The organ baths are kept at 37°C and gassed continuously with 95% O_2/5% CO_2. Strips are attached to isometric transducers connected to a polygraph and a resting tension of 1 g is applied to each strip. Changes in contraction are analyzed with a digital computer. Aortic strips are allowed to equilibrate for 1 h and washed every 15 min. Two consecutive contractile-response curves to cumulative addition of ATII (0.1–300 mM) are constructed. After each curve the strips are washed 4 times and allowed to relax to the baseline tension. Afterward, each strip is incubated for 30 min with the vehicle or with a single concentration of the antagonist (1 – 10 – 100 – 1000 mM) before a third concentration-response curve to angiotensin II is obtained.

EVALUATION

The result of each concentration is expressed as a percentage of maximum response to AII. The pA_2 and pD'_2 values are calculated (van Rossum 1963).

MODIFICATIONS OF THE METHOD

Isolated guinea pig aortas were used by Mizuno et al. (1995).

Cirillo et al. (1995) evaluated the antagonism against AII-induced vasoconstriction in rat isolated perfused kidney.

Chang et al. (1992, 1994) determined AII-induced aldosterone release in rat adrenal cells and AII-induced [^{3}H]inositol phosphate accumulation in cultured rat aorta smooth muscle cells.

Shibouta et al. (1993) described the pharmacological profile of a highly potent and long-acting Ang II receptor antagonist and its prodrug.

Ojima et al. (1997) studied the mechanisms of the insurmountable antagonism of candesartan, an angiotensin AT_1 receptor antagonist, on Ang-II-induced rabbit aortic contraction in contraction and binding studies.

REFERENCES AND FURTHER READING

Aiyar N, Baker E, Vickery-Clark L, Ohlstein EH, Gellai M, Fredrickson TA, Brooks DP, Weinstock J, Weidley EF, Edwards EM (1995) Pharmacology of a potent long-acting imidazole-5-acrylic acid angiotensin AT_1 receptor antagonist. Eur J Pharmacol 283:63–72
Chang RLS, Siegl PKS, Clineschmidt BV, Mantlo NB, Chakravarty PK, Greenlee WJ, Patchett AA, Lotti VJ (1992) *In vitro* pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. J Pharmacol Exp Ther 262:133–138
Chang RSL, Bendesky RJ, Chen TB, Faust KA, Kling PJ, O'Malley SA, Naylor EM, Chakravarty PK, Patchett AA,

Greenlee WJ, Clineschmidt BV, Lotti VJ (1994) *In vitro* pharmacology of MK-996, a new potent and selective angiotensin II (AT_1) receptor antagonist. Drug Dev Res 32:161–171

Cirillo R, Renzetti AR, Cucchi P, Guelfi M, Salimbeni A, Caliari S, Castellucci A, Evangeklista S, Subissi A, Giachetti A (1995) Pharmacology of LR-B/081, a new highly potent, selective and orally active, nonpeptide angiotensin II AT_1 receptor antagonist. Br J Pharmacol 114:1117–1124

Hong KW, Kim CD, Lee SH, Yoo SE (1998) The *in vitro* pharmacological profile of KR31080, a nonpeptide AT_1 receptor antagonist. Fundam Clin Pharmacol 12:64–69

Kawano KI, Fujishima K, Nagura J, Yasuda S, Hachisu SM, Konno F (1998) Nonpeptide angiotensin II receptor antagonist recognized inter-species differences in angiotensin AT_1 receptors. Eur J Pharmacol 357:33–39

Kushida H, Nomura S, Morita O, Harasawa Y, Suzuki M, Nakano M, Ozawa K, Kunihara M (1995) Pharmacological characterization of the nonpeptide angiotensin II receptor antagonist, U-97018. J Pharmacol Exp Ther 274:1042–1053

Liu YJ (1993) Evidence that [Sar^1]angiotensin II behaves differently from angiotensin II at angiotensin AT_1 receptors in rabbit aorta. Eur J Pharmacol 235:9–15

Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanikasawa H, Koike H (1995) Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. Eur J Pharmacol 285:181–188

Mochizuki S, Sato T, Furuta K, Hase K, Ohkura Y, Fukai C, Kosakai K, Wakabayashi S, Tomiyama A (1995) Pharmacological properties of KT3–671, a novel nonpeptide angiotensin receptor antagonist. J Cardiovasc Pharmacol 25:22–29

Noda M, Shibouta Y, Inada Y, Ojima M, Wada T, Sanada T, Kubo K, Kohara Y, Naka T, Nishikawa K (1993) Inhibition of rabbit aortic angiotensin II (AII) receptor by CV-1197, a new nonpeptide AII antagonist. Biochem Pharmacol 46:311–318

Ojima M, Inada Y, Shibouta Y, Wada T, Sanada T, Kubo K, Nishikawa K (1997) Candesartan (CV-11974) dissociates slowly from the angiotensin AT_1 receptor. Eur J Pharmacol 319:137–146

Renzetti AR, Cucchi P, Guelfi M, Cirillo R, Salimbeni A, Subissi A, Giachetti A (1995) Pharmacology of LR-B/057, a novel orally active AT_1 receptor antagonist. J Cardiovasc Pharmacol 25:354–360

Shibouta Y, Inada Y, Ojima M, Wada T, Noda M, Sanada T, Kubo K, Kohara Y, Naka T, Nishikawa K (1993) Pharmacological profile of a highly potent and long-acting angiotensin II receptor antagonist, 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4- yl]methyl]-1H-benzimidazole-7-carboxylic acid (CV-11974), and its prodrug, (+/-)-1-(cyclohexyloxycarbonyloxy)-ethyl 2-ethoxy-1-[[2′-(1H-tetrazol-5- yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate (TCV-116). J Pharm Exp Ther 266:114–120

Van Rossum JM (1963) Cumulative dose-response curves. II. Techniques for the making of dose-response curves in isolated organs and evaluation of drug parameters. Arch Int Pharmacodyn 143:299–330

Wong PC, Quan ML, Saye JM, Bernard R, Crain EJ Jr, McCall DE, Watson CA, Zaspel AM, Smith RD, Wexler RR, Timmermans PBMVM, Chiu AT (1995) Pharmacology of XR510, a potent orally active nonpeptide angiotensin II AT_1 receptor antagonist with high affinity for the AT_2 receptor subtype. J Cardiovasc Pharmacol 26:354–362

A.1.1.14.4 Angiotensin II Antagonism in Vivo

PURPOSE AND RATIONALE

The effect of ATII antagonists on blood pressure has been measured in anesthetized (Olins et al. 1993; Beauchamp et al. 1995; Kawano et al. 1998), in pithed (Cazes et al. 1995; Christophe et al. 1995; Cirillo et al. 1995; Deprez et al. 1995; Häuser et al. 1998) and in conscious (Junggren et al. 1996; Nozawa et al. 1997; Shibasaki et al. 1997; Hashimoto et al. 1998) normotensive and hypertensive rats.

PROCEDURE

Male Sprague-Dawley rats are anesthetized with 100 mg/kg i.p. Inactin and placed on servo-controlled heating pads to maintain body temperature between 37°C and 38°C. PE50 catheters are implanted in the femoral artery and vein to measure arterial blood pressure and administer compounds, respectively. A catheter is placed in the trachea to ensure airway patency. Arterial pressure is measured continuously by connecting the arterial catheter to transducer coupled to a Gould pressure transducer. The output is recorded on a polygraph. Mean arterial pressure is derived electronically. After a 30–45 min stabilization period, autonomic transmission is blocked by treatment with mecamylamine (3 mg/kg i.v.) and atropine (0.4 mg/kg i.v.). After arterial pressure has stabilized, angiotensin is infused i.v. in isotonic saline with a syringe pump. When the pressure response to angiotensin has stabilized, angiotensin II antagonists are given in increasing doses. The doses are given intravenously in a cumulative fashion, i. e., the next highest dose is given at the time of maximum response to the prior dose.

EVALUATION

Data are presented as percent inhibition of the angiotensin pressor response to each dose of the antagonists and plotted against the log of the cumulative doses of antagonist. Linear regression is used to calculate the dose at which the response to angiotensin is inhibited 50% (ID_{50}) for each rat. Means ±SEM are calculated.

MODIFICATIONS OF THE METHOD

Olins et al. (1993), Cirillo et al. (1995) determined also the antihypertensive effects in conscious spontaneously hypertensive rats and in conscious sodium-deficient dogs.

Stasch et al. (1997) studied the long-term blockade of the angiotensin II receptor in renin trangenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats.

Nishioka et al. (1998), Richter et al. (1998) used the (mRen-2)27 transgenic (Tg^+) rat, a hypertensive model dependent on increased expression of the renin angiotensin system, to explore the role of angiotensin AT_2 receptors in the control of cardiovascular and renal excretory function.

Simoes e Silva et al. (1998) evaluated the effects of chronic administration of an angiotensin antagonist on diuresis and natriuresis in normotensive and spontaneously hypertensive rats.

Kai et al. (1998) examined the effects of an angiotensin II type 1 antagonist on cardiac hypertrophy and nephropathy using Tsukuba hypertensive mice (THM) carrying both human renin and angiotensinogen genes.

Kivlighn et al. (1995a, b), Gabel et al. (1995), studied angiotensin II antagonists in conscious rats, dogs, rhesus monkeys and chimpancees.

Keiser et al. (1995) studied arterial blood pressure in conscious renal hypertensive rats, conscious sodium-depleted dogs, conscious sodium-depleted monkeys and conscious renal hypertensive monkeys.

Kim et al. (1997) examined the effects of an angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. Cardiac volume overload was prepared by abdominal aortocaval shunt. Cardiac tissue mRNA was measured by Northern blot analysis with specific probes.

Yamamoto et al. (1997), Ogilvie et al. (1998), studied angiotensin II receptor antagonists in acute heart failure induced by coronary artery ligation in anesthetized dogs and in chronic heart failure induced by left ventricular rapid-pacing in conscious dogs.

Massart et al. (1998) evaluated the cumulative hypotensive effects of angiotensin II- and endothelin-1-receptor antagonists in a model renovascular hypertension in dogs.

Hayashi et al. (1997) examined the hemodynamic effects of an angiotensin II type 1 receptor antagonist in rats with myocardial infarction induced by coronary ligation.

Kivlighn et al. (1995c) studied the effects of a nonpeptide that mimics the biological actions of angiotensin II in anesthetized rats.

Huckle et al. (1996) evaluated angiotensin II receptor antagonists for their ability to inhibit vascular intimal thickening in a porcine coronary artery model of vascular injury.

REFERENCES AND FURTHER READING

Beauchamp HT, Chang RSL, Siegl PKS, Gibson RE (1995) *In vivo* receptor occupancy of the angiotensin II receptor by nonpeptide antagonists: relationships to *in vitro* affinities and *in vivo* pharmacologic potency. J Pharmacol Exp Ther 272:612–618

Cazes M, Provost D, Versigny A, Cloarec A (1995) *In vivo* pharmacological characterization of UP 269–6, a novel nonpeptide angiotensin II receptor antagonist. Eur J Pharmacol 284:157–170

Christophe B, Libon R, Cazaubon C, Nisato D, Manning A, Chatelain B (1995) Effects of irbesartan (SR 47436/BMS-186295) on angiotensin II-induced pressor responses in the pithed rat: Potential mechanisms of action. Eur J Pharmacol 281:161–171

Cirillo R, Renzetti AR, Cucchi P, Guelfi M, Salimbeni A, Caliari S, Castellucci A, Evangelista S, Subissi A, Giachetti A (1995) Pharmacology of LR-B/081, a new highly potent, selective and orally active, nonpeptide angiotensin II AT_1 receptor antagonist. Br J Pharmacol 114:1117–1124

Deprez P, Guillaume J, Becker R, Corbier A, Didierlaurent S, Fortin M, Frechet D, Hamon G, Heckmann B, Heitsch H, Kleemann HW, Vevert JP, Vincent JC, Wagner A, Zhang J (1995) Sulfonylureas and sulfonycarbamates as new nontetrazole angiotensin II antagonists. Discovery of a highly potent orally active (imidazolylbiphenylyl)-sulfonylurea (HR 720). J Med Chem 38:2357–2377

Gabel RA, Kivlighn SD, Zingara GJ, Schorn TW, Schaffer LW, Ashton WT, Chang LL, Flanagan K Greenlee WJ, Siegl PKS (1995) *In vivo* pharmacology of L-159,913, a new highly potent and selective nonpeptide angiotensin II receptor antagonist. Clin Exp Hypertens 17:931–953

Häuser W, Denhofer A, Nguyen T, Dominiak P (1998) Effects of the AT1 antagonist HR 720 in comparison to losartan on stimulated sympathetic outflow, blood pressure, and heart rate in pithed spontaneously hypertensive rats. Kidney Blood Press Res 21:29–35

Hashimoto Y, Ohashi R, Kurosawa Y, Minami K, Kaji H, Hayashida K, Narita H, Murata S (1998) Pharmacologic profile of TA-606, a novel angiotensin II receptor antagonist in the rat. J Cardiovasc Pharmacol 31:568–575

Hayashi N, Fujimura Y, Yamamoto S, Kometani M, Nakao K (1997) Pharmacological profile of valsartan, a non-peptide angiotensin II type 1 receptor antagonist. 4th communication: Improvement of heart failure of rats with myocardial infarction by valsartan. Arzneim Forsch/Drug Res 47:625–629

Huckle WR, Drag MD, Acker WR, Powers M, McFall RC, Holder DJ, Fujita T, Stabilito II, Kim D, Ondeyka DL, Mantlo NB, Chang RSL, Reilly CF, Schwartz RS, Greenlee WJ, Johnson RG Jr (1996) Effects of subtype-selective and balanced angiotensin II receptor antagonists in a porcine coronary artery model of vascular restenosis. Circulation 93:1009–1019

Junggren IL, Zhao X, Sun X, Hedner T (1996) Comparative cardiovascular effects of the angiotensin II type 1 receptor antagonists ZD 7155 and losartan in the rat. J Pharm Pharmacol 48:829–833

Kai T, Sigimura K, Shimada S, Kurooka A, Takenaka T, Ishikawa K (1998) Inhibitory effects of a subdepressor dose of I-158,809, an angiotensin II type 1 antagonist, on cardiac hypertrophy and nephropathy via the activated human renin-angiotensin system in double transgenic mice with hypertension. Jpn Circ J 62:599–603

Kawano KI, Fujishima K, Nagura J, Yasuda S, Hachisu SM, Konno F (1998) Nonpeptide angiotensin II receptor an-

tagonist recognized inter-species differences in angiotensin AT_1 receptors. Eur J Pharmacol 357:33–39
Keiser JA, Ryan MJ, Panek RL, Hodges JC, Sircar I (1995) Pharmacological characterization of CI-996, a new angiotensin receptor antagonist. J Pharmacol Exp Ther 272:963–969
Kim S, Sada T, Mizuno M, Ikeda M, Yano M, Miura K, Yamanaka S, Koike H, Iwao H (1997) Effects of angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. Hypertens Res Clin Exp 20:133–142
Kivlighn SD, Zingaro GJ, Gabel RA, Broten TP, Schorn TW, SchafferLW, Naylor EM, Chakravaty PK, Patchett AA, Greenlee WJ, Siegl PKS (1995a) *In vivo* pharmacology of an novel AT_1 selective angiotensin antagonist, MK-996. Am J Hypertens 8:58–66
Kivlighn SD, Zingaro GJ, Gabel RA, Broten TP, Chang RSL, Ondeyka DL, Mantlo NB, Gibson RE, Greenlee WJ, Siegl PKS (1995b) *In vivo* pharmacology of an angiotensin AT_1 receptor antagonist with balanced affinity for angiotensin AT_2 receptors. Eur J Pharmacol 294:439–450
Kivlighn SD, Huckle WR, Zingaro GJ, Rivero RA, Lotti VJ, Chang RSL, Schorn TW, Kevin N, Johnson RG Jr, Greenlee WJ, Siegl PKS (1995) Discovery of L-162,313: a nonpeptide that mimics the biological actions of angiotensin II. Am J Physiol 286, Regul Integr Comp Physiol 37:R820–R823
Massart PE, Hodeige DG, van Mechelen H, Charlier AA, Ketelslegers JM, Heyndrickx GR, Donckier JE (1998) Angiotensin II and endothelin-1 receptor antagonists have cumulative hypotensive effects in canine Page hypertension. J Hypertens 16:835–841
Nishioaak T, Morris M, Li P, Ganten D, Ferrario CM, Callahan MF (1998) Depressor role of angiotensin AT_2 receptors in the (mRen-2)27 transgenic rat. Am J Hypertens 11:357–362
Nozawa Y, Haruno A, Oda N, Yamasaki Y, Matsuura N, Miyaka H, Yamada S, Kimura R (1997) Pharmacological profile of TH-142177, a novel orally active AT_1 receptor antagonist. Fundam Clin Pharmacol 11:395–401
Ogilvie RI, Zborowska-Sluis D (1998) Captopril and angiotensin II receptor agonist therapy in a pacing model of heart failure. Can J Cardiol 14:1025–1033
Olins GM, Smits GJ, Koepke JP, Huang HC, Reitz DB, Manning RE, Blaine EH (1993) *In vivo* pharmacology of SC-51316, a nonpeptidic angiotensin II receptor antagonist. Am J Hypertens 6:619–625
Richter C, Bruneval P, Menard J, Giudicelli JF (1998) Additive effects of enalapril and losartan in (mREN-2)27 transgenic rats. Hypertension 31:692–698
Shibasaki M, Fujimori A, Kusayama T, Tokioka T, Satoh Y, Okazaki T, Uchida W, Inagaki O, Yanagisawa I (1997) Antihypertensive activity of a nonpeptide angiotensin II receptor antagonist, YM358, in rats and dogs. Eur J Pharmacol 335:175–184
Simoes e Silva AC, Bello APC, Baracho NCV, Khosla MC, Santos RAS (1998) Diuresis and natriuresis produced by long term administration of a selective angiotensin-(1–7) antagonist in normotensive and hypertensive rats. Regul Pept 74:177–184
Stasch JP, Knorr A, Hirth-Dietrich C, Kramer T, Hubsch W, Dressel J, Fey P, Beuck M, Sander E, Frobel K, Kazda S (1997) Long-term blockade of the angiotensin II receptor in renin transgenic rats, salt-loaded Dahl rats, and stroke-prone spontaneously hypertensive rats. Arzneim Forsch/Drug Res 47:1016–1023
Yamamoto S, Hayashi N, Kometani M, Nakao K (1997) Pharmacological profile of valsartan, a non-peptide angiotensin II type 1 receptor antagonist. 5th communication: Hemodynamic effects of valsartan in dog heart failure models. Arzneim Forsch/Drug Res 47:630–634

A.1.1.15
Renin-Inhibitory Activity Using Human Kidney Renin and a Synthetic Substrate

PURPOSE AND RATIONALE

In contrast to other enzymes, renin shows a rather high species specificity. To be relevant for humans human renin has to be used. One of the reasons that human renin is specific for human angiotensinogen lies in the sequence of human angiotensinogen itself. Inhibition of renin is measured by angiotensinogen formed in the presence of angiotensinase inhibitors. The following procedure is used to determine the effect of potential renin inhibitors on purified human kidney renin without interference from plasma proteins or lipids.

PROCEDURE

The synthetic substrate represents the first fourteen amino acids of the N-terminus of human angiotensinogen: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn. The assay mixture is composed of phosphate buffer (pH 7.5), bovine serum albumin, 3 mM EDTA, 0.01 mM phenylmethylsulfonyl fluoride (PMSF), 0.002% Genapol PF 10, test compound (dissolved in DMSO), substrate (3 µM) and purified human kidney renin (Calbiochem GmbH, Frankfurt/M., Germany; cat. no. 553861). The mixture is incubated for two hours at 37°C. Then the reaction is stopped by transfer of 450 µl into preheated (95°C) Eppendorf tubes. The amount of angiotensin I liberated is measured by RIA (Renin MAIA kit, Serono Diagnostika GmbH, Freiburg, Germany).

Human angiotensinogen (0.2 µM) may be used as a substrate instead of the tetradekapeptide. The pH value of the incubation mixture may be lowered to 6.0 by using a maleic acid buffer; this results in higher renin activity. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) may be substituted for phosphate in the pH 7.5 buffer.

EVALUATION

Renin activity, i. e. angiotensin I production (ng/ml × 2 h), is corrected for an angiotensin I – like immunoreactivity which can be measured in the assay samples even in the absence of added renin. IC_{50} values are determined from a plot of renin activity (as per cent of control) vs. molar concentration of the test compound.

MODIFICATIONS OF THE METHOD

Wang et al. (1993) described a continuous fluorescence assay of renin activity employing a new fluorigenic peptide substrate.

Inhibition of plasma renin activity in blood samples from various species can be determined in order to evaluate the species specificity of a renin inhibitor (Linz et al. 1994)

Blood samples are obtained from dogs, sheep and rhesus monkeys by venipuncture. Wistar rats and guinea pigs are anesthetized with Nembutal (60 mg/kg intraperitoneally) and the blood is collected by puncture of the abdominal aorta. Human blood is collected from volunteers (Donafix blood collecting set, Braun Melsungen AG, Melsungen, FR Germany) in cooled bottles. All blood samples are anticoagulated with Na-EDTA (final concentration 10–15 mM). The renin is dissolved in DMSO as 10^{-2} M stock solution and diluted before each experiment in DMSO. The endogenous formation of ANG I in plasma during incubation at 37°C is determined as the measure of renin activity. Generation and quantitation of ANG I are performed using a commercial radio immunoassay kit (Renin-MAIA, Serono Diagnostika GmbH, Freiburg, FR Germany). Plasma samples are thawed on ice and centrifuged after addition of 100 µl PMSF solution (kit) per 10 ml. The assay mixture contains 450 µl plasma plus 1% (v/v) PMSF solution, 45 µl buffer (phosphate buffer, pH = 7.4, + 10^{-5} M ramiprilate) and 5 µl renin inhibitor solution (diluted in DMSO as required) or pure DMSO for controls. The assay is incubated for an appropriate time (2–3 hours) at 37°C. ANG I is measured in 100 µl samples (triplicate determinations). Basal ANG I immunoreactivity of the plasma is determined from an unincubated control assay (0°C). This pre-incubation value is subtracted from all measurements. The renin activity in the presence of the renin inhibitor is calculated as percent activity in relation to control samples containing only DMSO. The IC_{50} value is determined from a semilogarithmic plot of percent renin activity versus concentration of the renin inhibitor.

Wood et al. (1990) determined the activity of a synthetic renin inhibitor against rat, mouse, dog, guinea pig, rabbit, cat, marmoset and human renin using plasma pools from these species. Plasma from each species was collected using EDTA as an anticoagulant. Samples of plasma were incubated at 37°C in the presence or absence of varying concentrations of test compound. The ANG I formed was measured by radioimmunoassay.

Shibasaki et al. (1991) used squirrel monkeys to study the *in vivo* activity of a specific renin inhibitor after intravenous and oral application.

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Salimbeni et al. (1996) tested the *in vitro* inhibition of human plasma renin activity by two synthetic angiotensinogen transition state analogues.

Wood et al. (2003) determined the inhibitory potency of an orally effective renin inhibitor *in vitro* against human renin. Human recombinant renin (0.33 ng/ml) was incubated with a synthetic tetradecapeptide substrate (TDP, 13.33 µM) corresponding to the 14 terminal amino acids of human angiotensinogen, in 0.33 M Tes buffer, pH 7.2, containing 1% human serum albumin and 0.1% neomycin sulphate for 1 h at 37°C. The enzymatic reaction was stopped by adding 1 ml ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1% HSA. The angiotensin generated during the incubation was measured by radioimmunoassay. The oral activity was confirmed in hypertensive patients (Gradman et al. 2005).

REFERENCES AND FURTHER READING

Bohlender J, Ménard J, Wagner J, Luft FC, Ganten D (1996) Développement chez le rat d'un modèle d'hypertension à la rénine humaine. Arch Mal Cœur 89:1009–1011

Bolis G, Fung AKL, Greer J, Kleinert HD, Marcotte PA, Perun TJ, Plattner JJ, Stein HH (1987) Renin inhibitors. Dipeptide analogues of angiotensinogen incorporating transition state, nonpeptidic replacements at the scissile bond. J Med Chem 30:1729–1737

Corvol P, Menard J (1989) Renin inhibition: Immunological procedures and renin inhibitor peptides. Fundam Clin Pharmacol 3:347–362

Freedlender AE, Goodfriend TL (1979) Renin and the angiotensins. In: Jaffe BM, Behrman HR (eds) Methods of Hormone Radioimmunoassay. Academic Press, New York, pp 889–907

Gradman AH, Schmieder RE, Lins RL, Nussberger J, Chiang Y, Bedigian MP (2005) Aliskiren, a novel orally effective renin inhibitor, provides dose-dependent antihypertensive efficacy and placebo-like tolerability in hypertensive patients. Circulation 111:1012–1018

Greenlee WJ (1990) Renin inhibitors. Med Res Rev 10:173–236

Heitsch H, Henning R, Kleemann HW, Linz W, Nickel WU, Ruppert D, Urbach H, Wagner A (1993) Renin inhibitors containing a pyridylaminodiol derived C-terminus. J Med Chem 36:2788–2800

Kokubu T, Hiwada K, Murakami E, Muneta S, Morisawa Y, Yabe Y, Koike H, Iijima Y (1987) *In vitro* inhibition of human renin by statine-containing tripeptide inhibitor. J Cardiovasc Pharmacol 10 (Suppl. 7) S88–S90

Linz W, Heitsch H, Henning R, Jung W, Kleemann HW, Nickel WU, Ruppert D, Urbach H, Wagner A, Schölkens BA (1994) Effects of the renin inhibitor *N*-[*N*-(3-

(4-amino-1-piperidinyl-carbonyl)-2(*R*)-benzylpropionyl)-L-histidinyl]-(2*S*,3*R*,4*S*)-1-cyclohexyl-3,4-dihydroxy-6(2-pyridyl)-hexane-2-amide acetate (S 2864) in anesthetized rhesus monkeys. Arzneim Forsch/Drug Res 44:815–820

Murakami K, Ohsawa T, Hirose S, Takada K, Sakakibara S (1981) New fluorogenic substrates for renin. Analyt Biochem 110:232–239

Salimbeni A, Paleari F, Poma D, Criscuoli M, Scolastico C (1996) Synthesis and renin inhibitory activity of novel angiotensinogen transition state analogues modified at the P_2-histidine position. Eur J Med Chem 31:827–832

Shibasaki M, Asano M, Fukunaga Y, Usui T, Ichihara M, Murakami Y, Nakano K, Fujikura T (1991) Pharmacological properties of YM-21095, a potent and highly specific renin inhibitor. Am J Hypertens 4:932–938

Wang GT, Chung CC, Holzman TF, Krafft GA (1993) A continuous fluorescence assay of renin activity. Analyt Biochem 210:351–359

Wood JM, Criscione L, de Gasparo M, Bühlmayer P, Rüeger H, Stanton JL, Jupp RA, Kay J (1989) CGP 38 560: Orally active, low-molecular weight renin inhibitor with high potency and specificity. J Cardiovasc Pharmacol 14:221–226

Wood JM, Mah SC, Baum HP, de Gasparo M, Cumin F, Rüeger H, Nussberger J (1990) Evaluation of a potent inhibitor of subprimate and primate renins. J Pharmacol Exp Ther 253:513–517

Wood JM, Maibaum J, Rahuel J, Grütter MG, Cohen NC, Rasetti V, Rüger H, Göschke R, Stutz S, Fuhrer W, Schilling W, Rigollier P, Yamaguchi Y, Cumin F, Baum HP, Sdchnell CR, Herold P, Mah R, Jensen C, O'Brien E, Stanton A, Bedigian MP (2003) Structure-based design of aliskiren, a novel orally effective renin inhibitor. Biochem Biophys Res Commun 308:698–705

A.1.1.16 PAF Binding Assay

PRINCIPLE AND RATIONALE

Injection of platelet activating (PAF) factor induces a wide range of potent and specific effects on target cells, including aggregation of platelets and shock symptoms like systemic hypotension, pulmonary hypertension, increased vascular permeability, neutropenia and thrombocytopenia. Inhalation of PAF causes immediate bronchoconstriction followed by inflammation of the airways (further information see Sect. B.1.9).

Hikiji et al. (2004) showed in PAF-deficient mice that the absence of platelet-activating factor receptor protects mice from osteoporosis following ovariectomy, a model of postmenopausal osteoporosis.

PAF is also implicated in estrogen-induced angiogenesis via nuclear factor-κB activation (Seo et al. 2004) and in delaying corneal wound healing (Bazan 2005).

The PAF receptor belongs to the superfamily of G protein-coupled receptors (Chao and Olson 1993, Izumi and Shimizu 1995). Cloning studies have indicated a single human PAF receptor gene containing an intron at the 5′ flanking region, providing alternative sequences (Ishi and Shimizu 2000).

The following procedure is used to detect compounds that inhibit binding of ^{3}H-PAF (platelet activating factor) in rabbit platelets (PAF receptor).

PROCEDURE

Crude rabbit platelets are incubated in plastic tubes for 15 min at 25°C in a buffer solution (0.54 g/l KH_2PO_4, 0.6 g/l Na_2HPO_4, 5.8 g/l NaCl, 1.0 g/l BSA, pH 7.1) with 1 nM synthetic ^{3}H-labeled PAF (1-*O*-[1,2-$^3H_2$]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and various concentrations of test compound. Non-specific binding is determined in the presence of 10 µM CV 3988. Bound ligand is separated from the incubation medium by rapid filtration through Whatman GF/C glass fibre filters. Following rinsing with ice-cold buffer (3 × 5 ml), the filters are placed in 10 ml scintillation cocktail for radioactivity determination.

EVALUATION

The following parameters are calculated:

- total binding of ^{3}H-PAF
- non-specific binding in the presence of 10 µM CV 3988
- specific binding = total binding–non-specific binding
- % inhibition: 100–specific binding as percentage of the control value

Compounds are first tested at a single high concentration (5000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound. Binding potency of compounds is expressed either as a "relative binding affinity" (RBA) with respect to the standard compound (CV 3988) which is tested in parallel or as an IC_{50}.

$$\text{RBA} = \frac{\text{IC}_{50}\text{ standard compound}}{\text{IC}_{50}\text{ compound}} \times 100\%$$

Standard data:

- CV 3988 IC_{50}: 276 nM ± 24 ($n = 20$)

MODIFICATIONS OF THE METHOD

Several authors (Casals-Stenzel et al. 1987; Dent et al. 1989a, b; Ring et al. 1992; Ukena et al. 1988) used the specific platelet activating factor receptor antagonist [^{3}H]WEB-2086 or [^{3}H]Apafant to identify and

characterize the PAF-receptors expressed on the cell surface of platelets, macrophages, and eosinophils.

Balsa et al. (1996) characterized [^{3}H]Apafant binding to the PAF receptor on rabbit platelet membranes and compared a microplate filtration system with the standard method.

REFERENCES AND FURTHER READING

Balsa D, Merlos M, Giral M, Ferrando R, García-Rafanell J, Forn J (1996) Characterization of [^{3}H]Apafant binding to PAF receptor on rabbit platelet membranes: A comparison of a microplate filtration system and a standard method. J Pharmacol Toxicol Meth 36:53–62

Bazan HE (2005) Cellular and molecular events in corneal wound healing: significance of lipid signalling. Exp Eye Res 80:453–463

Casals-Stenzel J, Muacevic G, Weber KH (1987) Pharmacological actions of WEB-2086, a new specific antagonist of platelet activating factor. J Pharmacol Exp Ther 241:974–981

Chao W, Olson MS (1993) Platelet-activating factor: Receptors and signal transduction. Biochem J 292:617–629

Dent G, Ukena D, Sybrecht GW, Barnes PJ (1989a) [^{3}H]WEB-2086 labels platelet activating factor in guinea pig and human lung. Eur J Pharmacol 169:313–316

Dent G, Ukena D, Chanez P, Sybrecht GW, Barnes J (1989b) Characterization of PAF receptors on human neutrophils using the specific antagonist WEB-2086: Correlation between binding and function. FEBS Lett 244:365–368

Handley DA (1990) Preclinical and clinical pharmacology of platelet-activating factor receptor antagonists. Medicin Res Rev 10:351–370

Hikiji H, Ishii S, Shindou H, Takato T, Shimizu T (2004) Absence of platelet-activating factor receptor protects mice from osteoporosis following ovariectomy. J Clin Invest 114:85–93

Hwang SB, Lee CSC, Cheah MJ, Shen TY (1983) Specific receptor sites for 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and guinea pig smooth muscle membrane. Biochemistry 22:4756–4763

Ishii S, Shimizu T (2000) Platelet-activating factor (PAF) receptor and genetically engineered PAF mutant mice. Progr Lipid Res 39:41–82

Izumi T, Shimizu T (1995) Platelet-activating factor: Gene expression and signal transduction. Biochem Biophys Acta 34:317–333

Ring PC, Seldon PM, Bernes PJ, Giembycz MA (1992) Pharmacological characterization of a receptor for platelet activating factor on guinea pig peritoneal macrophages using [^{3}H]Apafant, a selective and competitive platelet activating factor antagonist: Evidence that the noncompetitive behavior of apafant in functional studies relates to slow kinetics of dissociation. Molec Pharmacol 43:302–312

Seo KH, Lee HS, Jung B, Ko HM, Cvhoi JH, Park SJ, Choi IH, Lee KH, Im SY (2004) Estrogen enhances angiogenesis through a pathway involving platelet-activating factor-mediated nuclear factor-κB activation. Cancer Res 64:6482–6488

Terashita ZI, Imura Y, Nishikawa K (1985) Inhibition by CV-3988 of the binding of [^{3}H]-platelet activating factor (PAF) to the platelet. Biochem Pharmacol 34:1491–1495

Ukena D, Dent G, Birke F, Robaut C, Sybrecht G, Barnes PJ (1988) Radioligand binding of antagonists of platelet activating factor to intact human platelets. FEBS Lett 228:285–289

A.1.1.17 Endothelin

A.1.1.17.1 General Considerations

Endothelin is an endothelium-derived peptide family consisting of three peptides (ET-1, ET-2, and ET-3) with very potent and long-lasting vasoconstrictive activity (Yanagisawa et al. 1988a, b; King et al. 1989; Miller et al. 1989; Yanagisawa and Masaki 1989; Inoue et al. 1989; Shinmi et al. 1989; Vanhoutte et al. 1992; Davenport 2002; Masaki 2004).

ET-1 is processed from prepro ET-1, pro-ET-1 to big ET-1, which is converted to ET-1 by the endothelin-converting-enzyme (ECE).

Subtypes of endothelin receptors have been described (Takayanagi et al. 1991; Miyazaki et al. 1992).

Molecular characterization of the ET_A and ET_B receptors was reported by Miyazaki et al. (1992), Sakuarai et al. (1992).

In addition, the existence of a third type, ET_C, was found in *Xenopus laevis* (Karne et al. 1993).

The comparison of recombinant endothelin receptors shows different affinity rank orders to the three endothelins (Masaki et al. 1994).

Grant et al. (1997) reported the *in vitro* expression of endothelin-1 (ET-1) and the ET_A and ET_B ET receptors by prostatic epithelium and stroma.

The ET peptides not only elicit potent and long-lasting contractions of isolated strips of various blood vessels *in vitro* but also increase blood pressure *in vivo* suggesting that this peptide family may be involved in the pathogenesis of cardiovascular diseases (Simonson and Dunn 1990; Masaki 1991; Doherty 1992; Goto et al. 1996; Gray and Webb 1996; Douglas and Ohlstein 1997). Sarafotoxin S6c, originally isolated from snake venom, is an agonist which distinguishes between endothelin subtypes (Williams et al. 1991).

Endothelin is degradeted by vascular smooth muscle cells (Bernmek et al. 1996).

REFERENCES AND FURTHER READING

Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S (1990) Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348:730–732

Bernmek H, Peng KC, Angelova K, Ergul A, Puett D (1996) Endothelin degradation by vascular smooth muscle cells. Regul Pept 66:155–162

Davenport AP (2002) International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. Pharmacol Rev 54:219–226
Doherty AM (1992) Endothelin: A new challenge. J Med Chem 35:1493–1508
Douglas SA, Ohlstein EH (1997) Signal transduction mechanisms mediating the vascular actions of endothelin. J Vasc Res 34:152–164
Goto K, Hama H, Kasuya Y (1996) Molecular pharmacology and pathophysiological significance of endothelin. Jpn J Pharmacol 72:261–290
Gray GA, Webb DJ (1996) The endothelin system and its potential as a therapeutic target in cardiovascular disease. Pharmacol Ther 72:109–148
Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989) The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci, USA, 86:2863–2867
Karne S, Jayawickreme CK, Lerner MR (1993) Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. J Biol Chem 268:19126–19133
King AJ, Brenner BM, Anderson S (1989) Endothelin: a potent renal and systemic vasoconstrictor peptide. Am J Physiol 256 (Renal Fluid Electrolyte Physiol 25): F1051–F1058
Lerman A, Hildebrand FL, Margulies KB, O'Morchu B, Perrella MA, Heublein DM, Schwab TR, Burnett JC (1990) Endothelin: A new cardiovascular regulatory peptide. Mayo Clin Proc 65:1441–1455
Masaki T, Yanagisawa M, Goto K, Kimura S, Takuwa Y (1991) Cardiovascular significance of endothelin. In: Rubanyi GM (ed) Cardiovascular Significance of Endothelium-Derived Vasoactive Factors. Futura Publ Co, Inc., Mount Kisco, NY, pp 65–81
Masaki T, Vane JR, Vanhoutte PM (1994) V. International union of pharmacology nomenclature of endothelin receptors. Pharmacol Rev 46:137–142
Masaki T (2004) Historical review: endothelin. Trends Pharmacol Sci 25:219–224
Miller WL, Redfield MM, Burnett JC, Jr (1989) Integrated cardial, renal, and endocrine actions of endothelin. J Clin Invest 83:317–320
Miyazaki H, Kondoh M, Masuda Y, Watanabe H, Murakami K (1992) Endothelin receptors and receptor subtypes. In: Rubanyi GM (ed) Endothelin. Oxford University Press, New York, Oxford, pp 58–71
Rubanyi GM, Bothelho LHP (1991) Endothelins. FASEB J 5:2713–2720
Sakuarai T, Yanigasawa M, Masaki T (1992) Molecular characterization of endothelin receptor. Trends Pharmacol Sci 13:103–108
Shinmi O, Kimura S, Sawamura T, Sugita Y, Yoshizawa T, Uchiyama Y, Yanagisawa M, Goto K, Masaki T, Kanazawa I (1989) Endothelin-3 is a novel neuropeptide: Isolation and sequence determination of endothelin-1 and endothelin-3 in porcine brain. Biochem Biophys Res Commun 164:587–593
Simonson MS, Dunn MJ (1990) Cellular signaling by peptides of the endothelin gene family. FASEB J 4:2989–3000
Takayanagi R, Ohnaka K, Takasaki C, Ohashi M, Nawata H (1991) Multiple subtypes of endothelin receptors in porcine tissues: characterization by ligand binding, affinity labeling and regional distribution. Regul Peptides 32:23–37
Vanhoutte PM, Gräser T, Lüscher TF (1992) Endothelium-derived contracting factors. In: Rubanyi GM (ed) Endothelin. Oxford University Press, New York, Oxford. pp 3–16
Williams DL Jr, Jones KL, Pettibone DJ, Lis EV, Clineschmidt BV (1991) Sarafotoxin S6c: an agonist which distinguishes between endothelin subtypes. Biochem Biophys Res Commun 175:556–561
Yanagisawa M, Masaki T (1989) Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible role in cardiovascular control. Biochem Pharmacol 38:1877–1883
Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsiu Y, Yasaki Y, Goto K, Masaki T (1988a) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411–415
Yanagisawa M, Inoue A, Ishikawa T, Kasuya Y, Kimura S, Kumagaye SI, Nakasjima K, Watanabe TX, Sakakibara S, Goto K, Masaki T (1988b) Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. Proc Natl Acad Sci USA 85:6964–6967

A.1.1.17.2 Evaluation of Endothelin Activity

PURPOSE AND RATIONALE

Most investigators used isolated arteries to evaluate the activity of endothelins and derivatives. Rodman et al. (1989) compared the potency and efficacy of porcine and rat endothelin in rat aortic and pulmonary rings.

PROCEDURE

Arterial rings are obtained from male Sprague Dawley rats weighing from 300–400 g. Rats are anesthetized with 50 mg/kg i.p. pentobarbital, the chest is opened, 100 units heparin sulfate are injected into the right ventricle, and the rats are exsanguinated. Rings are then isolated from either the descending thoracic aorta or the right main pulmonary artery, cleaned of adventitia, and suspended from Grass FT03 force-displacement transducers in muscle baths containing 10 ml of physiologic salt solution of the following composition ($\times 10^{-3}$ M): $CaCl_2$ 1.80, $MgSO_4$ 0.83, KCl 5.3.6, NaCl 116.34, NaH_2PO_4 0.40, D-glucose 5.50, and $NaHCO_3$ 10.04. The solution is maintained at 37°C and bubbled with 21% O_2 and 5% CO_2. Endothelium-denuded rings are prepared by gently rubbing the intima with a roughened steel rod. Denudation is confirmed by the absence of relaxation to 10^{-5} M acetylcholine in rings precontracted with 10^{-7} M norepinephrine. Resting force is adjusted to the optimum resting tension of 0.75 g for pulmonary artery rings and 1.0 g for aortic rings. Maximum contraction to 8×10^{-2} M KCl is determined and subsequent responses to endothelin are expressed as a percentage of maximum KCl contraction for determination of maximum effectiveness or as a percentage of maximum endothelin contraction for determination of potency.

EVALUATION

Concentration-response curves are compared using the method of Carpenter (1986). Data are expressed as means ± SEM and statistical comparisons are performed using Student's *t*-test, with $P < 0.05$ considered significant.

MODIFICATIONS OF THE METHOD

Lembeck et al. (1989) studied the effects of endothelin on the cardiovascular system and on smooth muscle preparations in different species.

Reynolds and Mok (1990) studied the role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of **rat aorta** to endothelin.

Pang et al. (1990) studied the cellular mechanisms of action of endothelin in **isolated canine coronary arteries**.

Lüscher et al. (1992) used **perfused and pressurized mesenteric resistance arteries of rats** and **human internal mammary arteries** to study the interaction between endothelin and endothelium-derived relaxing factors.

Michel et al. (2003) studied the endothelin system in various animal models of pulmonary hypertension.

Advenier et al. (1990) studied the contractile activity of three endothelins (ET-1, ET-2 and ET-3) on the **human isolated bronchus**.

Wallace et al. (1989) compared the effects of endothelin-1 and endothelin-3 on the **rat stomach**.

Aldosterone secretion in cultured calf zona glomerulosa cells was stimulated by ET-1 and sarafotoxin S6b to a similar degree, but less than by angiotensin II (Gomez-Sanchez 1990).

Brock and Danthuluri (1992) used **cultured vascular smooth muscle cells** to study the cellular actions of endothelin.

Pigment dispersion in cultured dermal melanophores from *Xenopus laevis* was used as indicator of ET_C receptor mediated responses (Karne et al. 1993).

REFERENCES AND FURTHER READING

Advenier C, Sarria B, Naline E, Puybasset L, Lagente V (1990) Contractile activity of three endothelins (ET-1, ET-2 and ET-3) on the human isolated bronchus. Br J Pharmacol 100:168–172

Brock TA, Danthuluri AR (1992) Cellular actions of endothelin in vascular smooth muscle. In: Rubanyi GM (ed) Endothelin. Oxford University Press, New York, Oxford. pp 103–124

Carpenter JR (1986) A method for presenting and comparing dose-response curves. J Pharmacol Meth 15:283–287

Gomez-Sanchez CE, Cozza EN, Foecking MF, Chiou S, Ferris MW (1990) Endothelin receptor subtypes and stimulation of aldosterone secretion. Hypertension 15:744–747

Karne S, Jayawickreme CK, Lerner MR (1993) Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. J Biol Chem 268:19126–19133

Lembeck F, Decrinis M, Pertl C, Amann R, Donnerer J (1989) Effects of endothelin on the cardiovascular system and on smooth muscle preparations in different species. Naunyn-Schmiedeberg's Arch Pharmacol 340:744–751

Lüscher TF, Boulanger C, Yang Z, Dohi Y (1992) Interaction between endothelin and endothelium-derived relaxing factor(s) In: Rubanyi GM (ed) Endothelin. Oxford University Press, New York, Oxford. pp 125–136

Michel PR, Langleben D, Dupuis J (2003) The endothelin system in pulmonary hypertension. Can J Physiol Pharmacol 81:542–554

Pang DC, Johns A, Patterson K, Parker Botelho LH, Rubanyi GM (1990) Cellular mechanisms of action of endothelin in isolated canine coronary arteries. In: Rubanyi GM, Vanhoutte PM (eds) Endothelin-Derived Contracting Factors. Karger Basel, pp 66–72

Reynolds EE, Mok LLS (1990) Role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of rat aorta to endothelin. J Pharmacol Exp Ther 252:915–921

Rodman DM, McMurty IF, Peach JL, O'Brien RF (1989) Comparative pharmacology of rat and porcine endothelin in rat aorta and pulmonary artery. Eur J Pharmacol 165:297–300

Wallace JL, Keenan CM, MacNaughton WK, McKnight GW (1989) Comparison of the effects of endothelin-1 and endothelin-3 on the rat stomach. Eur J Physiol 167:41–47

A.1.1.17.3 Endothelin Receptor Antagonism in Vitro

PURPOSE AND RATIONALE

Competitive endothelin antagonists are of therapeutic interest (Ihara et al. 1991; Fujimoto et al. 1992; Fukuroda et al. 1992; Urade et al. 1992; Breu et al. 1993; Mihara and Fujimoto 1993; Sogabe et al. 1993; Warner 1994; Opgenorth 1995; Brunner 1998).

A sensitive sandwich-enzyme immunoassay for human endothelin has been established by Suzuki et al. (1989).

PROCEDURE

The ventricles of rat hearts are minced with scissors and homogenized in 7 vol of ice-cold 20 mM $NaHCO_3$ containing 0.1 mM PMSF (Phenylmethylsulfonyl fluoride), pH 7.4, with a Polytron homogenizer (Brinkman Instruments Inc., Westberg, NY). The homogenates are centrifuged at 1000 *g* for 10 min, and then the pellet discarded. The supernatant is centrifuged at 30,000 *g* for 30 min. The pellet is washed once and resuspended in Tris buffer (50 mM, pH 7.4 at 25°C) containing 0.1 mM PMSF, and stored at –80°C until use.

For binding studies (Gu et al. 1989) cardiac membranes (0.21 mg/ml as protein) are incubated

with 25 pM [^{125}I]ET-1 or [^{125}I]ET-3 (New England Nuclear) in a final assay volume of 0.1 ml in borosilicated glass tubes, containing 50 mM Tris-HCl, 0.1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 250 µg/ml bacitracin, and 10 µg/ml soybean trypsin inhibitor (pH 7.4). Binding is performed for 60 min at 37°C. The binding reaction is terminated by the addition of 2.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), followed by a rapid filtration through a Whatman GF/C glass fibre filter (presoaked in 1% polyethyleneimine) under reduced pressure. The filters are then quickly washed 4 times with 2.5 ml of the buffer. Radioactivity retained on the filter is counted.

EVALUATION

Non-specific binding is defined in the presence of ET-1. Specific binding is the difference between total and non-specific binding. K_i-values and Scatchard plots are calculated.

MODIFICATIONS OF THE METHOD

The nomenclature of endothelin receptors has been reviewed by Alexander et al. (2001).

Cain et al. (1991) described an endothelin-1 receptor binding assay for high throughput chemical screening using the clonal cell line A10 of smooth muscle cells, derived from embryonic rat thoracic aorta.

Functional endothelin/sarafotoxin receptors were described in rat heart myocytes (Galron et al. 1989) and in the rat uterus (Bousso-Mittler 1989).

Mihara and Fujimoto (1993) cultured rat aortic smooth muscle A7r5 cells expressing ET_A receptors (Takuwa et al. 1990) and human Girardi heart cells expressing ET_B receptors (Mihara and Fujimoto 1992). Receptor specificity could be demonstrated.

Mihara et al. (1994) characterized the nonpeptide endothelin receptor antagonist 97–139, both *in vitro* (rat aortic smooth muscle cells and Girardi heart cells) and *in vivo* (ET1- antagonism in pithed rats) and compared it with another endothelin receptor antagonist (BQ-123). Discrepancies between *in vitro* and *in vivo* data were explained by different plasma binding.

Aramori et al. (1993) studied the receptor-binding properties and the antagonistic activities of an endothelin antagonist in transfected Chinese ovary hamster cells permanently expressing the two ET receptor subtypes (ET_A and ET_B).

De Juan et al. (1993) characterized an endothelin receptor subtype B in the retina of rats.

Clozel et al. (1994) performed binding assay on cells or membranes from baculovirus infected insect cells that expressed recombinant ET_A or ET_B receptor, CHO cells that expressed recombinant ET_A or ET_B receptor, cultured human vascular smooth muscle cells from umbilical veins, rat mesangial cells (for ET_A), microsomal membranes from human placenta and from porcine cerebellum (for ET_{B1}) and from porcine trachea (for ET_{B2}, using BQ-3020 or sarafotoxin S6C as ligand).

Williams et al. (1995) used CHO cells expressing cloned ET_A or ET_B receptors directly in binding and functional assays without preparing membranes from them.

Reynolds et al. (1995) used CHO-K1 cells expressing recombinant human ET_B receptor, Ltk^- cells expressing human ET_A receptor and rabbit renal artery vascular smooth muscle cells expressing rabbit ET_A receptor for evaluation of an ET_A receptor antagonist.

Rat or bovine cerebella were used for differentiation of receptor subtypes (Williams et al. 1991).

Peter and Davenport (1995) proposed a selective ligand for ET_A receptors.

Ihara et al. (1992), Watakabe et al. (1992) described radioligands for endothelin (ET_B) receptors.

Vigne et al. (1996) described the properties of an endothelin-3-sensitive Eta-like endothelin receptor in brain capillary endothelial cells.

The human type-B endothelin receptor was cloned from human lung poly A+RNA and expressed in CHO cells by Chiou et al. (1997). Dissociation characteristics of endothelin receptor agonists and antagonists were determined.

Stables et al. (1997) described a bioluminescent assay for agonist activity at G-protein-coupled receptors, such as the endothelin ET_A receptor. Transient expression of apoaequorin in CHO cells and reconstitution with the cofactor coelenterazine resulted in a large, concentration dependent agonist-mediated luminescent response following cotransfection with the endothelin ET_A, angiotensin AT_{II}, TRH and neurokinin NK_1 receptors, all of which interact predominantly with the $G\alpha_q$-like phosphoinositidase-linked G-proteins.

REFERENCES AND FURTHER READING

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Aramori I, Nirei H, Shoubo M, Sogabe K, Nakamura K, Kojo H, Notsu Y, Ono T, Nakanishi S (1993) Subtype selectivity of a novel endothelin antagonist, FR139317, for the two endothelin receptors in transfected Chinese hamster ovary cells. Mol Pharmacol 43:127–131

Banasik JL, Hosick H, Wright JW, Harding JW (1991) Endothelin binding in brain of normotensive and spontaneously hypertensive rats. J Pharmacol Exp Ther 257:302–306
Bousso-Mittler D, Kloog Y, Wollberg Z, Bdolah A, Kochva E, Sokolovsky M (1989) Functional endothelin/sarafotoxin receptors in the rat uterus. Biochem Biophys Res Commun 162:952–957
Breu V, Löffler BM, Clozel M (1993) *In vitro* characterization of RO 46–2005, a novel synthetic non-peptide endothelin antagonist of ET_A and ET_B receptors. FEBS 334:210–214
Brunner HR (1998) Endothelin inhibition as a biologic target for treating hypertension. Am J Hypertens 11, Suppl III:103S-109S
Cain MJ, Garklick RK, Sweetman PM (1991) Endothelin-1 receptor binding assay for high throughput chemical screening. J Cardiovasc Pharmacol 17 [Suppl 7]:S150–S151
Chiou WJ, Magnuson SR, Dixon D, Sundy S, Opgenorth TJ, Wu-Wong JR (1997) Dissociation characteristics of endothelin receptor agonists and antagonists in cloned human type-B endothelin receptor. Endothelium 5:179–189
Clozel M, Breu V, Gray GA, Kalina B, Löffler BM, Burri K, Cassal JM, Hirth G, Müller M, Neidhart W, Ramuz H (1994) Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. J Pharmacol Exp Ther 270:228–235
De Juan JA, Moya FJ, Garcia de Lacoba M, Fernandez-Cruz A, Fernandez-Durango R (1993) Identification and characterization of endothelin receptor subtype B in rat retina. J Neurochem 61:1113–1119
Fujimoto M, Mihara S, Nakajima S, Ueda M, Nakamura M, Sakurai K (1992) A novel, non-peptide endothelin antagonist, isolated from bayberry, *Myrica cerifera*. FEBS Lett 305:41–44
Fukuroda T, Nishikibe M, Ohta Y, Ihara M, Yano M, Ishikawa K, Fukami T, Ikemoto F (1991) Analysis of responses to endothelins in isolated porcine blood vessels by using a novel endothelin antagonist, BQ-153. Life Sci 50: PL107–PL112
Galron R, Klog Y, Bdolah A, Sokolovsky M (1989) Functional endothelin/sarafotoxin receptors in rat heart myocytes: structure-activity relationships and receptor subtypes. Biochem Biophys Res Commun 163:936–943
Gu XH, Calsey D, Nayler W (1989) Specific high-affinity binding sites for ^{125}I-labelled porcine endothelin in rat cardiac membranes. Eur J Pharmacol 167:281–290
Hiley CR (1995) Endothelin receptor ligands. Neurotransmissions/2:1–6
Ihara M, Saeki T, Fukuroda T, Kimura S, Ozaki S, Patel AC, Yano M (1992) A novel radioligand [^{125}I]BQ-3020 selective for endothelin (ET_B) receptors. Life Sci 51:PL47–52
Mihara SI, Fujimoto M (1992) Non-isopeptide-selective endothelin receptors in human Girardi heart cells. Life Sci 50:219–226
Mihara S, Fujimoto M (1993) The endothelin ET_A receptor-specific effects of 50–235, a nonpeptide endothelin antagonist. Eur J Pharmacol 246:33–38
Mihara S, Nakajima S, Matumura S, Kohnoike T, Fujimoto M (1994) Pharmacological characterization of a potent nonpeptide endothelin receptor antagonist, 97–139. J Pharmacol Exp Ther 268:1122–1128
Opgenorth TJ (1995) Endothelin receptor antagonism. Adv Pharmacol 33:1–65
Peter MG, Davenport AP (1995) Selectivity of [^{125}I]-PD151242 for human rat and porcine ET_A receptors in the heart. Br J Pharmacol 114:297–302
Reynolds EE, Keiser JA, Haleen SJ, Walker DM, Olszewski B, Schroeder RL, Taylor DG, Hwang O, Welch KM, Flynn MA, Thompson DM, Edmunds JJ, Berryman KA, Plummer M, Cheng XM, Patt WC, Doherthy AM (1995) Pharmacological characterization of PD 156707, an orally active ET_A receptor antagonist. J Pharmac Exp Ther 273:1410–1417
Sogabe K, Nirei H, Shoubo M, Nomoto A, Ao S, Notsu Y, Ono T (1993) Pharmacological profile of FR139317, a novel, potent endothelin ET_A receptor antagonist. J Pharm Exp Ther 264:1040–1046
Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M, Rees S (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal Biochem 252:115–126
Suzuki N, Matsumoto H, Kitada C, Msaki T, Fujino M (1989) A sensitive sandwich-enzyme immunoassay for human endothelin. J Immunol Meth 118:245–250
Takuwa Y, Kasuya Y, Takuwa N, Kudo M, Yanagisawa M, Goto K, Masaki T, Yamashita K (1990) Endothelin receptor is coupled to phospholipase C via a pertussis toxin-insensitive guanine nucleotide-binding regulatory protein in vascular smooth muscle cells. J Clin Invest 85:653–658
Urade Y, Fujitani Y, Oda K, Watakabe T, Umemura I, Takai M, Okada T, Sakata K, Karaki H (1992) An endothelin receptor-selective antagonist: IRL 1038, [Cys^{11}-Cys^{15}]-endothelin-1(11–21) FEBS Lett 311:12–16
Vigne P, Desmarets J, Guedin D, Frelin Ch (1996) Properties of an endothelin-3-sensitive Eta-like endothelin receptor in brain capillary endothelial cells. Biochem Biophys Res Commun 220:839–842
Warner TD (1994) Endothelin receptor antagonists. Cardiovasc Drug Dev 12:105–122
Watakabe T, Urade Y, Takai M, Umemura I, Okada T (1992) A reversible radioligand specific for the ET_B receptor: [^{125}I]Tyr^{13}-Suc-[Glu^{9},$Ala^{11,15}$]-endothelin-1(8–21), [^{125}I]IRL 1620. Biochem Biophys Res Commun 185:867–873
Williams DL Jr, Murphy KL, Nolan NA, O'Brien JA, Pettibone DJ, Kivlighn SD, Krause SM, Lis EV Jr, Zingaro GJ, Gabel RA, Clayton FC, Siegl PKS, Zhang K, Naue J, Vyas K, Walsh TF, Fitch KJ, Chakravarty PK, Greenlee WJ, Clineschmidt BV (1965) Pharmacology of L-754,142, a highly potent, orally active, nonpeptidyl endothelin antagonist. J Pharmac Exp Ther 275:1518–1526
Williams DL Jr, Jones KL, Pettibone DJ, Lis EV, Clineschmidt BV (1991) Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem Biophys Res Commun 175:556–561

A.1.1.17.4 Endothelin Receptor Antagonism in Vivo

PURPOSE AND RATIONALE

Various pharmacological models have been used for the characterization of endothelins and endothelin antagonists, such as the isolated porcine coronary artery. (Hickey et al. 1985; Yanagisawa et al. 1988, 1989; Inoue et al. 1989; Kimura et al. 1989; Ihara et al. 1991; Fukuroda et al. 1991).

Since the smooth musculature is considered to contain mainly ET_A receptors the preparation is used to test ET_A antagonists.

PROCEDURE

Left anterior descending coronary arteries are isolated from fresh porcine hearts. Connective tissues and adherent fat are removed. For removal of vascular endothelium, the intimal surface of spiral strips is rubbed gently with filter paper. The endothelium-denuded arteries are cut into spiral strips about 10 mm long and 1 mm wide. Each strip is suspended in an organ bath containing Krebs-Henseleit solution bubbled with 95% O_2/5% CO_2 at 37°C. After equilibration, reference contraction is isometrically obtained with 50 mM KCl. Concentration-response curves for ET-1 are obtained by cumulative additions of ET-1. Antagonists are added 20 min before the cumulative additions of ET-1.

EVALUATION

The pA_2 values and slopes are obtained by analysis of Schild plots.

MODIFICATIONS OF THE METHOD

Opgenorth et al. (1996) characterized an orally active and highly potent ET_A-selective receptor antagonist by *in vitro* and *in vivo* methods.

Calo et al. (1996) investigated three **rabbit vessels, the carotid, the pulmonary artery, and the jugular vein** to identify vascular monoreceptor systems, either ET(A) or ET(B), for structure-activity studies of endothelins and their antagonists.

Vedernikov et al. (1993) used **rings of the left circumflex coronary artery** from **dogs** which were denuded of endothelium and exposed to anoxic periods. August et al. (1989), Urade et al. (1992) used **rat aortic smooth muscle denuded of the epithelium** and Sogabe et al. (1993) **spirally cut strips of rabbit aorta**.

Williams et al. (1995) used **rat aorta, rabbit iliac and pulmonary artery** for contractile assays, and **anesthetized ferrets and conscious normotensive dogs** as *in vivo* models to characterize a nonpeptidyl endothelin antagonist.

Itoh et al. (1993) studied the preventive effect of an ET_A receptor antagonist on **experimental cerebral vasospasm in dogs** using a two-hemorrhage model of subarachnoid hemorrhage. Clozel et al. (1993) performed similar experiments in rats.

The **vasodilating** effect in the **isolated perfused rat mesentery** which is found after infusion of rat endothelin (Warner et al. 1989) and after the selective ET_B receptor agonist sarafotoxin S6c (Williams et al. 1991) can be antagonized by an endothelin receptor antagonist (Clozel et al. 1993).

Ercan et al. (1996) found an increase of digoxin-induced ectopic ventricular complexes by endothelin peptides in **isolated guinea pig hearts**, which could be antagonized by an endothelin-A receptor antagonist.

The **endothelin-induced sustained increase of blood pressure** in anesthetized rats was studied by Yanagisawa et al. (1988), Inoue et al. (1989), Ihara et al. (1991). Intravenous bolus injection of endothelin causes a biphasic blood pressure response: a transient decrease, probably mediated from the release of vasodilator mediators (prostacyclin and EDRF), and a sustained increase (Rubanyi and Bothelho 1991).

Nishikibe et al. (1993) examined the **antihypertensive effect** of an endothelin antagonist in a genetic hypertensive model (**stroke-prone spontaneously hypertensive rats**).

Watanabe et al. (1995) characterized the pharmacological profile of a non-selective endothelin receptor antagonist and studied the **inhibition of myocardial infarct size** in rats.

The contractile activity of the **isolated guinea pig trachea without epithelium** and of the guinea pig longitudinal muscle was used by Urade et al. (1992) for determination of **ET_B receptor** mediated responses.

Spinella et al. (1991) assessed bioactivity of a specific endothelin-1 antagonist in an **isolated perfused guinea pig lung** preparation in which pulmonary artery pressure was monitored.

Gosselin et al. (2002) demonstrated the effects of a selective ET_A-receptor antagonist in murine models of allergic asthma.

Tabrizchi and Ford (2003) studied the haemodynamic effects of the endothelin receptor antagonist tezosentan in anaesthetized rats treated with tumor necrosis factor-alpha.

REFERENCES AND FURTHER READING

August M, Delaflotte S, Chabrier PE, Braquet P (1989) Comparative effects of endothelin and phorbol 12–13- dibutyrate in rat aorta. Life Sci 45:2051–2059

Calo G, Gratton JP, Orleans-Juste P, Regoli D (1996) Pharmacology of endothelins: vascular preparations for studying ET(A) and ET(B) receptors. Mol Cell Biochem 154:31–37

Clozel M, Breu V, Burri K, Cassal JM, Fischli W, Gray GA, Hirth G, Löffler BM, Müller M, Neidhart W, Ramuz H (1993) Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. Nature 365:759–761

Ercan ZS, Ilhan M, Kilinç M, Türker RK (1996) Arrhythmogenic action of endothelin peptides in isolated perfused whole hearts from guinea pigs and rats. Pharmacology 53:234–240

Gosselin M, Goulet S, Wu-Wong JR, Wessale JL, Opgenorth TJ, Boulet LP, Battistini B (2002) Effects of a selec-

tive ET_A-receptor antagonist (ABT-627), in murine models of allergic asthma: demonstration of mouse strain specificity. Clin Sci 103 [Suppl 48]:367S–370S
Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, Fukami TG, Ishikawa K, Nishikibe M, Yano M (1991) Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. Life Sci 50:247–255
Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989) The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci, USA, 86:2863–2867
Itoh S, Sasaki T, Ide K, Ishikawa K, Nishikibe M, Yano M (1993) A novel ET_A receptor antagonist, BQ-485, and its preventive effect on experimental cerebral vasospasm in dogs. Biochem Biophys Res Commun 195:969–975
Kimura S, Kasuya Y, Sawamura T, Shinmi O, Sugita Y, Yanagisawa M, Goto K, Masaki T (1989) Conversion of big endothelin-1 to 21-residue endothelin-1 is essential for expression of full vasoconstrictor activity: Structure-activity relationships of big endothelin-1. J Cardiovasc Pharmacol 13 (Suppl 5):S5–S7
Nishikibe M, Tsuchida S, Okada M, Fukuroda T, Shimamoto K, Yano M, Ishikawa K, Ikemoto F (1993) Antihypertensive effect of a newly synthesized endothelin antagonist, BQ123, in a genetic hypertensive model. Life Sci 52:717–724
Opgenorth TJ, Adler AL, Calzadilla SV, Chiou WJ, Daytin BT, Dixon DB, Gehrke LJ, Hernandez L, Magnuson SR, Marsh KC, Novosad EI, von Geldern TW, Wessale JL, Winn M, Wu-Wong JR (1996) Pharmacological characterization of A-127722: an orally active and highly potent ET_A-selective receptor antagonist. J Pharmacol Exp Ther 276:473–481
Shimamoto H, Kwa CY, Daniel EE (1992) Pharmacological assessment of Ca^{2+}-dependence of endothelin-1-induced response in rat aorta. Eur J Pharmacol 216:225–233
Spinella MJ, Malik AB, Evertitt J, Andersen TT (1991) Design and synthesis of a specific endothelin-1 antagonist: effects on pulmonary vasoconstriction. Proc Natl Acad Sci USA 88:7443–7446
Tabrizchi R, Ford CA (2003) Haemodynamic effects of endothelin receptor antagonist, tezosentan, in tumour necrosis factor-alpha treated anaesthetized rats. Naunyn-Schmiedebergs Arch Pharmacol 367:156–167
Urade Y, Fujitani Y, Oda K, Watakabe T, Umemura I, Takai M, Okada T, Sakata K, Karaki H (1992) An endothelin receptor-selective antagonist: IRL 1038, [Cys^{11}-Cys^{15}]-endothelin-1(11–21). FEBS Lett 311:12–16
Vedernikov YP, Goto K, Vanhoutte PM (1993) The ET_A antagonist BQ-123 inhibits anoxic contractions of canine coronary arteries without endothelium. J Cardiovasc Pharmacol 22, Suppl 8:S252–S265
Warner TD, de Nucci G, Vane JR (1989) Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. Eur J Pharmacol 159:325–326
Watanabe T, Awane Y, Ikeda S, Fujiwara S, Kubo K, Kikuchi T, Kusomoto K, Wakimasu M, Fujino M (1995) Pharmacological profile of a non-selective ET_A and ET_B receptor antagonist, TAK-044 and the inhibition of myocardial infarct size in rats. Br J Pharmacol 114:949–954
Wilkes LC, Boarder MR (1991) Characterization of the endothelin binding site on bovine adrenomedullary chromaffin cells: Comparison with vascular smooth muscle cells. Evidence for receptor heterogeneity. J Pharmacol Exp Ther 256:628–633
Williams DL Jr, Murphy KL, Nolan NA, O'Brien JA, Pettibone DJ, Kivlighn SD, Krause SM, Lis EV Jr, Zingaro GJ, Gabel RA, Clayton FC, Siegl PKS, Zhang K, Naue J, Vyas K, Walsh TF, Fitch KJ, Chakravarty PK, Greenlee WJ, Clineschmidt BV (1965) Pharmacology of L-754,142, a highly potent, orally active, nonpeptidyl endothelin antagonist. J Pharmac Exp Ther 275:1518–1526
Yanagisawa M, Masaki T (1989) Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible role in cardiovascular control. Biochem Pharmacol 38:1877–1883
Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsiu Y, Yasaki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411–415

A.1.1.17.5 Quantitative Autoradiographic Localization of Endothelin-1 Receptor

PURPOSE AND RATIONALE

The endothelin-1 (ET-1) receptor can be quantified in tissue, such as in rat hearts with chronic infarction after left coronary ligation, by computerized *in vitro* autoradiography (Kohzuki et al. 1996)

PROCEDURE

Myocardial infarction is induced in Wistar rats by left coronary artery ligation (see A.3.2.2). After various time intervals (1–8 months) the animals are decapitated, the hearts rapidly removed, and snap-frozen in isopentane at –40°C. Frozen section (20 µm) are cut in a cryostat at –20°C. The sections are thaw-mounted onto gelatin-coated slides, dried in a desiccator for 2 h at 4°C and then stored at –80°C.

Quantitative autoradiography

Radioligand: Endothelin-1 is iodinated with ^{125}Iodine using Iodogen (Pierce Chemical Co, IL, USA)

^{125}I-ET-1 binding: The sections are preincubated for 15 min at 20°C in 20 mmol/L Hepes buffer, pH 7.4, containing 135 mmol/L NaCl, 2 mmol/L $CaCl_2$, 0.2% BSA, and 0.01% bacitracin. The sections are then incubated with 11.1 KBq/ml ^{125}I-ET-1 in the same buffer for 60 min at 20°C. Nonspecific binding is determined in the presence of 10^{-6} mol/L ET-1. Binding isotherms are determined using a set of serial sections incubated with 10^{-12} to 10^{-6} mol/L unlabelled ET-1 for 60 min.

After incubation, the sections are rapidly dried under a stream of cold air, placed in X-ray cassettes, and exposed to Agfa Scopix CR3 X-ray film for 12–72 h at room temperature. After exposure, the sections are fixed in formaldehyde and stained with hematoxylin and eosin. The optical density of the X-ray films is

quantified using an imaging device controlled by a personal computer.

EVALUATION

The optical density of the autoradiographs is calibrated in terms of the radioactivity density in dpm/mm^2 with reference standards maintained through the procedure. The apparent binding site concentration (B_{max}) and binding affinity constant (K_A) in all the areas (excluding coronary arteries) of the right ventricle, intraventricular septum, the infarcted area in the left ventricle and the non-infarcted area in the left ventricle are estimated by an iterative non-linear model-fitting computer program LIGAND (Munson and Rodbard 1980).

REFERENCES AND FURTHER READING

Kohzuki M, Johnston CI, Chai SY, Casley DJ, Mendelson FAO (1989a) Localization of endothelin receptors in rat kidney. Eur J Pharmacol 160:193–194

Kohzuki M, Johnston CI, Chai SY, Casley DJ, Rogerson F, Mendelson FAO (1989b) Endothelin receptors in rat adrenal gland visualized by quantitative autoradiography. Clin Exp Pharmacol Physiol 16:239–242

Kohzuki M, Chai SY, Paxinos J, Karavas A, Casley DJ, Johnston CI, Mendelson FAO (1989c) Localization and characterization of endothelin receptor binding sites in rat brain visualized by *in vitro* autoradiography. Neuroscience 42:245–260

Kohzuki M, Kanazawa M, Yoshida K, Kamimoto M, Wu XM, Jiang ZL, Yasujima M Abe K, Johnston CI, Sato T (1996) Cardiac angiotensin converting enzyme and endothelin receptor in rats with chronic myocardial infarction. Jpn Circ J 60:972–980

Munson PJ, Rodbard D (1980) LIGAND, a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239

A.1.1.17.6 Inhibition of Endothelin Converting Enzyme

PURPOSE AND RATIONALE

Endothelin converting enzyme inhibitors suppress the biosynthesis of endothelin are therefore potential antihypertensive drugs (De Lombaert et al. 1994; Trapani et al. 1995; Morita et al. 1994; Bihovsky et al. 1995; Claing et al. 1995; Descombes et al. 1995; Chackalamannil et al. 1996; Jeng 1997; Jeng and De Lombaert 1997; Brunner 1998).

Purification of rat and porcine endothelin converting enzyme (ECE) was reported by Ohmaka et al. (1993), Takahashi et al. 1993). Molecular cloning and characterization of the enzyme ECE-1 was performed from rat (Shimada et al. 1994), bovine (Ikura et al. 1994; Schmidt et al. 1994; Xu et al. 1994), and human tissue (Schmidt et al. 1994; Shimada et al. 1995; Yorimitsu et al. 1995).

A second enzyme, termed ECE-2, was cloned (Emoto and Yanagisawa 1995).

Walkden and Turner (1995) described the expression of endothelin converting enzyme and related membrane peptidases, e. g., the endopeptidase E-24.11, in the human endothelial cell line EA.hy926.

IN VITRO ASSAY

A rapid and selective *in vitro* assay for endothelin-converting enzyme was described by Fawzi et al. (1994). The assay is based on the quantitative determination of [^{125}I]endothelin-1 released from (3-[^{125}I]iodotyrosyl^{13})big endothelin-1 by binding to the membrane bound endothelin receptor.

PROCEDURE

For the **preparation of lung membranes**, frozen guinea pig lungs are weighed and homogenized in 10 times gram tissue weight of solution A (50 mM Tris-HCl, pH 7.4, 025 M sucrose, and 2 mM EDTA) using a Polytron tissue homogenizer. Homogenization is repeated 4 times with 5- to 8-min intervals between homogenization. Homogenates are spun for 30 min at 2000 *g*. Supernatants containing membranes are carefully decanted and saved. Pellets are rehomogenized in solution A and homogenates are spun at 2000 *g* for 30 min. Supernatants are removed, mixed with supernatants from the first spin and spun at 100,000 *g* for 60 min. Pellets containing membranes are suspended in solution B (10 mM Tris-HCl, pH 7.4, and 0.125 M sucrose) using a Dounce homogenizer. Samples are divided into 1-ml fractions, rapidly frozen in a dry ice-methanol bath, and stored at −80°C.

Rat liver membranes are prepared with the same method and further purified over a sucrose step gradient. The membranes are suspended in solution C (10 mM Tris-HCl, pH 7.4) containing 44% sucrose at a protein concentration of 2 mg/ml. Samples of 25 ml are placed in ultraclear centrifuge tubes for the Beckman SW 28 rotor, overlaid with 10 ml solution C containing 42.3% sucrose, and spun for 2 h at 27,000 rpm (100,000 *g*). Top layers containing membrane are collected and diluted with solution C to obtain an 8% sucrose concentration. Samples are spun in a 45 Ti rotor (100,000 *g*) for 1 h. Supernatants are discarded. Pellets containing membrane are suspended in solution B, divided into 1-ml samples, rapidly frozen in dry ice-methanol bath, and stored at −80°C.

For the **endothelin (ET) binding assay**, membrane preparations are incubated with selected concentrations of [^{125}I]endothelin-1 (final reaction volume = 500 µl) in a solution D containing 60 mM Tris-

HCl, pH 7.4, 150 mM NaCl and 6 mg/ml BSA for 90 min at 37°C. Reactions are terminated by the addition of 4 ml of solution E containing 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl at 4°C followed by rapid filtration on Whatman GF/B glass microfiber filters. Filters are presoaked for 1 h at 4°C in a solution containing 50 mM Tris-HCl, pH 7.4, 10 mg/ml BSA and 0.1% sodium azide. Test tubes and filters are washed four times with 4 ml of solution E at 4°C, and radioactivity retained on the filters is counted in a gamma counter. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture.

For the **endothelin converting enzyme assay**, samples containing 10 µg of protein are incubated in a solution containing 50 mM Tris-HCl, pH 7.0, 100 mM NaCl and 5 mg/ml BSA in a final volume of 100 µl. Conversion reactions are initiated by addition of [^{125}I]big endothelin-1 to obtain a final concentration of 500 pM. Samples are incubated for 2 h at 37°C. To measure [^{125}I]endothelin-1 released from [^{125}I]big endothelin-1 conversion, 50 µg of purified rat liver membranes (as a source of ET receptors) is added to the reaction mixture, and reaction volume is adjusted to 500 µl and solution composition is adjusted to that of solution D of the endothelin binding assay. Following a 90-min incubation at 37°C to reach equilibrium in binding, reactions are terminated by addition of a solution E at 4°C followed by rapid filtration on Whatman GF/B glass microfiber filters. Nonspecific binding is determined in the presence of 1 µM unlabeled ET-1 in the reaction mixture. Specific ET-1 binding is used as an index of endothelin converting enzyme activity.

To test the effect of endothelin converting enzyme inhibitors, endothelin converting enzyme assays are carried out in the presence of desired concentrations of the compounds.

EVALUATION

Endothelin converting enzyme activity in the presence of compounds is expressed as a percentage of control endothelin converting enzyme activity in the membrane preparation which is determined simultaneously. The concentration of compounds producing a 50% inhibition of endothelin converting enzyme activity (IC_{50} values) is determined from a plot of the percentage of control endothelin converting enzyme activity versus log concentration of compounds.

IN VIVO ASSAY

Procedure

Male Sprague Dawley rats weighing 300–400 g are anesthetized with ether, spinalized and placed under artificial respiration. The vagus nerves are cut and the carotid arteries ligated. A catheter is placed in one of the carotid arteries to allow measurement of arterial blood pressure. The second catheter in placed into the penile vein to allow infusion or injection of drugs. After stabilization, the animals receive a first injection of either ET-1, big ET-1, norepinephrine, angiotensin I or AT II, The pressor responses are recorded and after return to the baseline, a second injection of the agonist is given either in the presence or the absence of the inhibitor.

EVALUATION

Data are calculated as mean ± SEM. Student's *t*-test for paired and unpaired observations is used to analyze the results.

MODIFICATIONS OF THE METHOD

Little et al. (1994) developed a two-step protocol for high-throughput assays of endothelin converting enzyme activity. Human umbilical vein and human aorta endothelial cells were found to preferentially convert the big endothelin-1 isopeptide through a membrane-bound, thiorphan-insensitive, and phophoramidon-sensitive zinc metalloendopeptidase. Endothelins are quantified by a separate step using either enzyme immunoassays or radioceptor assays in 96-well formats. The method can be used to either characterize ECE from different tissues or screen for inhibitors of a specific ECE activity.

McMahon et al. (1993) tested the effects of endothelin converting enzyme inhibitors and endothelin receptor subtype A antagonists on blood pressure in spontaneously and renal hypertensive rats.

Changes of vascular resistance in isolated perfused kidneys were used by Descombes et al. (1995) to characterize a selective inhibitor of big ET-1 responses. The studies were performed on kidneys taken from adult male Wistar rats (300–400 g). The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the left kidney was prepared for infusion with Tyrode solution. The changes in renal vascular resistance were recorded as changes in perfusion pressure monitored at constant flow (6 ml/min). After stabilization, a bolus injection of ET-1 or big ET-1 was administered and the resulting pressure responses were recorded. On return to baseline levels, a second injection of the endothelins was given either under control conditions or in presence of the putative enzyme inhibitor.

Because increasing evidence implicates that endothelin plays a role in the pathophysiology of cerebral insults, Kwan et al. (1997) studied the prevention

and reversal of cerebral vasospasm in an experimental model of subarachnoid hemorrhage. Three ml of arterial blood was withdrawn from the ear artery of rabbits and injected into the cisterna magna under anesthesia. Drugs were administered either before or 24 h after this procedure. Forty-eight hours later, the animals were anesthetized again and perfusion fixation was performed with Hank's balanced salt solution followed by a mixture with 2% paraformaldehyde and 2.5% glutaraldehyde. Cross sections of the basilar arteries were analyzed by computer-assisted morphometry.

A review on the knowledge of molecular pharmacology of endothelin converting enzymes was given by Turner and Murphy (1996).

Johnson and Ahn (2000) developed an internally quenched fluorescent substrate selective for endothelin-converting enzyme-1.

Luciani et al. (2001) described highly sensitive and selective fluorescence assays for rapid screening of endothelin-converting enzyme inhibitors.

REFERENCES AND FURTHER READING

Bigaud M, Hauss B, Schalk C, Jauch MF, D'Orchymont H (1994) Structure activity relationship of phosphoramidon derivatives for *in vivo* endothelin converting-enzyme inhibition. Fundam Clin Pharmacol 8:155–161

Bihovsky R, Levinson BL, Loewi R, Erhardt PW, Polokoff MA (1995) Hydroxamic acids as potent inhibitors of endothelin-converting enzyme from human bronchiolar smooth muscle. J Med Chem 38:2119–2129

Brunner HR (1998) Endothelin inhibition as a biologic target for treating hypertension. Am J Hypertens 11:103S–109S

Chackalamannil S, Chung S, Stamford AW, McKittrick BA, Wang Y, Tsai H, Cleven R, Fawzi A, Czarniecki M (1996) Highly potent and selective inhibitors of endothelin converting enzyme. Bioorganic Medic Chem Lett 6:1257–1260

Claing A, Neugebauer W, Yano M, Rae GA, D'Orléans-Juste P (1995) [Phe^{22}]-big endothelin-1[19–37]: a new and potent inhibitor of the endothelin-converting enzyme. J Cardiovasc Pharmacol 26/Suppl 3:S72–S74

De Lombaert S, Ghai RD, Jeng AY, Trapani AJ, Webb RL (1994) Pharmacological profile of a non-peptide dual inhibitor of neutral endopeptidase 24.11 and endothelin converting enzyme. Biochem Biophys Res Commun 204:407–412

Descombes JJ, Mennecier P, Versluys D, Barou V, de Nanteuil G, Laubie M, Verbeuren TJ (1995) S 17162 is a novel selective inhibitor of big ET-1 responses in the rat. J Cardiovasc Pharmacol 26/Suppl 3:S61–S64

Emoto N, Yanagisawa M (1995) Endothelin-converting enzyme is a membrane bound, phosphoramidon sensitive metalloprotease with acidic pH optimum. J Biol Chem 270:15262–15268

Fawzi AB, Cleven RM, Wright DL (1994) A rapid and selective endothelin-converting enzyme assay: characterization of a phosphoramidon-sensitive enzyme from guinea pig lung membrane. Anal Biochem 222:342–350

Ikura T, Sawamura T, Shiraki T, Hosokawa H, Kido T, Hoshikawa H, Shimada K, Tanzawa T, Kobayashi S, Miwa S, Masaki T (1994) cDNA cloning and expression of bovine endothelin-converting enzyme. Biochem Biophys Res Commun 203:1417–1422

Jeng AY (1997) Therapeutic potential of endothelin converting enzyme inhibitors. Exp Opin Ther Patents 7:1283–1295

Jeng AY, DeLombaert S (1997) Endothelin converting enzyme inhibitors. Curr Pharmac Design 3:597–614

Johnson GD, Ahn K (2000) Development of an internally quenched fluorescent substrate selective for endothelin-converting enzyme-1. Anal Biochem 286:112–118

Kwan AL, Bavbek M, Jeng AY, Maniara W, Toyoda T, Lappe RW, Kassell NF, Lee KS (1997) Prevention and reversal of cerebral vasospasm by an endothelin converting enzyme inhibitor, CGS 26303, in an experimental model of subarachnoid hemorrhage. J Neurosurg 87:281–286

Little DK, Floyd DM, Tymiak AA (1994) A rapid and versatile method for screening endothelin converting enzyme activity. J Pharm Toxicol Meth 31:199–205

Luciani N, de Rocquigny H, Turcaud S, Romieu A, Roques BP (2001) Highly sensitive and selective fluorescence assays for rapid screening of endothelin-converting enzyme inhibitors. Biochem J 356:813–819

McMahon EG, Palomo MA, Brown MA, Bertenshaw SR, Carter JS (1993) Effect of phosphoramidon (endothelin converting enzyme inhibitor) and BQ-123 (endothelin receptor subtype A antagonist) on blood pressure in hypertensive rats. Am J Hypertens 6:667–673

Morita A, Nomizu M, Okitsu M, Horie K, Yokogoschi H, Roller PP (1994) D-Val^{22} containing human big endothelin-1 analog, [D-Val^{22}]Big ET-1[16–38], inhibits the endothelin converting enzyme. FEBS Lett 353:84–88

Ohnaka K, Takayanagi R, Nishikawa M, Haji M, Nawata H (1993) Purification and characterization of phosphoramidon sensitive endothelin-converting enzyme in porcine aortic endothelium. J Biol Chem 268:26759–26766

Opgenorth JJ, Wu-Wong JR, Shiosaki K (1992) Endothelin-converting enzymes. FASEB J 6:2653–2659

Schmidt M, Kröger B, Jacob E, Seulberger H, Subkowski T, Otter R, Meyer T, Schmalzling G, Hillen H (1994) Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). FEBS Lett 356:238–243

Shimada K, Takahashi M, Tanzawa K (1994) Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. J Biol Chem 269:18275–18278

Shimada K, Matsushita Y, Wakabayashi K, Takahashi M, Matsubara A, Iijima Y, Tanzawa K (1995) Cloning and functional expression of human endothelin-converting enzyme cDNA. Biochem Biophys Res Commun 207:807–812

Takahashi M, Matsiushita Y, Iijima Y, Tanzawa K (1993) Purification and characterization of endothelin-converting enzyme from rat lung. J Biol Chem 268:21394–21398

Turner AJ, Murphy LJ (1996) Molecular pharmacology of endothelin converting enzymes. Biochem Pharmacol 51:91–102

Trapani AJ, De Lombaert S, Kuzmich S, Yeng AY (1995) Inhibition of big ET-1-induced pressure response by an orally active dual inhibitor of endothelin-converting enzyme and neutral endopeptidase 24.11. J Cardiovasc Pharmacol 26/Suppl 3:S69–S71

Walkden BJ, Turner AJ (1995) Expression of ECE and related membrane peptidases in the EA.hy926 cell line. J Cardiovasc Pharmacol 26/Suppl 3:S50–S60

Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M (1994) ECE-1: a membrane bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell 78:473–485

Yorimitsu K, Moroi K, Inagaki N, Saito T, Masuda Y, Masaki T, Seino S, Kimura S (1995) Cloning and sequencing of a human endothelin converting enzyme in renal adenocarcinoma (ACHN) cells producing endothelin-2. Biochem Biophys Res Commun 208:721–927

A.1.1.18 Nitric Oxide

A.1.1.18.1 General Considerations on Nitric Oxide

PURPOSE AND RATIONALE

The endothelium releases a labile, diffusable vasorelaxing substance that has been termed endothelium-derived relaxing factor = EDRF endothelium-derived relaxing factor. (Furchgott and Zawadszki 1980). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor (Palmer et al. 1987; Vanhoutte 1999).

Nitric oxide plays a role in a wide range of physiological processes including regulation of blood flow and arterial pressure via endothelium-dependent relaxation of blood vessels (Rees et al. 1989; Moncada et al. 1991; Umans and Levi 1995; Huraux et al. 1999; McIntyre et al. 1999; Zanzinger 1999; Hropot et al. 2003), ischemia/reperfusion injury (Gao et al. 2002; Schulz et al. 2004), peripheral nitregric transmission at smooth muscle (Rand and Li 1995), intracellular communication in the CNS with activation of guanylyl cyclase in target neurons (Southam and Garthwaite 993), experimental stroke (Willmot et al. 2005), learning and memory (Susswein et al. 2004), in neurogenic inflammation (Kajekar et al. 1995), in the regulation of leukocyte recruitment (Hickey 2001), and macrophage defense mechanisms following exposure to bacterial products (Förstermann et al. 1992; Förstermann and Kleinert 1995; Knowles and Moncada 1994). Fiorucci et al. (2002) discussed the effects of nitric oxide-releasing NSAIDs.

NO-donor drugs, such as sodium nitrite, sodium nitroprusside, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), 3-morpholino-sydnonimine (SIN-1) are used as vasodilators (Schör et al. 1989; Megson 2000). N^G-Nitro-L-arginine was described as an antagonist of endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release (Moore et al. 1990; Lamontagne et al. 1991). Ribero et al. (1992) proposed inhibition of nitric oxide synthesis by long-term treatment of rats with nitro-L-arginine as a new model of arterial hypertension.

Excessive production of NO damages DNA and activates poly(ADP-ribose)polymerase (PARP) (Pieper et al. 1999). In cases of massive NO production, neurons enter the PARP-suicide pathway. NO damages DNA via to major pathways: the first involves nitrosation of primary or secondary amines and nucleic acid bases, whereas the second involves the combination of NO with superoxide to from peroxinitrite (Szabó et al. 1996, 1997). The most likely reactive oxidant intermediate responsible for DNA breakage is peroxynitrous acid which rapidly oxidizes sulphydryl groups, and also nitrates and hydroxylates aromatic compounds including tyrosine, tryptophan, and guanosine (Halliwell 1997). Downstream DNA damage that follows excessive NO production results in significant activation of poly(ADP-ribose)polymerase which leads to rapid energy depletion and cell death (Feihl et al. 2001).

Davis et al. (2001) reviewed the non-3′,5′-cyclic-guanosine-monophosphate-mediated effects of NO including modifications of proteins, lipids, and nucleic acids.

Andreadis et al. (2003) described oxidative and nitrosative events in asthma.

REFERENCES AND FURTHER READING

Andreadis AA, Hazen SL, Comhair SAA, Erzurum SC (2003) Oxidative and nitrosative events in asthma. Free Radical Biol Med 15:213–225

Davis KL, Martin E, Turko IV, Murad F (2001) Novel effects of nitric oxide. Annu Rev Pharmacol Toxicol 41:203–236

Feihl F, Waeber B, Liaudet L (2001) Is nitric overproduction the target of choice for management of septic shock? Pharmacol Ther 91:179–213

Fiorucci S, Antonelli E, Burgaud JL, Morelli A (2002) Nitric oxide-releasing NSAIDs. Drug Safety 24:801–811

Förstermann U, Kleinert H (1995) Nitric oxide synthase: Expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch Pharmacol 352:351–364

Förstermann U, Schmidt HHHW, Pollock JS, Sheng H, Mitchell JA, Warner TD, Murad F (1992) Characterization and classification of constitutive and inducible isoforms of nitric oxide synthase in various cell types. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 21–23

Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL (2002) Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion. The roles of PI3-kinase, *Akt*, and endothelial nitric oxide synthase phosphorylation. Circulation 105:1497–1502

Halliwell B (1997) What nitrates tyrosine? Is nitrotyrosine specific as biomarker of peroxynitrite? FFEBS Lett 411:157–160

Hickey MJ (2001) Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. Clin Sci 100:1–11

Hropot M, Langer KH, Wiemer G, Grötsch H, Linz W (2003) Angiotensin II subtype AT_1 receptor blockade prevents hy-

pertension and renal insufficiency induced by chronic NO-synthase inhibition in rats. Naunyn-Schmiedebergs Arch Pharmacol 367:312–317
Huraux C, Makita T, Kurz S, Yamaguchi K, Szlam F, Tarpey MM, Wilcox JN, Harrison DG, Levy JH (1999) Superoxide production, risk factors, and endothelium-dependent relaxations in human internal mammary arteries. Circulation 99:53–59
Kajekar R, Moore PK, Brain SD (1995) Essential role for nitric oxide in neurogenic inflammation in rats cutaneous microcirculation. Evidence for endothelium-independent mechanism. Circ Res 76:441–447
Lamontagne D, Pohl U, Busse R (1991) N^G-Nitro-L-arginine antagonizes endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release in the isolated rabbit heart. Pflüger's Arch 418:266–270
McIntyre M, Bohr DF, Dominiczak AF (1999) Endothelial function in hypertension. The role of superoxide anion. Hypertension 34:539–545
Megson IL (2000) Nitric oxide donor drugs. Drugs Future 25:701–715
Moncada S, Palmer RMJ, Higgs SA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142
Moore PK, al-Swayeh OA, Chong NWS, Evans RA, Gibson A (1990) L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. Br J Pharmacol 99:408–412
Palmer RMJ, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524–526
Pieper AA, Verma A, Zhang J, Snyder SH (1999) Poly(ADP-ribose)polymerase, nitric oxide and cell death. Trends Pharmacol Sci 20:171–181
Rand MJ, Li CG (1995) Nitric oxide as a neurotransmitter in peripheral nerves: Nature of transmitter and mechanism of transmission. Ann Rev Physiol 57:659–682
Rees DD, Palmer RMJ, Moncada S (1989) Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 86:3375–3378
Ribero MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R (1992) Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20:298–303
Schrör K, Förster S, Woditsch I, Schröder H (1989) Generation of NO from molsidomine (SIN-1) *in vitro* and its relationship to changes in coronary vessel tissue. J Cardiovasc Pharmacol 14, Suppl 11:S29–S34
Schulz R, Kelm M, Heusch G (2004) Nitric oxide in myocardial ischemia/reperfusion injury. Cardiovasc Res 61:L402–L413
Southam E, Garthwaite J (1993) The nitric oxide-cyclic GMP signalling pathway in the rat brain. Neuropharmacol 32:1267–1277
Susswein AJ, Katzoff A, Miller N, Hurwitz I (2004) Nitric oxide and memory. Neuroscientist 10:153–162
Szabó C (1996) DNA strand breakage and activation of poly-ADP ribosyltransferase: A cytotoxic pathway triggered by peroxynitrite. Free Radic Biol Med 21:855–869
Szabó C, Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL (1997) Endothelial dysfunction in a rat model of endotoxic shock. Importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. J Clin Invest 100:723–735
Umans JG, Levi R (1995) Nitric oxide in the regulation of blood flow and arterial pressure. Ann Rev Physiol 57:771–790
Vanhoutte PM (1999) How to assess endothelial function in human blood vessels. J Hypertens 17:1047–1058
Willmot M, Gray L, Gibson C, Murphy S, Bath PMW (2005) A systematic review of nitric oxide donors and l-arginine in experimental stroke; effects on infarct size and cerebral blood flow. Nitric Oxide 12:141–149
Zanzinger J (1999) Role of nitric oxide in the neural control of cardiovascular function. Cardiovasc Res 43:639–649

A.1.1.18.2 Bioassay of EDRF Release

PURPOSE AND RATIONALE

EDRF release from arterial endothelium can be studied by a sandwich technique using donor tissue with intact endothelium facing with its intimal side the intimal side of a detector tissue.

PROCEDURE

Rabbits are subjected to various kinds of treatment, e. g., atherogenic diet or drug treatment for prevention of arteriosclerosis. Aorta segments, about 2 cm in length, are prepared, cut open along their longitudinal axis and pinned to a tissue suspender without damaging the endothelium. These segments serve as donor tissue for EDRF. Circumferential aorta strips from the abdominal aorta of untreated control rabbits are de-endothelialized by gently blotting their luminal surfaces on wet filter paper. These denuded abdominal aorta strips are pinned opposite the donor segments (intimal surface facing intimal surface) and function as detector for luminally released EDRF. Each sandwich preparation is suspended in a 40-ml organ bath, filled with oxygenated Krebs-Ringer buffer at 37°C containing 10 mM indomethacin. After connecting the detector strip to a force transducer, the angle between the detector strip and the donor segment is minimized and the distance between donor and detector tissue standardized. After one hour stabilization, the strips are brought to their optimum length-tension relationship by repeated exposure to 80 mM KCl. When a stable contractile response is established, the strips are precontracted with phenylephrine to 80–100% of their KCl-induced contraction. After stabilization of plateau phase, cumulative doses of acetylcholine (0.01–10 mM) are added to induce EDRF release from donor tissues.

EVALUATION

Relaxations of the detector strip induced by EDRF release from treated donor rabbit aortas are compared with aortas from control rabbits.

MODIFICATIONS OF THE METHOD

An other bioassay for measuring function of cultured endothelial cells using a computer system for the acquisition and analysis of vascular contractility has been published by Winn et al. (1992).

REFERENCES AND FURTHER READING

Riezebos J, Vleeming W, Beems RB, van Amsterdam JGC, Meijer GW, de Wildt DJ, Porsius AJ, Wemer J (1994) Comparison of Israpidine and Ramipril in cholesterol-fed rabbits: effect on progression of atherosclerosis and endothelial dysfunction. J Cardiovasc Pharmacol 23:415–423

Winn MJ, Panus PC, Norton P, Dai J (1992) Computer system for the acquisition and analysis of vascular contractility. Application to a bioassay of endothelial cell function. J Pharmacol Toxicol Meth 28:49–55

A.1.1.18.3 Isolated Arteries with and Without Endothelium

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and release potent vasoconstrictive agents, such as endothelin and angiotensin as well as vasodilating agents, such as EDRF. In isolated arterial segments the endothelial surface can be functionally destroyed allowing a differentiation between a direct action of drugs on the smooth muscle cells and an indirect effect via the endothelium. Isolated rings of rabbit or rat aorta are useful models to study the effects of endothelium derived factors such as EDRF or endothelins and their antagonists (Linz et al. 1986; Tracey et al. 1990; Fujimoto et al. 1992; Fukuroda et al. 1992; Wiemer et al. 1992). A survey of the history and on techniques leading to the discovery of endothelium-dependent relaxation was given by Furchgott (1993).

PROCEDURE

The descending thoracic aorta from rabbits of either sex (weighing 2.5–3.5 kg) is excised and dissected free from connective tissue. Care is taken to avoid damage of the endothelium. The aorta is divided into 2 mm wide rings and cut off in small strips. From some strips, the endothelium is removed by gently rubbing the intimal surfaces between the fingers for approximately 30 s. The strips are suspended in a 25 ml organ bath containing Krebs-bicarbonate solution at 37°C being gassed continuously with 5% CO_2/95% O_2. Contractions of the strips are recorded isometrically with a load of 2 g on the tissues. After an equilibration period of 2 h a stable baseline tone is reached.

To study the vasodilating effects of a compound, the strips are contracted with norepinephrine (10^{-8} M), or angiotensin II (10^{-7} M), or potassium chloride (20 mM). When a stable contraction plateau has been reached, the vasodilating agent is added in various concentrations. In these concentrations, norepinephrine, angiotensin II and KCl evoke a response of 60–80% of maximal contraction in intact rings of rabbit aorta. Rings without endothelium exhibit a response which is significantly enhanced in comparison with the response of the intact preparation after norepinephrine and angiotensin II precontraction.

To indicate the functional removal of the endothelium, the responsiveness of each preparation is tested with the known endothelium-dependent dilator, acetylcholine. In endothelium-intact rings, acetylcholine relaxes contractions induced by norepinephrine or angiotensin II. In precontracted rings devoid of endothelium, acetylcholine does not show any relaxing effect or causes contractions by itself at higher concentrations (Furchgott and Zawadzki 1980). As an example, atriopeptin III causes a similar concentration-dependent relaxation of all precontracted preparations with intact and with functionally destroyed endothelium indicating a direct effect on the smooth muscle cells. The relaxation is accompanied by an increase of cGMP.

EVALUATION

Statistical analyses are performed by regression analysis of dose response curves to determine EC_{50} values. Data are given as means ± standard deviation.

CRITICAL ASSESSMENT OF THE METHOD

The isolated aortic ring of rabbits with and without functionally intact endothelium is a useful tool to differentiate direct effects on the arterial smooth musculature from effects mediated by the endothelium.

MODIFICATIONS OF THE METHOD

Fujimoto et al. (1992) used the thoracic aorta from rats to study the effects between endothelin and an endothelin receptor antagonist. In transverse strips from **rat**thoracic aorta, 2 mm wide and 4–5 mm long, the endothelium was removed by gently rubbing the interior surface of the aorta. Concentration-response curves of contractions after ET-1 in the presence and the absence of the inhibitor were compared.

Pellisier et al. (1992) perfused the isolated mesenteric vascular bed of the **rat** with Tyrode solution and measured the perfusion pressure after injection of graded doses of norepinephrine and the dose-dependent relaxation due to acetylcholine in the vascular bed precontracted by norepinephrine infusion. In order to destroy the endothelial layer, the perfusate was

changed to a hypotonic Tyrode solution containing all of the constituents present in normal Tyrode solution but in one-tenth of the concentration resulting in disruption of more than 95% of the endothelial cells. The effect of norepinephrine was enhanced, whereas the effect of acetylcholine was abolished.

Legan and Sisson (1990) described a method to denude **rat** aortic endothelium *in vitro* with saponin.

Bohn and Schönafinger (1989) used helical strips of pulmonary arteries of **guinea pigs** in which the endothelium has been removed for biological detection of NO.

Fukuroda et al. (1992) used spiral strips from **porcine** coronary artery and vein and from intrapulmonary artery and vein removing the intimal surface by lightly rubbing with wet filter paper. Concentration-contraction curves for ET-1 and ET-3 were obtained with and without an endothelin antagonist.

Hayashi et al. (1988) described functional and anatomical recovery of endothelium after balloon denudation of the left circumflex coronary artery in **dogs**.

Endothelial denudation of the left circumflex coronary artery was used by Chu and Cobb (1987) to study the vasoactive effects of serotonin on proximal coronary arteries in awake **dogs**.

Experiments in isolated rings of the left circumflex or left anterior descending coronary artery of **dogs** with and without endothelium were performed by Desta et al. (1995).

Terrón (1996) analyzed the effects of 5-HT_1-receptor antagonists on 5-HT and sumatriptan induced isometric contractions in endothelium denuded segments of **canine** coronary arteries.

Ren et al. (1993) isolated coronary arteries from Japanese **monkeys** (*Macaca fuscata*) with and without endothelium to study muscarinic receptor subtypes mediating vasodilatation and vasoconstriction.

REFERENCES AND FURTHER READING

Bohn H, Schönafinger K (1989) Oxygen and oxidation promote the release of nitric oxide from sydnonimines. J Cardiovasc Pharmacol 14 (Suppl 11):S6–S12

Chu A, Cobb FR (1987) Vasoactive effects of serotonin on proximal coronary arteries in awake dogs. Circ Res 61 (Suppl II):II81–II87

Desta B, Nakashima M, Kirchengast M, Vanhoutte PM, Boulanger CM (1995) Previous exposure to bradykinin unmasks an endothelium-dependent relaxation to the converting enzyme inhibitor Trandolaprilat in isolated canine coronary arteries. J Pharm Exp Ther 272:885–891

Fujimoto M, Mihara S, Nakajima S, Ueda M, Nakamura M, Sakurai K (1992) A novel, non-peptide endothelin antagonist, isolated from bayberry, Myrica cerifera. FEBS Lett 305:41–44

Fukuroda T, Nishikibe M, Ohta Y, Ihara M, Yano M, Ishikawa K, Fukami T, Ikemoto F (1992) Analysis of responses to endothelins in isolated porcine blood vessels by using a novel endothelin antagonist, BQ-153. Life Sci 50:PL-107–PL-112

Furchgott RF (1993) The discovery of endothelium-dependent relaxation. Circulation 87: Suppl V:V3–V8

Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376

Hayashi Y, Tomoike H, Nagasawa K, Yamada A, Nishijima H, Adachi H, Nakamura M (1988) Functional and anatomical recovery of endothelium after denudation of coronary artery. Am J Physiol 254:H1081–H1090

Jeremy JY, Dandona P (1989) Effect of endothelium removal on stimulatory and inhibitory modulation of rat aortic prostaglandin synthesis. Br J Pharmacol 96:243–250

Legan E, Sisson JA (1990) Method to denude rat aortic endothelium with saponin for phosphoinositide analysis in vascular smooth muscle. J Pharmacol Meth 23:31–39

Linz W, Albus U, Wiemer G, Schölkens BA, König W (1986) Atriopeptin III induces endothelium-independent relaxation and increases cGMP levels in rabbit aorta. Klin Wschr 64 (Suppl VI):27–30

Peach MJ, Singer HA, Loeb AL (1985) Mechanism of endothelium-dependent vascular smooth muscle relaxation. Biochem Pharmacol 34:1867–1874

Pelissier T, Miranda HF, Bustamante D, Paelle C, Pinardi G (1992) Removal of the endothelial layer in perfused mesenteric vascular bed of the rat. J Pharmacol Meth 27:41–44

Pörsti I, Bara AT, Busse R, Hecker M (1994) Release of nitric oxide by angiotensin-(1–7) from porcine coronary endothelium: implications for a novel angiotensin receptor. Br J Pharmacol 111:652–654

Ren LM, Nakane T, Chiba S (1993) Muscarinic receptor subtypes mediating vasodilation and vasoconstriction in isolated, perfused simian coronary arteries. J Cardiovasc Pharmacol 22:841–846

Reynolds EE, Mok LLS (1990) Role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of rat aorta to endothelin. J Pharmacol Exp Ther 252:915–921

Scivoletto R, Carvalho MHC (1984) Cardionatrin causes vasodilation *in vitro* which is not dependent on the presence of endothelial cells. Eur J Pharmacol 101 :143–145

Terrón JA (1996) GR127935 is a potent antagonist of the 5-HT_1-like receptor mediating contraction in the canine coronary artery. Eur J Pharmacol 300:109–112

Tracey WR, Linden J, Peach MJ, Johns RA (1990) Comparison of spectrophotometric and biological assays for nitric oxide (NO) and endothelium-derived relaxing factor (EDRF): Nonspecificity of the diazotization reaction for NO and failure to detect EDRF. J Pharmacol Exp Ther 252:922–928

Wiemer G, Becker RHA, Jablonka B, Rosenkranz G, Schölkens BA, Linz W (1992) Effects of converting enzyme inhibitors and the calcium antagonist nifedipine alone and in combination on precontracted isolated rabbit aortic rings. Arzneim Forsch /Drug Res 42:795–797

A.1.1.18.4 Nitric Oxide Formation by Cultured Endothelial Cells

PURPOSE AND RATIONALE

Endothelial cells are able to synthesize and to release not only potent vasoconstrictor peptides such as an-

giotensin and endothelin but also potent dilators such as nitric oxide (NO), ATP, substance P, and bradykinin.

NO-formation can be assessed by determination of intracellular cyclic GMP in cultured endothelial cells, whereas release of NO from these cells can be measured by the stimulatory effect of NO on the activity of soluble guanylyl cyclase (Lückhoff et al. 1988; Wiemer et al. 1991; Linz et al. 1992; Bogle et al. 1992; review by Moncada et al. 1991).

PROCEDURE

Endothelial cell culture

Bovine or porcine aorta is obtained from local slaughter houses. Endothelial cells are isolated by digestion with dispase (Lückhoff et al. 1988). The cells are seeded on 6- or 24-well plates (e. g., Nunc Intermed, Wiesbaden, Germany) and grown to confluence. Dulbecco's modified Eagle's/Ham's F-12 medium containing 20% fetal calf serum is supplemented with penicillin (10 U/ml), streptomycin (10 µg/ml), L-glutamate (1 mM/l), glutathione (5 mg/ml), and L(+)ascorbic acid (5 mg/ml); (Biotect protection medium).

Measurement of cyclic GMP

Primary cultures of endothelial cells are used. After removal of the culture medium by aspiration, the monolayer is washed twice with 2 ml HEPES-Tyrode's solution (37°C). Thereafter, the cells are preincubated for 15 min at 37°C with 3-isobutyl-1-methylxanthine (IBMX), (10^{-4} M/l). After this time, drugs or solvents are added. After predetermined periods, the incubation medium is quickly removed. The cells are then immediately extracted with 0.6 ml 6% trichloroacetic acid and scraped off with a rubber scraper. The cell suspension is sonicated for 10 s before being centrifuged for 5 min at 4000 *g*. The supernatants are extracted with four volumes of water saturated diethylether, and the samples frozen (–20°C) until analysis. The protein contents of the samples are measured according to Lowry et al. (1951). Cyclic GMP can be determined in the acetylated samples by various methods (Heath et al. 1992), e. g., using a commercially available radioimmunoassay (New England Nuclear). Cyclic GMP content is expressed as picomoles GMP per milligram protein.

Measurement of NO release

Release of NO from endothelial cells is assayed on the basis of the stimulatory effect of NO on the activity of soluble guanylyl cyclase (purified from bovine lung) (Gerzer et al. 1981). The activity of the enzyme is determined in terms of the formation of cyclic [^{32}P]GMP from α-[^{32}P]GTP. Reactions are carried out in a reaction mixture containing 30 mM triethanolamine-HCl (pH 7.4), 1 mM reduced glutathione, 4 mM $MgCl_2$, 1 mM cGMP and 0.1 mg/ml bovine γ-globulin (total volume of 0.18 ml) at 37°C in the presence of α-[^{32}P]GTP (0.03 mM; 0.2 µCi) and soluble guanylyl cyclase (4 µg). Ten-µl samples are quickly transferred to the reaction mixture. Enzymatic formation of cGMP is allowed to proceed for 60 s and then stopped by the addition of 450 µl zinc acetate (120 mM) and 500 µl sodium carbonate (120 mM). A complete inhibition of cGMP formation can be achieved by preincubation of the monolayers for 30 min with the stereospecific inhibitor of NO synthase, N^G-nitro-L-arginine.

EVALUATION

Time-response curves and dose-response curves after addition of various activators or inhibitors of NO synthase are established. Data are reported as mean values ±SEM of cGMP (pmol/mg protein) or guanylyl cyclase activity (nmol/mg/min). Statistical evaluation is performed with Student's *t*-test.

MODIFICATIONS OF THE METHOD

The clinical pharmacology of L-arginine has been reviewed by Böger and Bode-Böger (2001).

Isolation of porcine cerebral capillary endothelial cells has been described by Wiemer et al. (1994).

Feelisch and Noack (1987) and Nakazawa et al. (1992) used chemiluminescence techniques for determination of NO.

A method for on-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy was described by Mordvintcev et al. (1991). Similar methods were used by Ichimori et al. (1992), Lancester et al. (1992), Steel-Goodwin et al. (1992).

Hecker et al. (1995) used a cascade superfusion bioassay to characterize a stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells.

Electrochemical microprobes for direct measurement of NO in tissues have been developed (Shibuki 1990; Ishida et al. 1996; Smits and Lefebvre 1997).

Malinski and Taha (1992), Linz et al. (1999) measured nitric oxide release by a porphyrinic-based microsensor with a detection limit of 10 nmol/L. The amperometric signal at a constant potential of 0.67 V was measured with a voltametric analyzer (PAR model 273, Princeton Applied Research) interfaced

with an IBM 80486 computer with data acquisition and software.

Gabriel et al. (1997) developed a method for the detection of intracellular nitric oxide generation in dissociated cerebellar granule cells using dichlorofluorescin diacetate and flow cytometry.

Sumpio et al. (1987) found that cyclic mechanical stress stimulates cultured bovine aortic endothelial cells to proliferate.

Using this method, Rosales et al. (1997) found that exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools.

REFERENCES AND FURTHER READING

Böger RH, Bode-Böger SM (2001) The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol 41:79–99

Bogle RG, Coade SB, Moncada S, Pearson JD, Mann GE (1992) Bradykinin stimulates L-arginine transport and nitric oxide release in vascular endothelial cells. formation in cytokine-treated rat hepatocytes and in blood and liver during sepsis. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 1 Physiological and Clinical Aspects. Portland Press, London and Chapel Hill, pp 80–84

Busse R, Lamontagne D (1991) Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. Naunyn-Schmiedeberg's Arch Pharmacol 344:126–129

Feelisch M, Noack E (1987) Nitric oxide (NO) formation from nitrovasodilators occurs independently of hemoglobin or non-heme iron. Eur J Pharmacol 142:465–469

Gabriel C, Camins A, Sureda FX, Aquirre L, Escubedo E, Pallàs M, Camarasa J (1997) Determination of nitric oxide generation in mammalian neurons using dichlorofluorescin diacetate and flow cytometry. J Pharmacol Toxicol Meth 38:93–98

Gerzer R, Hofmann F, Schultz G (1981) Purification of a soluble, sodium-nitroprussidestimulated guanylate cyclase from bovine lung. Eur J Biochem 116:479–486

Griffiths MJD, Messent M, McAllister RJ, Evans TW (1993) Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br J Pharmacol 110:963–968

Heath R, Brynat B, Horton JK (1992) Which cyclic GMP assay? In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 98–102

Hecker M, Boese M, Schini-Kerth VB, Mülsch A, Busse R (1995) Characterization of the stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells as an N^G-hydroxy-L-arginine-nitric oxide adduct. Proc Natl Acad Sci USA 92:4671–4675

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J, Schölkens BA (1991) Hoe 140 a new potent and long acting bradykinin antagonist: *in vitro* studies. Br J Pharmacol 102:769–773

Holzmann S, Kukovetz WR, Windischhofer W, Paschke E, Graier WF (1994) Pharmacologic differentiation between endothelium-dependent relaxations sensitive and resistant to nitro-L-arginine in coronary arteries. J Cardiovasc Pharmacol 23:747–756

Ichimori K, Pronai L, Fukahori M, Arroyo CM, Nakzawa H (1992) Spin trapping/electron paramagnetic spectroscopy analysis of endothelium-derived relaxing factors and their intermediates in human platelets. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 68–69

Ishida Y, Hashimoto M, Fukushima S, Masumura S, Sasaki T, Nakayama K, Tamura K, Murakami E, Isokawa S, Momose K (1996) A nitric oxide-sensitive electrode: requirement of lower oxygen concentration for detecting nitric oxide from the tissue. J Pharmacol Toxicol Meth 35:19–24

Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. J Clin Invest 52:2745–2756

Lancester JR Jr, Stadler J, Billiar TR, Bergonia HA, Kim YM, Piette LH, Simmons RL (1992) Electron-paramagnetic resonance detection of iron-nitrosyl formation in cytokine-treated rat hepatocytes and in blood and liver during sepsis. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 76–80

Linz W, Wiemer G, Schölkens BA (1992) ACE-inhibition induces NO-formation in cultured bovine endothelial cells and protects isolated ischemic rat hearts. J Mol Cell Cardiol 24:909–919

Linz W, Wohlfart P, Schoelkens BA, Becker RHA, Malinski T, Wiemer G (1999) Late treatment with ramipril increases survival in old spontaneously hypertensive rats. Hypertension 34:291–295

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with phenol reagent. J Biol Chem 193:265–275

Lückhoff A, Pohl U, Mülsch A, Busse R (1988) Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. Br J Pharmacol 95:189–196

Malinski T, Taha Z (1992) Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor. Nature 358:676–678

Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–142

Mordvintcev P, Mülsch A, Busse R, Vanin A (1991) On-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy. Anal Biochem 19:142–146

Mülsch A, Böhme E, Busse R (1987) Stimulation of soluble guanylate cyclase by endothelium-derived relaxing factor from cultured endothelial cells. Eur J Pharmacol 135:247–250

Nakazawa H, Fukahori M, Murata T, Furuya T (1992) On-line monitoring of nitric oxide generation from isolated perfused rat lung using decrease in superoxide-dependent chemiluminescence. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 69

Nathan C (1992) Nitric oxide as a secretory product of mammalian cells. FASEB J 6:3051–3064

Rosales OR, Isales CM, Barrett PQ, Brophy C, Sumpio BE (1997) Exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools. Biochem J 326:385–392

Shephard JT, Vanhoutte PM (1991) Endothelium-derived relaxing (EDRF) and contracting factors (EDCF) in the control of cardiovascular homeostasis: the pioneering observations. In: Rubanyi GM (ed) Cardiovascular Significance of Endothelium-Derived Vasoactive Factors. Futura Publ Comp, Inc., Mount Kisco, NY, pp 39–64

Shibuki K (1990) An electrochemical microprobe for detecting nitric oxide release in brain tissue. Neurosci Res 9:69–76

Smits GJM, Lefebvre RA (1997) Evaluation of an electrochemical microprobe for direct NO measurement in the rat gastric fundus. J Pharmacol Toxicol Meth 37:97–103

Steel-Goodwin L, Arroyo CM, Gray B, Carmichael AJ (1992) Electron paramagnetic resonance detection of nitric oxide-dependent spin adducts in mouse jejunum. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 80–84

Sumpio BE, Banes AJ, Levin LG, Johnson G Jr (1987) Mechanical stress stimulates aortic endothelial cells to proliferate. J Vasc Surg 6:252–256

Wiemer G, Schölkens BA, Becker RHA, Busse R (1991) Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium derived bradykinin. Hypertension 18:558–563

Wiemer G, Popp R, Schölkens BA, Gögelein H (1994) Enhancement of cytosolic calcium, prostaglandin and nitric oxide by bradykinin and the ACE inhibitor ramiprilate in porcine brain capillary endothelial cells. Brain Res 638:261–266

A.1.1.18.5 Expression of Nitric Oxide Synthase

PURPOSE AND RATIONALE

Properties of various forms of nitric oxide synthase (NOS) have been described by Mayer et al. (1992), Leone et al. (1992), Hevel et al. (1992), Förstermann et al. (1992, 1995), (Salter et al. 1992), Pollock et al. (1992), Schmidt et al. (1992), and Mungrue et al. (2003), among them type I, which is constitutively expressed in neurons; the inducible type II which is found in macrophages and hepatocytes, but also in the brain (Moro et al. 1998), where it may contribute to NO-mediated neurotoxicity; and type III, which is constitutively expressed in endothelial cells (Knowles and Moncada 1994). NOS can be inhibited by several routes, e. g., competition with L-arginine, NADPH, flavin or tetrahydrobiopterin, interaction of the haeme group of NOS, interference with Ca^{2+} availability or calmodulin binding to the enzyme (Fukuto and Chaudhuri 1995). A widely used inhibitor is L-NAME (Vargas et al. 1991). Selective inhibition of constitutive NOS can by achieved by 7-nitroindazole (Moore et al. 1993); of the inducible NOS by aminoguanidine hydrochloride (Griffiths et al. 1993) and by 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (= AMT) (Nakane et al. 1995). Linz et al. (1999) determined NOS in the left cardiac ventricle of hypertensive rats.

PROCEDURE

Tissues are ground at the temperature of liquid nitrogen using a microdismembranator (Braun). The powders are extracted for 1 h on ice with 10 mmol/l Tris-HCl, pH 7.4, containing 1% SDS and protease inhibitors (complete, Boehringer Mannheim). Debris is removed by a 30-min centrifugation at 4°C (>100,000 *g*). 100 µg of total of the protein extracts are subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The eNOS protein is detected by use of a specific antibody (monoclonal anti-NOS III), Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). As a secondary antibody, an anti-mouse IgG antibody coupled to alkaline phosphatase is used (Jackson ImmunoResearch Laboratories). Chemifluorescence is analyzed and quantified by scanning with a Fluorimager 595-system (Molecular Dynamics).

EVALUATION

The data are given as mean ± SEM. ANOVA is used followed by Tuckey's test for post-ANOVA multiple pair comparisons.

MODIFICATIONS OF THE METHOD

Linz et al. (1997) measured expression of ecNOS in the carotid artery of hypertensive rats by Western blot analysis. Frozen (–70°C) vessels were thawed and extracted with guanidium isothiocyanate/phenol/chloroform (Chomczynski and Sacchi 1987). Crude protein fractions were obtained by alcohol precipitation of the phenol phase. A total of 100 µg of the protein extracts was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Ponceau staining was performed to verify the quality of the transfer and the equipartition of protein in each lane. EcNOS protein was detected with a specific antibody (mouse NOS III, Transduction Laboratories) and visualized by enhanced chemifluorescence with a commercially available kit (Amersham). The autoradiographs were analyzed by scanning densitometry.

McCall et al. (1991) identified N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase.

Bauersachs et al. (1998, 1999) measured vascular reactivity in isolated rat aortic rings mounted in an organ bath (Föhr Medical Instruments, Seeheim Germany) for isometric force measurement and determined superoxide anion production by lucigenin-enhanced chemiluminescence and endothelial nitric ox-

ide synthase and soluble guanylyl cyclase expression by reverse transcription-polymerase chain reaction.

Von der Leyen et al. (1995) reported gene therapy inhibiting neointimal vascular lesions in rats. After denudation of the endothelium of carotid arteries by balloon injury, endothelial cell nitric oxidase expression in the vessel wall was restored by using the Sendai virus/liposome *in vivo* gene transfer technique.

Lund et al. (2000) found that gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits.

Mungrue et al. (2002) discussed lessons from murine genetic models on the role of NOS in heart failure.

REFERENCES AND FURTHER READING

Bauersachs J, Bouloumié A, Fraccarollo D, Hu K, Busse R, Ertl G (1998) Hydralazine prevents endothelial dysfunction, but not the increase in superoxide production in nitric oxide-deficient hypertension. Eur J Pharmacol 362:77–81

Bauersachs J, Bouloumié A, Fraccarollo D, Hu K, Busse R, Ertl G (1999) Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression. Role of enhanced vascular superoxide production

Chomczynski P, Sacchi N (1987) Single-step method for RNA isolation by acid guanidium thiocyanatephenolchloroform extraction. Anal Biochem 162:156–159

Förstermann U, Schmidt HHHW, Pollock JS, Sheng H, Mitchell JA, Warner TD, Murad F (1992) Characterization and classification of constitutive and inducible isoforms of nitric oxide synthase in various cell types. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 21–23

Förstermann U, Kleinert H (1995) Nitric oxide synthase: Expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch Pharmacol 352:351–364

Fukuto JM, Chaudhuri G (1995) Inhibition of constitutive and inducible nitric oxide synthase: Potential selective inhibition. Annu Rev Pharmacol Toxicol 35:165–194

Hevel JM, White KA, Marletta MA (1992) Purification of the inducible murine macrophage nitric oxide synthase: identification as a flavoprotein and detection of enzyme-bound tetrahydrobiopterin. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 19–19–21

Knowles RG, Moncada S (1994) Nitric oxide synthases in mammals. Biochem J 298:249–258

Leone AM, Palmer RMJ, Knowles RG, Francis PL, Ashton DS, Moncada S (1992) Molecular oxygen is incorporated in nitric oxide and citrulline by constitutive and inducible nitric oxide synthases. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 7–14

Linz W, Jessen T, Becker RHA, Schölkens BA, Wiemer G (1997) Long-term ACE inhibition doubles lifespan of hypertensive rats. Circulation 96:3164–3172

Linz W, Wohlfart P, Schoelkens BA, Becker RHA, Malinski T, Wiemer G (1999) Late treatment with ramipril increases survival in old spontaneously hypertensive rats. Hypertension 34:291–295

Lund DD, Faraci FM, Miller FJ, Jr, Heistad DD (2000) Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. Circulation 101:1027–1033

Mayer B, John M, Heinzel B, Klatt P, Werner ER, Böhme E (1992) Properties of Ca^{2+}-regulated brain nitric oxide synthase. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 4–6

McCall TB, Feelisch M, Palmer RMJ, Moncada S (1991) Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br J Pharmacol 102:234–238

Moore PK, Wallace P, Gaffen Z, Hart SL, Babbedge RC (1993) Characterization of the novel nitric oxide synthase inhibitor 7-nitroindazole and related indazoles: Antinociceptive and cardiovascular effects. Br J Pharmacol 110:219–224

Moro MA, de Alba J, Leza JC, Lorenzo P, Fernández AP, Bentura ML, Boscá L, Lizasoain I (1998) Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. Eur J Neurosci 10:445–456

Mungrue IN, Husain M, Stewart DJ (2002) The role of NOS in heart failure. Lessons from murine genetic models. Heart Failure Rev 7:407–422

Mungrue IN, Bredt DS, Stewart DJ, Husain M (2003) From molecules to mammals: what's NOS got to do with it ? Acta Physiol Scand 179:123–135

Nakane M, Klinghöfer V, Kuk JE, Donnelly JL, Budzik GP, Pollock JS, Basha F, Carter GW (1995) Novel potent and selective inhibitors of inducible nitric oxide synthase. Mol Pharmacol 47:831–834

Pollock JS, Mitchell JA, Warner TD, Schmidt HHHW, Nakana M, Förstermann U, Murad F (1992) Purification of nitric oxide synthases from endothelial cells. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 108–111

Salter M, Knowles RG, Moncada S (1992) Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 193–197

Schmidt HHHW, Smith RM, Nakana M, Gagne GD, Miller MF, Pollock JS, Sheng H, Förstermann U, Murad F (1992) Type I nitric oxide synthase: purification, characterization and immunohistochemical localization. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA (eds) The Biology of Nitric Oxide. 2 Enzymology, Biochemistry and Immunology. Portland Press, London and Chapel Hill, pp 112–114

Vargas HM, Cuevas JM, Ignarro LJ, Chaudhuri G (1991) Comparison of the inhibitory potencies of N(G)-methyl-, N(G)-nitro- and N(G)-amino-L-arginine on EDRF formation in the rat: Evidence for continuous basal EDRF release. J Pharm Exp Ther 257:1208–1215

Von der Leyen HE, Gibbbons GH, Morishita R, Lewis NP, Zhang L, Nakajima M, Kaneda Y, Cooke JP, Dzau VJ (1995) Gene therapy inhibiting neointimal vascular lesion: *In vivo* transfer of endothelial cell nitric oxide synthase gene. Proc Natl Acad Sci USA 92:1137–1141

A.1.1.19 Inhibition of Rho Kinase

PURPOSE AND RATIONALE

Rho is a member of the Ras-related family of small molecular weight GTP-binding proteins, and Rho works as a molecular switch by shuttling between the GDP-bound inactive form and the GTP-bound active form. Rho is involved in cell motility, cell adhesion, cytokinesis, Ras-induced transformation, transcriptional activation, and cell cycle progression. These actions, through Rho signaling, are mediated by downstream Rho effectors, such as the ROCK family of Rho-associated serine/threonine protein kinases. Studies with ROCK-specific inhibitors indicate that the ROCK pathway works in the contraction of vascular smooth muscle. Several studies indicate that Rho-kinase may be a novel therapeutic target in the treatment of cardiovascular disease (Kobayashi et al. 2002; Shimokawa 2002; Ito et al. 2003, 2004; Nakakuki et al. 2005; Budzyn et al. 2006; Winaver et al. 2006).

Ishizaki et al. (2000) described the pharmacological properties of a specific inhibitor of Rho-associated kinases.

PROCEDURE

Kinase assay

Recombinant ROCK-I, ROCK-II, PKN, or citron kinase was expressed in HeLa cells as a Myc-tagged protein by transfection using lipofectamine, and was precipitated from the cell lysates by the use of 9E10 monoclonal anti-Myc antibody coupled to G protein-Sepharose (Ishizaki et al. 1997). Recovered immunocomplexes were incubated with various concentrations of [^{32}P]ATP and 10 µg of histone type 2 as substrates, in the absence or presence of various concentrations of test compounds at 30°C for 30 min in a total volume of 30 µl of the kinase buffer containing 50 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.02% Briji 35, and 2 mM dithiothreitol. PKCα was incubated with 5 µM [^{32}P]ATP and 200 µg/ml histone type 2 as substrates in the absence or presence of various concentrations of test compounds at 30°C for 10 min in a kinase buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, 5 mM magnesium acetate, 25 µg/ml phosphatidyl serine, 50 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate and 0.001% leupeptin in a total volume of 30 µl. Incubation was terminated by the addition of 10 µl of 4× Laemmli sample buffer. After boiling for 5 min, the mixture was subjected to SDS-polyacrylamide gel electrophoresis on a 16% gel. The gel was stained with Coomassie Brilliant Blue, and then dried. The bands corresponding to histone type 2 were excised, and the radioactivity was measured.

EVALUATION

K_i values were either determined by the double reciprocal plot or calculated from the equation $K_i = IC_{50}/(1 + S/K_m)$, where S and K_m are the concentration of ATP and the K_m value for ATP, respectively.

REFERENCES AND FURTHER READING

Budzyn K, Marley PD, Sobey CG (2006) Targeting Rho and Rho-kinase in the treatment of cardiovascular disease. Trends Pharmacol Sci 27:97–104

Ishizaki T, Naito M, Fujisawa K, Maekawa M, Watanabe N, Saito Y, Narumiya S (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett 404:118–124

Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S (2000) Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases. Mol Pharmacol 57:976–983

Ito K, Hirooka Y, Sakai K, Kishi T, Kaibuchi K, Shimokawa H, Takeshita A (2003) Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system. Possible involvement of neural mechanisms of hypertension. Circ Res 92:1337–13343

Ito K, Hirooka Y, Kishi T, Kimura Y, Kaibuchi K, Shimokawa H, Takeshita A (2004) Rho/Rho-kinase pathway in the brain stem contributes to hypertension caused by chronic nitric oxide synthase inhibition. Hypertension 43:156–162

Kobayashi N, Horinaka S, Mita SI, Nakano S, Honda T, Yoshida K, Kobayashi T, Matsuoka H (2002) Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. Cardiovasc Res 55:757–767

Nakakuki T, Ito M, Iwasaki H, Kureishi Y, Okamoto R, Moriki N, Kongo M, Kato S, Yamada N, Isaka N, Nakano T (2005) Rho/Rho-kinase pathway contributes to C-reactive protein-induced plasminogen activator inhibitor-1 expression in endothelial cells. Arterioscler Thromb Vasc Biol 25:2088–2093

Shimokawa H (2002) Rho-kinase as a novel therapeutic target in treatment of cardiovascular disease. J Cardiovasc Pharmacol 39:319–327

Winaver J, Ovcharenko E, Rubinstein I, Gurbanov K, Pollesello P, Bishara B, Hoffman A, Abassi Z (2006) Involvement of Rho kinase pathway in the mechanisms of renal vasoconstriction and cardiac hypertrophy in rats with experimental heart failure.. Am J Physiol 290:H2007–H2014

A.1.1.20 Inhibition of Na^+/H^+ Exchange

PURPOSE AND RATIONALE

Na^+/H^+ exchange was first described by Murer et al. (1976) in a study of intestinal and renal brush border vesicles. The plasma membrane Na^+/H^+ exchanger is an ubiquitous pH regulating cellular ion transport system. It is driven by the Na^+ gradient and extrudes

protons from the cytosol in exchange for extracellular Na^+ ions (Aronson 1985; Frelin et al. 1988; Fliegel and Dyck 1995; Orlowski and Grinstein 1997; Wakabayashi et al. 1997; Dibrov and Fliegel 1998). Six mammalian Na^+/H^+ exchangers: NHE1, NHE2, NHE3; NHE4, NHE5 (Attaphitaya et al. 1999; Szabo et al. 2000), and NHE6 have been described (Tse et al. 1994; Orlowski 1999; Counillon and Pouysségur 2000).

In cardiac tissue the exchanger has a major role in the control of intracellular pH. At the onset of cardiac ischemia and during reperfusion, Na^+/H^+ exchange is excessively activated by low intracellular pH. Since the deleterious Na^+ influx in this condition was found to originate mainly from Na^+/H^+ exchange (Frelin et al. 1984; Schömig et al. 1988), the exchanger seems to be responsible for an increase of cytosolic sodium in ischemic cells. The accumulation of intracellular Na^+ causes an activation of Na^+/K^+ ATPase (Frelin et al. 1984; Rasmussen et al. 1989) which in turn increases ATP consumption.

During ischemia the aerobic metabolism of glucose terminates in lactic acid. A vicious circle leads to a further decrease of intracellular pH and to a further activation of Na^+/H^+ exchange, resulting in energy depletion, cellular Na^+ overload and finally due to the coupling of Na^+ and Ca^{2+} transport via Na^+/Ca^{2+} exchange, cellular Ca^{2+} overload (Lazdunski et al. 1985; Tani and Neely 1990; Scholz and Albus 1993). Especially in ischemic cardiac tissue, where Na^+/H^+ exchange is the predominant pH regulating ion transport system (Weissenberg et al. 1989), these pathological events can lead to increased excitability and precipitation of cellular death. Therefore, it is desirable to find potent and well tolerated inhibitors of Na^+/H^+ exchange which should be able to interrupt this vicious cycle, to conserve cellular energy stores and to diminish cellular excitability and necrosis during cardiac ischemia. Such effects have been found with relatively weak inhibitors of Na^+/H^+ exchange at high toxic doses, such as amiloride and ethyl isopropyl amiloride (Scholz et al. 1992).

The myocardial Na^+/H^+ exchanger is regarded as a therapeutic target for the prevention of myocardial ischemic and reperfusion injury and attenuation of postinfarction heart failure (Karmazyn et al. 2001).

More potent Na^+/H^+ exchange inhibitors showed beneficial effects on ischemia/reperfusion injury (see A.5.0.7 and A.5.0.8) in rats (Aye et al. 1997; Myers et al. 1998; Aihara et al. 2000), dogs (Gumina et al. 1998, 2000) and pigs (Portman et al. 2001). Heart hypertrophy and heart failure after myocardial infarction is reduced (Yoshida and Karmazyn 2000; Kusumoto et al. 2001). Ischemia-induced apoptosis in isolated rat hearts is attenuated by sodium-hydrogen exchange inhibitors (Chakrabarti et al. 1997).

Linz and Busch (2003) demonstrated the effects of NHE-1 inhibition from protection during acute ischemia/reperfusion to prevention of myocardial remodeling.

REFERENCES AND FURTHER READING

Aihara K, Hisa H, Sato T, Yoneyama F, Sasamori J, Yamaguchi F, Yoneyama S, Mizuno Y, Takahashi A, Nagai A, Kimura T, Kogi K, Sato S (2000) Cardioprotective effect of TY-12533, a novel Na^+-H^+ exchange inhibitor on ischemia/reperfusion injury. Eur J Pharmacol 404:221–229

Aronson PS (1985) Kinetic properties of the plasma membrane Na^+-H^+ exchanger. Ann Rev Pharmacol 47:545–560

Attaphitaya S, Park K, Melvin JE (1999) Molecular cloning and functional expression of a rat Na^+/H^+ exchanger (NHE5) highly expressed in brain. J Biol Chem 274:4383–4388

Aye NN, Xue YX, Hashimoto K (1997) Antiarrhythmic effects of cariporide, a novel Na^+/H^+ exchange inhibitor, on reperfusion ventricular arrhythmias in rat hearts. Eur J Pharmacol 339:121–127

Chakrabarti S, Hoque ANE, Karmazy M (1997) A rapid ischemia-induced apoptosis in isolated rat hearts and its attenuation by the sodium-hydrogen exchange inhibitor HOE 642 (Cariporide) J Mol Cell Cardiol 29:3169–3174

Counillon L, Pouysségur J (2000) The expanding family of eukaryotic Na^+-H^+ exchangers. J Biol Chem 275:1–4

Dibrov P, Fliegel L (1998) Comparative molecular analysis of Na^+/H^+ exchangers: a unified model for Na^+/H^+ antiport? FEBS Letters 424:1–5

Fliegel L, Dyck JRB (1995) Molecular biology of the cardiac sodium/hydrogen exchanger. Cardiovasc Res 29:155–159

Frelin C, Vigne P, Lazdunski M (1984) The role of Na^+/H^+ exchange system in cardiac cells in relation to the control of the internal Na^+ concentration. J Biol Chem 259:8880–8885

Frelin C, Vigne P, Ladoux A, Lazdunski M (1988) The regulation of intracellular pH in cells from vertebrates. Eur J Biochem 174:3–14

Gumina RJ, Mizumura T, Beier N, Schelling P, Schultz JJ, Gross GJ (1998) A new sodium/hydrogen exchange inhibitor, EMD 85131, limits infarct size in dogs when administered before or after coronary artery occlusion. J Pharmacol Exp Ther 286:175–183

Gumina RJ, Daemmgen J, Gross GJ (2000) Inhibition of the Na^+/H^+ exchanger attenuates phase 1b ischemic arrhythmias and reperfuion-induced ventricular fibrillation. Eur J Pharmacol 396:119–124

Karmazyn M, Sostaric JV, Gan XT (2001) The myocardial Na^+/H^+ exchanger: a therapeutic target for the prevention of myocardial ischaemic and reperfusion injury and attenuation of postinfarction heart failure. Drugs 61:375–389

Kusumoto K, Haist JV, Karmazyn M (2001) Na^+/H^+ exchange inhibition reduces hypertrophy and heart failure after myocardial infarction in rats. Am J Physiol 280 (Heart Circulatory Physiol): H738–H745

Lazdunski M, Frelin C, Vigne P (1985) The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulation internal concentrations of sodium and internal pH. J Mol Cell Cardiol 17:1029–1042

Linz WJ, Busch AE (2003) NHE-1 inhibition: from protection during acute ischaemia/reperfusion to prevention of myocardial remodelling. Naunyn-Schmiedebergs Arch Pharmacol 368:239–246
Myers ML, Farhangkhoee P, Karmazyn M (1998) Hydrogen peroxide induced impairment of postischemic ventricular function is prevented by the sodium-hydrogen exchange inhibitor HOE &42 (cariporide). Cardiovasc Res 40:290–296
Murer H, Hopfer U, Kinne R (1976) Sodium/proton antiport in brush-border-membranes isolated from rat small intestine and Kidney. Biochem J 154:597–604
Orlowski J (1999) Na^+/H^+ exchangers. Molecular diversity and relevance to the heart. Ann NY Acad Sci 874:346–353
Orlowski J, Grinstein S (1997) Na^+/H^+ exchangers in mammalian cells. J Biol Chem 272:22373–22776
Portman MA, Panos AL, Xiao Y, Anderson DL, Ning X-H (2001) HOE-642 (cariporide) alters pH_i and diastolic function after ischemia during reperfusion in pig hearts *in situ*. Am J Physiol 280 (Heart Circulatory Physiol): H830–H834
Rasmussen HH, Cragoe EJ, Ten Eick RE (1989) Na^+-dependent activation of Na^+-K^+ pump in human myocardium during recovery from acidosis. Am J Physiol 256:H431–H439
Schömig A, Kurz T, Richardt G, Schömig E (1988) Neuronal sodium homeostasis and axoplasmic amine concentration determine calcium-independent noradrenaline release in nor-moxic and ischemic rat heart. Circ Res 63:214–226
Scholz W, Albus U (1993) Na^+/H^+ exchanger exchange and its inhibition in cardiac ischemia and reperfusion. Basic Res Cardiol 88:443–455
Szabo EZ, Numata M, Shull GE, Orlowski J (2000) Kinetic and pharmacological properties of a human brain Na^+/H^+ exchanger isoform 5 stably expressed in Chinese hamster ovary cells. J Biol Chem 275:6302–6307
Tse CM, Levine SA, Yun CHC, Brant SR, Nath S, Pouysségur J, Donowitz M (1994) Molecular properties, kinetics and regulation of mammalian Na^+/H^+ exchangers. Cell Physiol Biochem 4:282–300
Tani M, Neely JR (1990) Na^+ accumulation increases Ca^{+2} overload and impairs function in anoxic rat heart. J Mol Cell Cardiol 22:57–72
Wakabayshi S, Shigekawa M, Pouysségur J (1997) Molecular physiology of vertebrate Na^+/H^+ exchangers. Physiol Rev 77:51–74
Weissberg PL, Little PJ, Cragoe EJ, Bobik A (1989) The pH of spontaneously beating cultured rat hearts is regulated by an ATP-calmodulin-dependent Na^+/H^+ antiport. Circ Res 64:676–685
Yoshida H, Karmazyn M (2000) Na^+/H^+ exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. Am J Physiol 278 (Heart Circulatory Physiol): H300-H304

A.1.1.20.1 Inhibition of Na^+/H^+ Exchange in Thrombocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in platelets by measuring the optical density after osmotic cell swelling (Rosskopf et al. 1991).

PROCEDURE

About 5 ml blood is withdrawn by venipuncture from human donors or from the vena jugularis externa of Beagle dogs or from the aorta of anesthetized Wistar rats (weighing 250–350 g). Coagulation is inhibited by 0.8 ml citrate acid dextrose (65 mM citric acid, 11 mM glucose, 85 mM trisodium citrate). Platelet-rich plasma (PRP) is obtained by centrifugation of whole blood at 90 *g* for 10 min at room temperature. Platelet count is measured, e. g., with a Casey 1 multichannelyser (Schärfe System, Reutlingen, Germany).

Each of the experiments is performed with 10–50 µl PRP containing 20×10^6 platelets in a volume of 100 µl with saline. To activate Na^+/H^+ exchange in the platelets by intracellular acidification, 500 µl propionate buffer (135 mM Na-propionate, 1 mM HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 6.7, 22°C) are added to the PRP/NaCl solution. Swelling of the platelets results in a decrease of optical density which can be measured with an aggregometer, e. g. with a Turbitimer (Behringwerke, Marburg, Germany). The system is activated photometrically by the addition of the propionate buffer to the cuvette. The experiments are performed with and without the addition of the Na^+/H^+ exchange inhibitor to be tested. The inhibitors are added in concentrations between 10^{-4} and 10^{-8} mol/l. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) is used as standard. During the experiments all solutions are kept at 22°C in a temperature controlled water bath.

EVALUATION

Results are given as means ± SD. Student's *t*-test is employed for statistical evaluation. IC_{50} values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

Rosskopf D, Morgenstern E, Scholz W, Osswald U, Siffert W (1991) Rapid determination of the elevated Na^+-K^+exchange in platelets of patients with essential hypertension using an optical swelling assay. J Hypertens 9:231–238

A.1.1.20.2 Inhibition of Na^+/H^+ Exchange in Cholesterol Activated Rabbit Erythrocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in cholesterol activated rabbit erythrocytes by flame photometry of sodium (Scholz et al. 1992, 1993).

PROCEDURE

White rabbits (New Zealand strain, Ivanovas) are fed with a rabbit standard chow with 2% cholesterol for

6 weeks to increase the Na^+/H^+ exchange (Scholz et al. 1990) and to make the erythrocytes suitable for measurement of sodium influx via Na^+/H^+ exchange by flame photometry. Blood is drawn from the ear artery of the rabbits and coagulation prevented with 25 IU/ml potassium heparin. The hematocrit of the samples is determined in duplicate by centrifugation. Aliquots of 100 µl are taken to measure the initial sodium content of the erythrocytes.

To determine the amiloride sensitive sodium influx into erythrocytes, 100 µl of each blood sample are added to 5 ml of buffer made hyperosmolar by sucrose (140 mM NaCl, 3 mM KCl, 150 mM sucrose, 0.1 mM ouabain, 20 mM tris-hydroxymethylaminomethane, pH 7.4) and incubated for 60 min at 37°C. Subsequently, the erythrocytes are washed three times in ice-cold $MgCl_2$-ouabain-solution (112 mM $MgCl_2$, 0.1 mM ouabain).

For determination of intracellular sodium content, the cells are hemolyzed in distilled water, the cell membranes are centrifuged and the sodium concentration of the haemolysate is measured by flame photometry. Net influx of sodium into the erythrocytes is calculated from the difference between the initial sodium content and the sodium content after incubation. Amiloride-sensitive sodium influx is calculated from the difference between sodium content of erythrocytes incubated with and without amiloride (3×10^{-4} M). Each experiment is done with the erythrocytes from 6 different animals. In each case, the comparison of Na^+ contents is based on erythrocytes from the same animal. Doses between 10^{-4} and 10^{-7} M of the inhibitor are tested.

EVALUATION

Statistical analysis of the data obtained is performed with Student's *t*-test for paired groups. IC_{50} values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

Scholz W, Albus U, Hropot M, Klaus E, Linz W, Schölkens BA (1990) Zunahme des Na^+/H^+-Austausches an Kaninchenerythrozyten unter atherogener Diät. In: Assmann G, Betz E, Heinle H, Schulte H (eds) Arteriosklerose – Neue Aspekte aus Zellbiologie und Molekulargenetik. Epidemiologie und Klinik. Vieweg, Braunschweig, Wiesbaden. pp 296–302

Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Schölkens BA (1992) Effects of Na^+/H^+ exchange inhibitors in cardiac ischaemia. J Mol Cell Cardiol 24:731–740

Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Schölkens BA (1993) Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. Br J Pharmacol 109:562–568

A.1.1.20.3 Sodium Influx into Cultured Cardiac Myocytes

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in cultured cardiac myocytes (Scholz et al. 1992).

PROCEDURE

Rat myocardial cells are isolated from hearts of neonatal rats by trypsin digestion. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO) in an atmosphere containing 10% CO_2. After confluence, the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs-Ringer-solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, $CaCl_2$ 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37°C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM/l glucose. The culture dishes are then incubated for another 10 min with Na^+-propionate for cytosolic acidification and stimulation of Na^+/H^+ exchange. The compounds are dissolved in 500 µl/dish KRB in which 50% of the sodium chloride has been replaced by choline chloride containing additionally 2 µCi/ml $^{22}Na^+$-bicarbonate, and 5-(N-ethyl-N-isopropyl)amiloride (EIPA). After the stimulation period, sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lyzed with 250 µl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 3×10^{-4} and 10^{-8} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION

Mean values ± SD are compared with Student's *t*-test. IC_{50} values are calculated from dose-response curves.

REFERENCES AND FURTHER READING

Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Schölkens BA (1992) Effects of Na^+/H^+ exchange inhibitors in cardiac ischaemia. J Mol Cell Cardiol 24:731–740

A.1.1.20.4 Inhibition of Na^+/H^+ Exchange into Cultured Aortic Endothelial Cells

PURPOSE AND RATIONALE

The inhibition of Na^+/H^+ exchange has been studied in endothelial cells (Scholz et al. 1993) by measuring the $^{22}Na^+$ influx.

PROCEDURE

Bovine aortic endothelial cells (BAEC) are isolated by dispase digestion form bovine aorta obtained from animals killed at the local slaughter house. The cells are cultured in 35 mm dishes and grown to confluence in Dulbecco's Minimum Essential Medium (DMEM, GIBCO) in an atmosphere with 10% CO_2. Three days after confluence the cells are used for measurement of $^{22}Na^+$ influx. The cells are washed twice with Krebs-Ringer solution buffered with HEPES/Tris (KRB) in which sodium chloride has been replaced by choline chloride (Choline chloride 130 mM, $CaCl_2$ 1.5 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 20 mM, pH 7.0 with Tris) and then incubated for 20 min at 37°C in the same buffer with added 0.1% bovine serum albumin (BSA) and 10 mM glucose. To stimulate Na^+/H^+ exchange the culture dishes are incubated for another 10 min with 500 µl/dish KBR in which all sodium chloride has been replaced by 65 mM each of choline chloride and Na^+-propionate or with KBR in which 50% of the sodium chloride has been replaced by choline chloride for unstimulated controls. In addition, the buffer contains 2 µCi/ml $^{22}Na^+$ and the test compounds or the standard. After the stimulation period, the sodium influx is terminated by washing the cells twice with ice-cold stop solution (0.1 mM $MgCl_2$, 10 mM Tris, pH 7.0). Subsequently, the cells are lyzed with 250 µl trichloroacetic acid and scraped from the dishes. Radioactivity is determined in a Packard gamma counter. Doses between 10^{-5} and 10^{-7} mM/l of standard and new compounds are tested. Six dishes are used for each concentration of test compounds.

EVALUATION

Mean values ± SD are compared with Student's *t*-test. IC_{50} values are calculated from dose-response curves.

MODIFICATIONS OF THE METHOD

Ewart et al. (1997) studied lipoprotein lipase activity in cultured rat cardiomyocytes in the presence of insulin and dexamethasone.

REFERENCES AND FURTHER READING

Benos DJ, Cunningham S, Baker RR, Beason KB, Oh Y, Smith PR (1992) Molecular characteristics of amiloride-sensitive sodium channels. Rev Physiol Biochem Pharmacol 120:31–113

Ewart HS, Carroll R, Severson DL (1997) Lipoprotein lipase activity in rat cardiomyocytes is stimulated by insulin and dexamethasone. Biochem J 327:439–442

Frelin C, Vigne P, Lazdunski M (1985) The role of Na^+/H^+ exchange system in the regulation of internal pH in cultured cardiac cells. Eur J Biochem 149:1–4

Frelin C, Vigne P, Breittmayer JP (1990) Mechanism of the cardiotoxic action of palytoxin. Mol Pharmacol 38:904–909

Jean T, Frelin C, Vigne P, Lazdunski M (1986) The Na^+/H^+ exchange system in glial cell lines. Properties and activation by an hyperosmotic shock. Eur J Biochem 160:211–219

Scholz W, Albus U (1993) Na^+/H^+ exchange and its inhibition in cardiac ischemia and reperfusion. Bas Res Cardiol 88:443–455

A.1.1.20.5 NHE Activity Measured by Intracellular pH in Isolated Ventricular Myocytes

PURPOSE AND RATIONALE

Changes of the intracellular pH of cultured bovine endothelial cells have been fluorometrically monitored using the pH-dye 2′,7′-bis(carboxyethyl)carboxyfluorescein (BCECF) by Kitazano et al. (1988). This method has been used to study the activity of inhibitors of Na^+/H^+ exchange (Scholz et al. 1995).

PROCEDURE

For preparation of isolated rat ventricular muscular cells (Yazawa et al. 1990), hearts of male Wistar rats are dissected, mounted on a Langendorff apparatus and perfused first at 37°C for 3 min with Tyrode solution adjusted to pH 7.4, second for 5–7 min with nominally calcium free Tyrode solution and finally with calcium free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma type I). After 15–20 min collagenase treatment, the heart is washed with storage solution (composition in mmol/L: KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 20, HEPES 10, and EGTA 0.5, pH 7.4). The ventricles are cut into small pieces and myocytes are dispersed by gently shaking and finally by filtration through a nylon mesh (365 µm). Thereafter, the cells are washed twice by centrifugation at 600–1000 rpm for 5 min and kept at 4°C until use. For the pH recovery experiment the cells are loaded with the membrane permeable acetoxymethyl ester (AM) form of the fluorescent indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). BCECF-AM is dissolved in DMSO and diluted to a 1.25 µM storage solution. Cardiomyocytes are loaded in this solution for 30 min at room temperature and are then centrifuged and resuspended in storage solution. The measure-

ments are performed in bicarbonate-free NaCl solution (NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1, glucose 10, and HEPES 10 mM/L, pH 7.4) at 34°C using an apparatus according to Nitschke et al. (1991). The pH-dependent signal of BCECF is obtained by illuminating at 490 and 437 nm and dividing the emitted light signals (520–560 nm). The background signal, determined by closing the shutter, is subtracted from the total signal. The autofluorescence determined by illuminating unloaded cells can be ignored. In order to investigate the function of the Na^+/H^+ exchange system, the intracellular pH (pH_i) of the cells is decreased by the NH_4Cl prepulse technique and the rate of return to resting pH_i is determined. Test compounds are dissolved in the incubation medium. For each test concentration, the recovery of pH_i is first recorded in control NaCl solution.

EVALUATION

Data are analyzed by fitting a straight line to the initial (5 min) data points of the pH recovery curve. For statistical presentation, the slopes of the linear curves are demonstrated. All reported data are presented as means ± SEM. Statistical comparisons are made using either a paired or unpaired *t*-test.

MODIFICATIONS OF THE METHOD

The pH-sensitive fluorescence dye C-SNARF-1 (= carboxy-semi-naphtho-rhoda-fluor 1) was used by Yatsutake et al. (1996), Shipolini et al. (1997), and Yokoyama et al. (1998).

Fischer et al. (1999) tested new drugs for the Na^+/H^+ exchanger in Chinese hamster ovary cells which are enriched with the NHE-1 isoform of the Na^+/H^+ anti-porter. The Na^+/H^+ exchanger was stimulated with NaCl and the rate of extracellular acidification was quantified with the Cytosensor.

REFERENCES AND FURTHER READING

Fischer H, Seelig A, Beier N, Raddatz P, Seelig J (1999) New drugs for the Na^+/H^+ exchanger. Influence of Na^+ concentration and determination of inhibition constants with a microphysiometer. J Membr Biol 1:39–45

Kitazono T, Takeshige K, Cragoe EJ, Minakami S (1988) Intracellular pH changes of cultured bovine aortic endothelial cells in response to ATP addition. Biochem Biophys Res Commun 152:1304–1309

Nitschke R, Fröbe U, Greger R (1991) Antidiuretic hormone acts via V_1 receptor in intracellular calcium in the isolated perfused rabbit cortical thick ascending limb. Pflüger's Arch 417:622–632

Scholz W, Albus U, Counillon L, Gögelein H, Lang H-J, Linz W, Weichert A, Schölkens BA (1995) Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc Res 29:260–268

Shipolini AR, Yokoyama H, Galiñanes M, Edmondson SJ, Hearse DJ, Avkiran M (1997) Na^+/H^+ exchanger activity does not contribute to protection by ischemic preconditioning in the isolated rat heart. Circulation 96:3617–3625

Yasutake M, Haworth RS, King A, Avkiran M (1996) Thrombin activates the sarcolemmal Na^+/H^+ exchanger: evidence for a receptor-mediated mechanism involving protein kinase C. Circ Res 79:705–715

Yazawa K, Kaibara M, Ohara M, Kamayama M (1990) An improved method for isolating cardiac myocytes useful for patch-clamp studies. Jpn J Physiol 40:157–163

Yokoyama M, Yasutake M, Avkiran M (1998) α_1-adrenergic stimulation of sarcolemnal Na^+/H^+ exchanger activity in rat ventricular myocytes: evidence for selective mediation by the α_{1A}-adrenoceptor subtype. Circ Res 82:1078–1985

A.1.1.20.6 NHE Subtype Specificity

PURPOSE AND RATIONALE

Molecular identification of mammalian Na^+/H^+ exchanger subtypes has been pioneered by Pouysségour and coworkers (Sardet et al. 1989) who used genetic complementation of fibroblast cell lines that lack all endogenous NHEs. Schwark et al. (1998) studied an inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types.

PROCEDURE

cDNAs for the NHE subtypes human NHE1, rabbit NHE2, rat NHE3 (Pouysségour) or cloned by reverse transcriptase-polymerase chain reaction from human kidney mRNA are used. These cDNAs are cloned into the mammalian expression vector pMAMneo and transferred into the NHE-deficient mouse fibroblast cell line LAP1. Cells expressing the NHE subtypes are selected by the acid load survival method (Sardet et al. 1989). Clonal cell lines for each subtype are used for intracellular pH (pH_i) recovery after acid load. For studies of pH_i recovery (Faber et al. 1996), cells are scraped off the culture dishes washed and incubated with 5 µmol/l BCECF-AM [2′,7′-bis(2-carboxyethyl)-5,6-carboxy-fluorescein-acetoxy-methyl ester] for 20 min at 37°C in a buffer containing 20 mM NH_4Cl. The cells are then washed to remove extracellular dye and resuspended in the loading buffer without BCECF-AM. Intracellular acidification is induced by addition of 975 µl NH_4Cl-free and HCO_3^--free solution (so-called recovery medium: HCO_3^--free to inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger of LAP1 cells) to an 25-µl aliquot of cells (≅25,000 cells). The pH_i recovery is recorded with a dual-grating Deltascan single-photon counting fluorometer (Photon Technology International, South Brunswick, NJ, USA) with exci-

tation wavelength of 505 nm and 440 nm and an emission wavelength of 535 nm. The measurement time varies between subtypes (120 s for NHE1, 300 s for NHE2, 180 s for NHE3). The inhibitors are first dissolved in DMSO, diluted in recovery medium and added in a volume of 975 µl to this medium.

A cloned opossum kidney cell line (Helmle-Kolb et al. 1990) is used additionally. Cells are grown as a monolayer in growth medium (1:1 mixture of nutrient mixture Ham F12 and Dulbecco's modified medium Eagle with 10% fetal calf serum). For subcultivation and pH-recovery experiments, the cells are detached from the surface of the culture vessels with trypsin-EDTA solution (2.5 g trypsin+0.2 g EDTA per liter in Dulbecco's phosphate-buffered saline) and suspended in growth medium. Measurement time in pH_i recovery experiments is 400 s.

Porcine renal brush-border membrane vesicles (BBMV) prepared by a Mg^{2+} precipitation technique are loaded with 150 mmol/l NaCl, 5 mmol/l HEPES/Tris, pH 7.0, and pre-incubated for 10 min at 37°C with various concentrations of NHE inhibitors. Intravesicular acidification through Na^+/H^+ exchange is started by diluting BBMV into Na^+-free buffer (150 mmol/l tetramethylammonium chloride, 5 mmol HEPES/Tris, pH 7.0) containing the appropriate concentrations of the NHE inhibitors and the fluorescent Δ pH indicator acridine orange (12 µmol/l). The fluorescence changes of acridine orange are recorded continuously by a Hitachi F-2000 spectrofluorometer at 495 nm excitation and 525 nm emission wavelength. The initial acridine orange fluorescence quenching in controls (no inhibitor) is set to 100%.

EVALUATION

Values are presented as means ± SD (four measurement per concentration). The IC_{50} values and Hill coefficients are calculated using the Sigma plot software. Statistical significance is calculated by means of the distribution-independent *H*-test and non-parametric *U*-test. $P < 0.05$ is considered as significant.

MODIFICATIONS OF THE METHOD

Counillon et al. (1993), Scholz et al. (1995) determined the NHE subtype specificity of Na^+/H^+ antiporters by their ability to inhibit initial rates of amiloride sensitive $^{22}Na^+$ uptake in fibroblast cell lines separately expressing the NHE-1, NHE-2 and NHE-3 isoforms.

Ko et al. (2004) determined the inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships.

REFERENCES AND FURTHER READING

Counillon LT, Scholz W, Lang HJ, Pouysségour J (1993) Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. Mol Pharmacol 44:1041–1045

Faber S, Lang HJ, Hropot M, Schölkens BA, Mutschler E (1996) A novel screening assay of the Na^+-dependent $Cl^-/HCO3^-$ exchanger (NCBE) and its inhibitors. Cell Physiol Biochem 6:39–49

Helmle-Kolb C, Montrose MH, Stange G, Murer H (1990) Regulation of Na^+/H^+ exchange in opossum kidney cells by parathyroid hormone, cyclic AMP and phorbol esters. Pflüger's Arch 415:461–470

Ko WC, Shih CM, Lai YH, Chen JH, Huang HL (2004) Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships. Biochem Pharmacol 68:2087–2094

Sardet C, Franchi A, Pouysségour J (1989) Molecular cloning, primary structure, and expression of the human growth factor-activable Na^+/H^+ antiporter. Cell 56:271–280

Scholz W, Albus U, Counillon L, Gögelein H, Lang H-J, Linz W, Weichert A, Schölkens BA (1995) Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc Res 29:260–268

Schwark JR, Jansen HW, Lang HJ, Krick W, Burckhardt G, Hropot M (1998) S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. Pflüger's Arch Eur J Physiol 436:797–800

A.1.1.21 Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and/or cGMP. They function with adenyl and guanylyl cyclases to regulate the amplitude and duration of responses triggered by the second messengers cAMP and cGMP. The enzyme phosphodiesterase (PDE) exists in various forms. At least 11 families of phosphodiesterases have been identified (Torphy and Page 2000; Francis et al. 2001; Maurice et al. 2003; Lugnier 2006). The properties and functions of GAF domains in cyclic nucleotide phosphodiesterases are reviewed by Zoraghi et al. (2004).

Hofmann et al. (2006) reviewed the nomenclature and structure–function relationships of cyclic nucleotide-regulated channels.

Mongillo et al. (2004) reported fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes, revealing distinct functions of compartmentalized phosphodiesterases. Studying real-time monitoring of PDE2 activity in live cells, Nikolaev et al. (2005) found that hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis.

Snyder et al. (2005) studied the role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis.

Zhang et al. (2004) desribed a glutamine switch mechanism for nucleotide selectivity by phosphodiesterases.

REFERENCES AND FURTHER READING

Francis SH, Turko IV, Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. Prog Nucleic Acid Res Mol Biol 65:1–52

Hofmann F, Biel M, Kaupp UB (2006) International Union of Pharamcology. LI. Nomenclature and structure-function relationships of cyclic nucleotide-regulated channels. Pharmacol Rev 57:455–462

Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol Ther 109:366–398

Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, Jimmo SL (2003) Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol Pharmacol 64:533–546

Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95:67–75

Snyder PB, Esselstyn JM, Loughney K, Wolda SL, Florio VA (2005) The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis. J Lipid Res 46:494–503

Torphy TJ, Page C (2000) Phosphodiesterases: the journey towards therapeutics. Trends Pharmacol Sci 21:157–159

Zhang KYJ, Card GL, Suzuki Y, Artis DR, Fong D, Gillette S, Hsieh D, Neiman J, West BL, Zhang C, Milburn MV, Kim SH, Schlessinger J, Bollag G (2004) A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. Mol Cell 15:279–286

Zoraghi R, Corbin JD, Francis SH (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. Mol Pharmacol 65:267–278

A.1.1.21.1 Inhibition of Phosphodiesterase

PURPOSE AND RATIONALE

The inhibition of cAMP-PDE and cGMP-PDE by various test compounds can be measured using a two-step radioisotopic procedure.

PROCEDURE

Materials

[8-^{3}H]cAMP (28 Ci/mmol), [8-^{3}H]-cGMP (15 Ci/mmol) and [U^{14}C]guanosine (528 mCi/mmol) are obtained from Du Pont de Nemours (Paris, France). Unlabelled cyclic nucleotides, 5′-nucleotidase (Ophiophagus hannah venom) are from the Sigma Chemical Co. (La Verpillére, France).

Tissue preparations

Male Sprague-Dawley rats (250–300 *g*) are decapitated. Hearts are perfused with 0.15 M NaCl through the aorta to remove the blood. The ventricles are minced in 5 vol. of 10 mM Tris-HCl buffer containing 0.32 M sucrose, 1 mM EDTA, 5 nM DTT and 0.1 mM PMSF at pH 7.5. The suspension is homogenized in a glass-glass Potter-Elvejhem. The homogenate is then centrifuged at 105,000 *g* for 60 min. The 105,000 *g* supernatant is stored at –75°C until injection on the HPLC column.

Isolation of PDEs

The cytosolic fraction from rat ventricles (5–8 mg of protein) is loaded at the rate of 1 ml/min on a Mono Q HPLC column which has been previously equilibrated with buffer A (50 mM Tris-HCl, 2 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5). Under these conditions, greater than 95% of the PDE activity is bound to the column. PDE activity is eluted at a flow rate of 1 ml/min using the following step by step and linear gradients of NaCl in buffer A: 25 ml of 0.16 M NaCl, 20 ml of 0.23 M NaCl, 30 ml from 0.23 to 0.29 M NaCl, 15 ml of 0.29 M NaCl, 30 ml from 0.29 to 0.50 M NaCl. The separation is done at 4°C. Fractions of 1 ml are collected and stored at –75°C in the presence of 20% glycerol. The fractions are tested for PDE activity, and the peaks containing the different isoenzymes are identified. Fractions containing preferentially one isoenzyme are pooled.

PDE Assay

PDE activity is assayed by a two-step radioisotopic procedure according to Thompson et al. (1974), Boudreau and Drummond (1975), Prigent et al. (1981). cAMP-PDE and cGMP-PDE activities are measured with a substrate concentration of 0.25 µM. To evaluate the cGMP-stimulated PDE activity, assays are performed with 5 µM cAMP in the absence or presence of 5 µM cGMP. Xanthine derivates are dissolved in DMSO. The stock solutions are appropriately diluted with 40 mM Tris-HCl buffer so that the final DMSO concentration in the PDE assay does not exceed 1%. At this concentration, DMSO has no significant effect on the PDE activity of any of the fractions. The inhibitory potency of the xanthine derivates is examined on each separated isoform.

EVALUATION

The IC_{50} values (concentration of a drug which inhibit 50% of the enzymatic activity) are calculated by plotting the percentage of residual enzymatic activity ver-

sus the logarithmic concentration of the drug. Confidence limits (95%) for the IC_{50} values are determined by linear regression analysis.

MODIFICATIONS OF THE METHOD

Phosphodiesterase activity can be determined using ^{32}P-labelled GPT (Reinsberg 1999). The reaction is performed using an Eppendorf cup with 10 mM GTP, the test solution and [^{32}P]GTP. The reaction is started with 1 mM sodium nitroprusside solution. After an incubation period of 20 min at 37°C, the reaction is stopped with 500 µl of 120 mM sodium carbonate and 400 µl of zinc acetate, whereby the zinc carbonate and the substrate GTP are almost (about 90%) all precipitated, and so separated from cGMP. The precipitate is separated by centrifugation and the supernatant submitted to chromatography on an aluminum oxide column. The column is first treated with Tris HCl buffer. From the supernatant, 900 ml is applied. After two washing periods, the cGMP is eluted in five fractions. These fractions are measured and the highest activities are pooled.

The PDE activity is calculated from the difference between applied [^{32}P]GTP minus unaltered [^{32}P]GTP.

REFERENCES AND FURTHER READING

Boudreau RJ, Drummond GI (1975) A modified assay of 3′,5′-cyclic-AMP phosphodiesterase. Anal Biochem 63:388–399

Meskini N, Némoz G, Okyayuz-Baklouti I, Lagarde M, Prigent AF (1994) Phosphodiesterase inhibitory profile of some related xanthine derivatives pharmacologically active on the peripheral microcirculation. Biochem Pharmacol 47:781–788

Pichard AL, Cheung WY (1976) Cyclic 3′:5′-nucleotide phosphodiesterase. Interconvertible multiple forms and their effects on enzyme activity and kinetics. J Biol Chem 251:5726–5737

Prigent AF, Némoz G, Yachaoui Y, Pageaux JF, Pacheco H (1981) Cyclic nucleotide phosphodiesterase from a particulate fraction of rat heart. Solubilization and characterization of a single enzymatic form. Biochem Biophys Res Commun 102:355–364

Prigent AF, Fougier S, Nemoz G, Anker G, Pacheco H, Lugnier C, Lebec A, Stoclet JC (1988) Comparison of cyclic nucleotide phosphodiesterase isoforms from rat heart and bovine aorta. Separation and inhibition by selective reference phosphodiesterase inhibitors. Biochem Pharmacol 37:3671–3681

Reinsberg L (1999) Personal Communication

Terai M, Furihata C, Matsushima T, Sugimura T (1976) Partial purification of adenosine 3′, 5′-cyclic monophosphate phosphodiesterase from rat pancreas in the presence of excess protease inhibitors. Arch Biochem Biophys 176:621–629

Thompson WJ, Brooker G, Appleman MM (1974) Assay of cyclic nucleotide phosphodiesterases with radioactive substrates. In: Hardman JG, O'Malley BW (eds) Methods in enzymology, Vol 38. Academic Press, New York, pp 205–212

Description of Phosphodiesterase Isoforms

Phosphodiesterase 1

PDE1 family variants are activated upon Ca^{2+}/calmodulin binding (Kakkar et al. 1999; Goraya and Cooper 2005; Sharma et al. 2006). The PDE1 subfamily consists of three different gene products (PDFE1A, PDE1B, and PDE1C) which differ in their regulatory properties, substrate affinities, specific activities for calmodulin, tissue distribution, and molecular weights (Yan et al. 1996; Yu et al. 1997). PDE1 is present in brain, cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells (Maurice et al. 2003). Inhibition has been described for nimodipine (Epstein et al. 1982) and for vinpocetine (Hagiwara et al. 1984).

Phosphodiesterase 2

A single *PDE2* gene encodes three PDE2 variants (Rosman et al. 1997). Martinez et al. (2002) studied the crystal structure of murine PD2A and found that the two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and cGMP binding. PDE2 is found in various areas of the brain (Lugnier 2006), in adrenal medulla, heart, rat ventricle (Yanaka et al. 2003), liver, and brown adipose tissue (Coudray et al. 1999). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was shown to specifically act on PDE2 by inhibiting cCMP-activated PDE2 (Podzuweit et al. 1995).

Chambers et al. (2006) described a new chemical tool for exploring the physiological function of the PDE2 isozyme.

Phosphodiesterase 3

The PDE3 family is composed of two genes, *PDE3A* and *PDE3B*. *PDE3A* mRNA is enriched in blood vessels, heart, megakaryocytes and oocytes, whereas *PDE3B* is highest in in adipocytes, hepatocytes, brain, renal collecting duct epithelium, and developing spermatocytes (Reinhardt et al. 1995). PDE3 inhibitors have been extensively investigated and developed as non-glycoside, non-sympathomimetic, cardiotonic agents for the treatment of heart failure. Milrinone is the most studied and most extensively used PDE3 inhibitor and is used in the acute treatment of heart failure (Cruickshank 1993). Trequinsin (HL 725) inhibits PDE3 in a nanomolar range (Ruppert and Weithmann 1982); it also inhibits PDE1, PDE2, and PDE4 in submicromolar concentrations (Stoclet et al. 1995).

Boswell-Smith et al. (2006) studied the pharmacology of two long-acting trequinsin-like phosphodiesterase 3/4 inhibitors, RPL554 and RPL565.

Hambleton et al. (2005) studied isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium.

Masciarelli et al. (2004) described mice deficient of cyclic nucleotide phosphodiesterase 3A as a model of female infertility.

Adachi et al. (2005) reported the effects of a phosphodiesterase 3 inhibitor, olprinone, on rhythmical change in the tension of human gastroepiploic artery.

Abbott and Thompson (2006) performed analysis of anti-PDE3 activity of 2-morpholinochromone derivates revealing multiple mechanisms of antiplatelet activity.

Phosphodiesterase 4

PDE4 is mainly present in the brain (Houslay et al. 1998), inflammatory cells (Tenor and Schudt 1996), cardiovascular tissues (Stoclet et al. 1995), and smooth muscles, but is lacking in the platelets. Four *PDE4* genes (*PDE4A–D*) yield a large number of distinct PDE4 varaints. These enzymes, which result from the use of alternate promoters and extensive splicing of PDE4 mRNAs, are stratified into long or short forms (Conti et al. 2003).

One *PDE4A*, three *PDE4B* (PDE4B1, PDE4B2, and PDE4B3), and three *PDE4D* (PDE4D1, PDE4D2, PDE4D3) variants are expressed in rat and human cardiac tissue (Houslay and Adams 2003). Two *PDE4D* gene-derived variants, PDE4D3 and PDE4D6, are expressed in human and rat aortic, mesenteric, and femoral contractile/quiescent and synthetic/activated vascular smooth muscle cells (Liu et al. 2000).

The role of cAMP-specific PDE4 phosphodiesterases in cellular signaling was reviewed by Houslay and Adams (2003) and Conti et al. (2003).

Huai et al. (2003) studied the three-dimensional structures of PDE4D in a complex with roliprams and the implication for inhibitor selectivity.

Baillie et al. (2003) found that β-arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from G_s to G_i.

The antidepressant compound rolipram (ZK 62711) is a selective inhibitor of PDE4 (Schwabe et al. 1976; Komas et al. 1989). O'Donnell and Zhang (2004) described the antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Many analogs were studied as PDE4 inhibitors for treatment of asthma and chronic obstructive pulmonary disease (COPD) as well as anti-inflammatory drugs; however, most failed due to emetic side-effects (Giembycz 2002).

Phosphodiesterase 5

Selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (**PDE5**) were found to be effective in the treatment of erectile dysfunction in men (Jeremy et al. 1997; Ballard et al. 1998; Chuang et al. 1998; Stief et al. 1998; Corbin and Francis 1999; Turko et al. 1999; Wallis et al. 1999; Hosogai et al. 2001; Rotella 2001; Saenz de Tejada et al. 2001; Ukita et al. 2001).

The PDE5 family consists of a single *PDE5* gene that can encode three distinct proteins (PDE5A1–3) (Loughney et al. 1998).

Wang et al. (2001) characterized type 5 phosphodiesterases in the corpus cavernosum of several species.

Qiu et al. (2000) demonstrated that rabbit corpus cavernosum smooth muscle shows a different phosphodiesterase profile than human corpus cavernosum.

Kim et al. (2001) compared the inhibition of cyclic GMP hydrolysis in human corpus cavernosum smooth muscle cells by vardenafil with that by sildenafil.

The discovery of novel, potent, and selective PDE5 inhibitors was reported by Bi et al. (2001).

Corbin et al. (2004) described the structural basis for the higher potency of vardenafil compared with sildenafil in inhibiting cCMP-specific phosphodiesterase-5 (PDE5).

PDE5 inhibitors such as sildenafil can be used in the treatment of pulmonary arterial hypertension (Galié et al. 2005).

Cohen et al. (1996) found that inhibition of cGMP-specific phosphodiesterase selectively vasodilates the pulmonary circulation in rats made chronically hypoxic by exposure to a simulated high altitude. Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling (Jernigan et al. 2004).

Thompson et al. (2001) studied the effect of sildenafil on corpus cavernosal smooth muscle relaxation and cGMP formation in the diabetic rabbit.

Tantini et al. (2005) found an antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells.

Other Phosphodiesterases

The families of **PDE6 to PDE11** have been identified as a result of bioinformatics-based genomic screening (Soderling and Beavo 2000). The search for inhibitors is just beginning.

Gillespie and Beavo (1989) discussed inhibition and stimulation of photoreceptor phosphodiesterases (later on termed **PDE6**) by dipyridamole and M&B 22,948.

D'Armours et al. (1999) investigated the potency and mechanism of action of E4021, a type 5 phosphodiesterase isozyme-selective inhibitor, on the photoreceptor phosphodiesterase.

Zhang et al. (2005) described the efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (**PDE6**) in retinal photoreceptors.

Smith et al. (2004) reported the discovery of a selective inhibitor of **phosphodiesterase 7**.

The discovery of thiadiazoles as a novel structural class of potent and selective **PDE7 inhibitors** was reported by Vergne et al. (2004a, 2004b).

Lorthiois et al. (2004) and Bernadelli et al. (2004) described spiroquinazolinones as potent and selective **PDE7** inhibitors.

The first potent and selective **PDE9** inhibitor was characterized by Wunder et al. (2005) using a cGMP reporter cell line.

REFERENCES AND FURTHER READING

Abbott BM, Thompson PE (2006) Analysis of anti-PDE3 activity of 2-morpholinochromone derivates reveals multiple mechanisms of anti-platelet activity. Bioorg Med Chem Lett 16:969–973

Adachi H, Kakiki M, Kishi Y (2005) Effects of a phosphodiesterase 3 inhibitor, olprinone, on rhythmical change in tension of human gastroepiploic artery. Eur J Pharmacol 528:137–143

Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD (2003) β-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from G_s to G_i. Proc Natl Acad Sci USA 100:940–945

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM (1998) Effects of sildenafil on the ralaxation of human corpus cavernosum tissue in vitro and the activities of cyclic nucleotide phosphodiesterase isozymes. J Urol 159:2164–2171

Bernadelli P, Lorthiois E, Vergne F, Oliveira C, Mafroud AK, Proust E, Pham N, Ducrot P, Moreau F, Idrissi M, Tertre A, Bertin B, Coupe M, Chevalier E, Descours A, Berliozseux F, Berna P, Li M (2004) Spiroquinazolinones as novel, potent and selective PDE7 inhibitors. Part 2: Optimization of 5,8-disubstituted derivatives. Bioorg Med Chem Lett 14:4627–4631

Bi Y, Stoy P, Adam L, He B, Krupinski J, Normandin D, Pongrac R, Seliger L, Watson A, Macor JE (2001) The discovery of novel, potent and selective PDE5 inhibitors. Bioorg Med Chem Lett 11:2461–2464

Boswell-Smith V, Spina D, Oxford AW, Comer MB, Seeds EA, Page CP (2006) The pharmacology of two novel long-acting phosphodiesterase 3/4 inhibitors, RPL554 [9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(n-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one] and RPL565 [6,7-dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4H-pyrimido[6,1-a]isoquinolin-4-one]. J Pharmacol Exp Ther 318:840–848

Chambers RJ, Abrams K, Garceau NY, Kamath AV, Manley CM, Lilley SC, Otte DA, Scott DO, Sheils AL, Tess DA, Vellekoop AS, Zhang Y, Lam KT (2006) A new chemical tool for exploring the physiological function of the PDE2 isozyme. Bioorg Med Chem Lett 16:307–310

Chuang AT, Strauss JD, Murphy RA, Steers WD (1998) Sildenafil, a type-5 cGMP phosphodiesterase inhibitor, specifically amplifies cGMP-dependent relaxation in rabbit corpus cavernosum muscle *in vitro*. J Urol 160:257–261

Cohen AH, Hanson K, Morris K, Fouty B, MacMurty IF, Clarke W, Rodman DM (1996) Inhibition of cyclic 3′-5′-guanosine monophosphate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. J Clin Invest 97:172–179

Conti M, Richter W, Mehats C, Livera G, Park JY, Jin G (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem 278:5493–5496

Corbin JD, Francis SH (1999) Cyclic GMP phosphodiesterase-5: Target of sildenafil. J Biol Chem 274:13729–13732

Corbin JD, Beasley A, Blount MA, Francis SH (2004) Verdenafil: structural basis for higher potency over sildenafil in inhibiting cCMP-specific phosphodiesterase-5 (PDE5). Neurochem Int 45:859–863

Coudray C, Charon C, Komas N, Mory G, Diot-Dupuy F, Manganiello V, Ferre P, Bazin R (1999) Evidence for the presence of several phosphodiesterase isoforms in brown adipose tissue of Zucker rats: modulation of PDE2 by the fa gene expression. FEBS Lett 456:207–210

Criuckshank JM (1993) Phosphodiesterase III inhibitors: long-term risks and short-term benefits. Cardiovasc Drugs Ther 7:655–660

D'Armours MR, Granovsky AE, Artemyev NO, Cote RH (1999) Potency and mechanism of action of E4021, a type 5 phosphodiesterase isozyme-selective inhibitor, on the photoreceptor phosphodiesterase depend on the state of activation of the enzyme. Mol Pharmacol 3:508–514

Epstein PM, Fiss K, Hachisu R, Andrenyak DM (1982) Interaction of calcium antagonists with cyclic AMP phosphodiesterase and calmodulin. Biochem Biophys Res Commun 105:1142–1149

Galié N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simmoneau G (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. N Engl J Med 353:2148–2157

Giembycz MA (2002) Development status of second generation PDE4 inhibitors for asthma and COPD: the story so far. Monaldi Arch Chest Dis 57:48–64

Gillespie PG, Beavo JA (1989) Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948. Mol Pharmacol 36:773–781

Goraya TA, Cooper DMF (2005) Ca^{2+}-calmodulin-dependent phosphodiesterase (PDE1): current perspectives. Cell Sign 17:789–797

Hagiwara M, Endo T, Hidaka H (1984) Effects of vinpocetine on cyclic nucleotide metabolism in vascular smooth muscle. Biochem Pharmacol 33:453–457

Hambleton R, Krall, Tikishvili E, Honeggar M, Ahmad F, Manganiello VC, Movsesian MA (2005) Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium. J Biol Chem 280:39168–39174

Hosogai N, Hamada K, Tomita M, Nagashima A, Takahashi T, Sekizawa T, Mizutani T, Urano Y, Kuroda A, Sawada K,

Ozaki T, Seki J, Goto T (2001) FR226807: a potent and selective phosphodiesterase type 5 inhibitor. Eur J Pharmacol 428:295–302

Houslay MD, Sullivan M, Bolger GB (1998) The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective inhibition by compounds exerting anti-inflammatory and antidepressant actions. Adv Pharmacol 44:225–342

Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signaling cross-talk, desensitization and compartmentalization. Biochem J 370:1–18

Huai G, Wang H, Sun Y, Kim HY, Liu Y, Ke H ((2003) Three-dimensional structures of PDE4D in complex with roliprams and implication on inhibitor selectivity. Structure 11:865–873

Jeremy JY, Ballard SA, Naylor AM, Miller MAW, Angelini GD (1997) Effects of sildenafil, a type-5 cGMP phosphodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum *in vitro*. Br J Urol 79:958–963

Jernigan NL, Walker BR, Resta TC (2004) Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling. Am J Physiol 287:L1220–L1229

Kakkar R, Raju RVS, Sharma RK (1999) Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). Cell Mol Life Sci 55:1164–1185

Kim NN, Huang YH, Goldstein I, Bischoff E, Traish AM (2001) Inhibition of cyclic GMP hydrolysis in human corpus cavernosum smooth muscle cells by vardenafil, a novel, selective phosphodiesterase type 5 inhibitor. Life Sci 69:2249–2256

Komas N, Lugnier C, Le Bed A, Serradeil-Le Gal C, Barthelemy G, Stoclet JC (1989) Differential sensitivity to cardiotonic drugs of cyclic AMP phosphodiesterases isolated from canine ventricular and sinoatrial-enriched tissues. J Cardiovasc Pharmacol 14:213–220

Liu H, Palmer D, Jimmo SL, Tilley DG, Dunkerley HA, Pang SC, Maurice DH (2000) Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways. A potential mechanism allowing for the coordinated regulation of PDE4D activity and expression in cells. J Biol Chem 275:26615–26624

Lorthiois E, Bernadelli P, Vergne F, Oliveira C, Mafroud AK, Proust E, Heuze L, Moreau F, Idrissi M, Tertre A, Bertin B, Coupe M, Wrigglesworth R, Decours A, Soulard P, Berna P (2004) Spiroquinazolinones as novel, potent and selective PDE7 inhibitors. Part 1. Bioorg Med Chem Lett 14:4623–4626

Loughney K, Hill TR, Florio VA, Uher L, Rosman GJ, Wolda SL, Jones BA, Howard ML, McAllister-Lucas LM, Sonnenburg WK, Francis SH, Corbin JD, Beavo JA, Ferguson K (1998) Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding cGMP-specific 3′, 5′-cyclic nucleotide phosphodiesterase. Gene 216:139–147

Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol Ther 109:366–398

Martinez SE, Wu AY, Glavas NA, Tang XB, Turley S, Hol WGJ (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and cGMP binding. Proc Natl Acad Sci USA 99:13260–13265

Masciarelli S, Horner K, Liu C, Park SH, Hinckley M, Hockman S, Nedachi T, Jin C, Conti M, Manganiello V (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. J Clin Invest 114:196–205

Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, Jimmo SL (2003) Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol Pharmacol 64:533–546

Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95:67–75

Nikolaev VO, Gambaryan S, Engelhardt S, Walter U, Lohse MJ (2005) Real-time monitoring of the PDE2 activity of live cells. J Biol Chem 280:1716–1719

O'Donnell JM, Zhang HT (2004) Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Trends Pharmacol Sci 26:158–163

Podzuweit T, Nennstiel P, Müller A (1995) Isozyme selective inhibition of cCMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hdroxy-3-nonyl) adenine. Cell Signal 7:733–738

Qiu Y, Kraft P, Lombardi E, Clancy J (2000) Rabbit corpus cavernosum smooth muscle shows a different phosphodiesterase profile than human corpus cavernosum. J Urol 164:882–886

Reinhardt RR, Chin E, Zhou J, Taira M, Murata T, Manganiello VC, Bondy CA (1995) Distinctive anatomical patterns of gene-expression for cCMP-inhibited cyclic nucleotide phosphodiesterases. J Clin Invest 95:1528–1538

Rosman GJ, Martins TJ, Sonnenburg WK, Beavo JA, Ferguson K, Loughney K (1997) Isolation and characterization of human cDNAs encoding a cGMP-stimulated 3′, 5′-cyclic nucleotide phosphodiesterase. Gene 191:89–95

Rotella DP (2001) Phosphodiesterase type 5 inhibitors: discovery and therapeutic utility. Drugs Future 26:153–162

Ruppert D, Weithmann KU (1982) HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation. Life Sci 31:2037–2043

Saenz de Tejada I, Angulo J, Cuevas P, Fernández A, Moncada I, Allona A, Lledó E, Körschen I, Niewöhner U, Haning H, Pages E, Bischoff E (2001) The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor vardenafil. Int J Impot Res 13:282–290

Schwabe U, Miyake M, Ohga Y, Daly JW (1976) 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3′,5′-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. Mol Pharmacol 11:900–911

Sharma RK, Das SB, Lakshmikuttyamma A, Selvakumar P, Shrivastav A (2006) Regulation of calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1): Review. Int J Mol Med 18:95–105

Smith SJ, Cieslinski LB, Newton R, Donnelly LE, Fenwick PS, Nicholson AG, Barnes PJ, Barnette MS, Giembycz MA (2004) Discovery of BRL 50481 [3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: In vitro studies in human monocytes, lung macrophages, and $CD8^+$ T-lymphocytes. Mol Pharmacol 66:1679–1689

Soderling SH, Beavo SH (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr Opin Cell Biol 12:174–179

Stief CG, Uckert S, Becker AJ, Truss MC, Jonas U (1998) The effect of the specific phosphodiesterase (PDE) inhibitors on human and rabbit cavernous tissue in vitro and in vivo. J Urol 159:1390–1393

Stoclet JC, Keravis T, Komas N, Lugnier C (1995) Cyclic nucleotide phosphodiesterases as therapeutic targets in cardiovascular diseases. Expert Opin Invest Drugs 4:1081–1100

Tantini B, Manes A, Fiumana E, Pignatti C, Guarnieri C, Zannoli R, Branzi A, Galié N (2005) Antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells. Basic Res Cardiol 100:131–138

Tenor H, Schudt C (1996) Analysis of isoenzyme profiles in cells and tissues by pharmacological methods. In: Schudt C, Dent G, Rabe KF (eds) Phosphodiesterase inhibitors. Handbook of Immunopharmacology. Academic Press, London, pp 21–40

Thompson CS, Mumtaz FH, Khan MA, Wallis RM, Mikhailidis DP, Morgan RJ, Angelini CD, Jeremy JY (2001) The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. Eur J Pharmacol 425:57–64

Turko IV, Ballard SA, Francis SH, Corbin JD (1999) Inhibition of GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. Mol Pharmacol 56:124–130

Ukita T, Nakamura Y, Kubo A, Yamamoto Y, Moritani Y, Saruta K, Higashijima T, Kotera J, Kikawa K, Omori K (2001) Novel, potent, and selective phosphodiesterase 5 inhibitors: synthesis and biological activities of a series of 4-ary-1-isochinoline derivatives. J Med Chem 44:2204–2218

Vergne F, Bernadelli P, Lorthiois E, Pham N, Proust E, Oliveira C (2004a) Discovery of thiadiazoles: a novel structural class of potent and selective PDE7 inhibitors: Part 2: Metabolism-directed optimization studies towards orally bioavailable derivatives. Bioorg Med Chem Lett 14:4615–4621

Vergne F, Bernadelli P, Lorthiois E, Pham N, Proust E, Oliveira C, Mafroud AK, Royer F, Wrigglesworth R, Schellhaas JK, Barvian MR, Moreau F, Idrissi M, Tertre A, Bertin B, Coupe M, Berna P, Soulard P (2004a) Discovery of thiadiazoles as a novel structural class of potent and selective PDE7 inhibitors. Part 1: Design, synthesis and structure-activity relationship studies. Bioorg Med Chem Lett 14:4607–4613

Vergne F, Bernadelli P, Lorthiois E, Pham N, Proust E, Oliveira C, Mafroud AK, Ducrot P, Wrigglesworth R, Berlioz-Seux F, Coleon F, Chevalier E, Moreau F, Idrissi M, Tertre A, Descours A, Berna P, Li M (2004b) Discovery of thiadiazoles as a novel structural class of potent and selective PDE7 inhibitors. Part 2: Metabolism-directed optimization studies towards orally bioavailable derivatives. Bioorg Med Chem Lett 14:4615–4621

Wallis RM, Corbin JD, Francis SH (1999) Tissue distribution of phosphodiesterase families and the effect of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings *in vitro*. Am J Cardiol 83:3C–12C

Wang P, Wu P, Myers JG, Stamford A, Egan RW, Billah MM (2001) Characterization of human, dog and rabbit corpus cavernosum type 5 phosphodiesterases. Life Sci 68:1977–1987

Wunder F, Tersteegen A, Rebmann A, Erb C, Fahrig T, Hendrix M (2005) Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. Mol Pharmacol 68:1775–1781

Yan C, Zhao AZ, Bentley JK, Beavo JA (1996) The calmodulin-dependent phosphodiesterase gene *PDE1C* encodes several functionally different splice variants in a tissue-specific manner. J Biol Chem 271:25699–25706

Yanaka N, Kurosawa Y, Minami K, Kawai E, Omori K (2003) CGMP-phosphodiesterase activity is upregulated in response to pressure overload of rat ventricles. Biosci Biochem 67:073–079

Yu S, Wolda SL, Frazier ALB, Florio VA, Martins TJ, Snyder PB, Harris EAS, McCaw KN, Farell CA, Steiner B, Bentley JK, Beavo JA, Ferguson K, Gelinas R (1997) Identification and characterisation of a human calmodulin-stimulated phosphodiesterase PDEB1. Cell Signal 9:519–529

Zhang X, Feng Q, Cote RH (2005) Efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (PDE6) in retinal photoreceptors. Invest Ophthalmol Vis Sci 46:3060–3066

A.1.1.22
Stimulation of Heart Membrane Adenylate Cyclase

PURPOSE AND RATIONALE

Metzger and Lindner (1981) discovered that the positive inotropic and vasodilatory effects of forskolin were correlated with the stimulation of adenylate cyclase and cAMP-dependent protein kinase. Subsequent studies by Seamon (1981) demonstrated that forskolin, unlike hormones, guanine nucleotides, fluoride or cholera toxin could stimulate cyclase activity in the absence of the guanine nucleotide regulatory protein. Since those reports, hundreds of papers have been published on the effects of forskolin in numerous mammalian organ and cell systems. Several comprehensive review articles have also been published (Seamon and Daly 1981, 1983; Daly 1984).

While forskolin has proven to be an invaluable research tool for investigations of adenylate cyclase systems (Salomon et al. 1974; Seamon et al. 1981, 1983), reports on its effects on cardiovascular (Lindner et al. 1978), pulmonary (Chang et al. 1984) and ocular physiology (Caprioli and Sears 1983; Caprioli 1985) suggest a therapeutic potential (Seamon 1984) as well.

Described here is an *in vitro* assay which can be used to compare the potency of forskolin, forskolin analogs or other direct or indirect adenylate cyclase activators for the stimulation of adenylate cyclase in heart membranes. The purpose of this assay is to determine and compare the potency of direct or indirect activators of adenylate cyclase for an ability to stimulate heart membrane adenylate cyclase *in vitro*.

PROCEDURE

The hearts of the Wistar rat, Hartley guinea pig, golden Syrian hamster or cardiomyopathic hamster (CHF-146) are used as a source of adenylate cyclase for this assay.

Reagents

1. 0.5 M Tris buffer, pH 7.4
2. 0.05 M Tris buffer, pH 7.4, containing 0.1 M $CaCl_2$
3. Tris buffer mixture
   0.05 M Tris buffer, containing 1 mM IMBX (isobutylmethylxanthine), 0.2 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP ($Na_2ATP \times 3\,H_2O$) and 20 mM creatine phosphate (Na_2 Creatine-$PO_4 \times 5\,H_2O$) (final concentrations in the incubation media).
4. Creatine phosphokinase (ATP:Creatine N-Phosphotransferase, EC 2.73.2), type I, from rabbit muscle is obtained from Sigma Chemical Co.
   The concentration of enzyme in the incubation media is 40 U/ml.
5 Test compounds
   For most assays, a 30 mM stock solution is made up in a suitable solvent (ethanol, ethyl acetate or DMSO for most forskolin analogs). Serial dilutions are made such that the final concentration in the assay ranges from 3×10^{-4} to 3×10^{-7} M. The concentration of vehicle in the assay is 1%.

Tissue Preparation

One entire heart from rat, guinea pig or hamster is dissected, weighed and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH 7.4 containing 0.1 mM $CaCl_2$ (reagent 2) using a Brinkman Polytron (setting 7 for 15 s). The homogenate is centrifuged at 10,000 *g* for 20 min at 4°C. The resulting pellet is resuspended in 50 volumes of homogenizing buffer and recentrifuged as before. The supernatant of this spin is discarded and the resulting pellet is finally resuspended in 10 volumes of the homogenizing buffer, for rat and hamster and 60 volumes for guinea pig tissue. This final preparation is filtered through a thin layer of gauze and kept on ice until used in the assay. The protein concentration is approximately 250–350 mg/ml.

Protein concentrations from an aliquot of the tissue suspension are determined on the day of the experiment by the method of Bradford (1976) using the Bio-Rad assay kit.

Assay

300 µl	Tris buffer mixture, pH 7.4 (reagent 3)
50 µl	creatine phosphokinase
5 µl	vehicle or appropriate concentration of test drug
95 µl	H_2O
50 µl	tissue suspension

The tubes are incubated for 5 min at 37°C and the reaction is then stopped by placing the tubes into a boiling water bath for 4 min. The tubes are then centrifuged at 1000 *g* for 10 min and cAMP levels determined in a 15 µl aliquot of the supernatant using a radioimmunoassay kit (Code TRK432) obtained from Amersham according to the manufacturer's protocol.

Test principle: The method is based on the competition between unlabeled cAMP and a fixed quantity of ^{3}H-labelled cAMP for binding to a protein which has high specificity and affinity for cyclic AMP. The amount of labelled cAMP–protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. The concentration of cAMP in the unknown is determined by comparison with a linear standard curve.

EVALUATION

The data are expressed as pmol cAMP/mg protein/min and dose-response stimulation curves are subjected to logic analysis to determine the concentration of compound which exhibits 50% of maximal stimulation (ED_{50}).

The β-blocking activities of compounds can be determined by their activities, by which they counteract the isoproterenol and GTP induced stimulation of rat heart membrane bound adenylate cyclase (Greenslade et al. 1979).

REFERENCES AND FURTHER READING

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248–254

Caprioli J (1985) The pathogenesis and medical management of glaucoma. Drug Dev Res 6:193–215

Caprioli J, Sears M (1983) Forskolin lowers intraocular pressure in rabbits, monkeys and man. Lancet 1:958–960

Chang J, Hand JM, Schwalm S, Dervinis A, Lewis AJ (1984) Bronchodilating activity of forskolin *in vitro* and *in vivo*. Eur J Pharmacol 101:271–274

Daly JW (1984) Forskolin, adenylate cyclase and cell physiology: An overview. In: Greengard P et al (eds) Advances in Cyclic Nucleotide and Protein Phosphorylation Research 17:81–89

Greenslade FC, Tobia AJ, Madison SM, Krider KM, Newquist KL (1979) Labetalol binding to specific alpha- and beta-adrenergic sites *in vitro* and its antagonism of adrenergic responses *in vivo*. J Mol Cell Cardiol 11:803–811

Hubbard JW, Conway PG, Nordstrom LC, Hartman HB, Lebedinsky Y, O'Malley GJ, Kosley RW (1992) Cardiac adenylate cyclase activity, positive chronotropic and inotropic effects of forskolin analogs with either low, medium or high binding site activity. J Pharm Exp Ther 256:621–627

Kebabian JW (1992) The cyclic AMP cascade: A signal transduction system. Neurotransmiss 8(2):1–4

Lebedinsky Y, Nordstrom ST, Aschoff SE, Kapples JF, O'Malley GJ, Kosley RW, Fielding S, Hubbard JW (1992) Cardiotonic and coronary vasodilator responses to milrinone, forskolin, and analog P87–7692 in the anesthetized dog. J Cardiovasc Pharmacol 19:779–789

Lindner E, Dohadwalla AN, Bhattacharya BK (1978) Positive inotropic and blood pressure lowering activity of a diterpene derivative isolated from Coleus forskohli: Forskolin. Arzneim Forsch/Drug Res 28:284–289
Metzger H, Lindner E (1981) The positive inotropic-acting forskolin, a potent adenylate-cyclase activator. Arzneim Forsch/Drug Res 31:1248–1250
Salomon Y, Londos C, Rodbell M (1974) A highly sensitive adenylate cyclase assay. Analyt Biochem 58:541–548
Seamon KB (1984) Forskolin and adenylate cyclase: new opportunities in drug design. Ann Rep Med Chem 19:293–302
Seamon KB, Daly JW (1981a) Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. J Biol Chem 256:9799–9801
Seamon KB, Daly JW (1981b) Forskolin: A unique diterpene activator of cyclic AMP-generating systems. J Cycl Nucl Res 7:201–224
Seamon KB, Daly JW (1983) Forskolin, cyclic AMP and cellular physiology. Trends Pharmacol Sci 4:120–123
Seamon KB, Padgett W, Daly JW (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci USA 78:3363–3367
Seamon KB, Daly JW, Metzger H, de Souza NJ, Reden J (1983) Structure activity relationships for activation of adenylate cyclase by the diterpene forskolin and its derivates. J Med Chem 26:436–439
Seamon KB, Vaillancourt R, Edwards M, Daly JW (1984) Binding of [^{3}H]forskolin to rat brain membranes. Proc Natl Acad Sci USA 81:5081–5085

A.1.1.23 ^{3}H-Forskolin Binding Assay

PURPOSE AND RATIONALE

This assay is used to identify compounds which demonstrate high affinity [nM] for association with forskolin binding sites *in vitro* as a preliminary screen in conjunction with stimulation of adenylate cyclase to determine the potential for cardiac chronotropic and inotropic and other effects of forskolin. Guinea pig heart tissue and rat brain tissue are used as sources of binding assays.

PROCEDURE FOR GUINEA PIG HEART TISSUE

A. Reagents
1. 0.05 M Tris-HCl buffer, pH 7.4
2. 0.05 M Tris-HCl, pH 8.0 containing 1 mM EGTA, 1 mM $MgCl_2$ and 0.32 M sucrose
3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear.
   Final concentration in the assay is approximately 15 nM.
4. Forskolin and test compounds
   Forskolin and forskolin analogs are diluted to 40 µM. A 100 µl addition of this solution to the final incubate of 200 µl tissue and 100 µl ^{3}H-forskolin results in a final concentration of 10^{-5} M.
   Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation
Male Hartley guinea pigs (300–350 g) are sacrificed by decapitation. The heart is immediately removed, weighed, rinsed, diced and homogenized in 10 volumes of ice-cold 0.05 M buffer(reagent 2) using a Polytron homogenizer (setting 10, 30 s). The resulting homogenate is centrifuged at 12,000 *g* for 15 min at 4°C. The clear supernatant of this spin is discarded, the remaining pellet (P_2) is resuspended in 5 volumes of the same ice-cold buffer and rehomogenized. This final suspension (approximately 0.8–1.0 mg protein/ml) is kept on ice until use. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay
To generate a dose-response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.
200 µl tissue suspension
100 µl ^{3}H-forskolin
100 µl appropriate concentration of forskolin or forskolin analog, or buffer
Tubes are incubated at 30°C for 10 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 1) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed 3 times with 5 ml aliquots of Tris-HCl buffer (reagent 1), added to 10 ml scintillation cocktail and analyzed for radioactivity.

PROCEDURE FOR RAT BRAIN TISSUE

A. Reagents
1. 0.32 M sucrose buffer
2. 0.05 M Tris-HCl buffer, pH 7.5
3. [^{3}H]-Forskolin (specific activity 31.6 Ci/mmol) is obtained from New England Nuclear.
   Final concentration in the assay is approximately 10 nM.
4. Forskolin and test compounds
   Forskolin and forskolin analogs are diluted to 40 µM. A 100 µl addition of this solution to the final incubate of 200 µl tissue and 100 µl ^{3}H-forskolin results in a final concentration of 10^{-5} M.

Appropriate serial dilutions of this stock solution are made such that the final concentrations in the assay range from 10^{-5} to 10^{-11} M with each concentration being done in triplicate.

B. Tissue preparation

Male Sprague-Dawley rats (200–250 g) are sacrificed by decapitation. The brain is rapidly removed and dissected on ice. Striata are homogenized in 50 volumes of ice-cold 0.32 M sucrose buffer (reagent 1) using a Polytron homogenizer (setting 7, 15 s). The resulting homogenate is centrifuged at 1000 *g* for 10 min at 0–4°C. The supernatant is retained and recentrifuged at 20,000 *g* for 10 min at 0–4°C. The clear supernatant of this spin is discarded and the remaining pellet (P_2) is resuspended in ice-cold Tris-HCl buffer, pH 7.5 such that the final protein concentration is approximately 3.0–4.0 mg/ml. Protein concentrations are estimated according to the method of Lowry et al. (1951).

C. Binding assay

To generate a dose-response inhibition curve, ^{3}H-forskolin binding is performed according to the method of Seamon et al. (1984) with minor modifications.

200 µl tissue suspension

100 µl ^{3}H-forskolin

100 µl appropriate concentration of forskolin or forskolin analog or buffer

Tubes are incubated at 23°C for 60 min. The incubate is then diluted with 5 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 (reagent 2) and immediately vacuum filtered through glass fiber filters (Whatman GF/B) by using a Brandel Cell Harvester. The filters are rapidly washed three times with 5 ml aliquots of Tris-HCl buffer (reagent 2), added to 10 ml scintillation cocktail and analyzed for radioactivity.

EVALUATION

Specific binding is defined as the difference between binding of ^{3}H-forskolin in the absence and presence of 10 µM forskolin and represents 85–90% of total binding at 10 nM ^{3}H-forskolin.

Results of the dose-response inhibition curves are analyzed by determining the concentration of competing compound which inhibits 50% of the ^{3}H-forskolin binding sites (IC_{50}). This value is determined by computer-derived log-probit analysis.

The activity of various forskolin analogs are based on IC_{50} values and are categorized as follows:

	IC_{50}[M]
0 = Not determined	
1 = No activity	$>10^{-5}$
2 = Slight activity	10^{-5}–10^{-6}
3 = Moderate activity	10^{-6}–10^{-7}
4 = Marked activity	$<10^{-7}$

To compare the activity of various compounds from experiment to experiment, an inhibition constant (K_I) is determined as described by Cheng and Prusoff (1973). The K_I is determined from the equation:

$$K_I = IC_{50}/1 + LC/K_D$$

IC_{50} = concentration of competing compound which inhibits 50% of the ^{3}H-forskolin binding sites

LC = determined ^{3}H-forskolin concentration (approximately 10 nM)

K_D = dissociation of affinity constant for ^{3}H-forskolin determined previously to be approximately 13.4 nM for rat striatum and 196 nM for guinea pig heart

Standard data:

Binding inhibition values for forskolin

	Striatum	Heart
IC_{50}	43.1 ± 4.9 nM	34.2 ± 5.0 nM
K_I	26.0 ± 3.1 nM	31.6 ± 4.6 nM

REFERENCES AND FURTHER READING

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with phenol reagent. J Biol Chem 193:265–275

Seamon KB, Vaillancourt R, Edwards M, Daly JW (1984) Binding of [^{3}H]forskolin to rat brain membranes. Proc Natl Acad Sci USA 81:5081–5085

A.1.1.24 Patch Clamp Technique[2]

PURPOSE AND RATIONALE

The introduction of the patch clamp technique (Neher and Sakmann 1976) revolutionized the study of cellular physiology by providing a high-resolution method of observing the function of individual ionic channels in a variety of normal and pathological cell types. By

[2]Contributions by H. Gögelein.

the use of variations of the basic recording methodology, cellular function and regulation can be studied at a molecular level by observing currents through individual ionic channels (Liem et al. 1995; Sakmann and Neher 1995).

The most intriguing method is called the "on-cell" or "cell-attached" configuration, because ion channels can be recorded on an intact cell (Jackson 1993). This mode is well suited for investigation of ion channels that are activated by hormonal stimulation and triggered by intracellular second messengers.

Another versatile mode is the "cell-excised" configuration (Hamill 1993). It is obtained by suddenly removing the patch pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows the investigator to expose the channel proteins to drugs by changing the bath solution. The single channel currents are recorded on a videotape and are analyzed off-line by a computer system. Various parameters are evaluated, such as the single channel conductance, open and closed times of the channel, and the open-state probability, which is the percentage of time the channel stays in its open state.

In addition to these modes, which enable the recording of single channel currents, it is also possible to measure the current flowing through the entire cell. This "whole-cell mode" is obtained by rupturing the membrane patch in the cell-attached mode (Hamill et al. 1981; Dietzel et al. 1993). This is achieved by applying suction to the interior of the patch pipette. The "whole-cell mode" allows not only the recording of electrical current, but also the measurement of cell potential. Moreover, the cell interior is dialyzed by the electrolyte solution contained in the patch pipette.

The fabrication of patch clamp pipettes has been described by Sakmann and Neher (1995) and Cavalié et al. (1993).

Variations of the patch clamp technique have been used to study neurotransmitter transduction mechanisms (Smith 1995).

High throughput methods are required when developing drugs that work on ion channel function (Mathes 2003; Bennett and Guthrie 2003). Patch clamping suffers from low throughput, which is not acceptable for drug screening.

Fertig et al. (2002) and Brueggemann et al. (2004, 2006) presented nanopatch clamp technology, which is based on a planar, microstructured glass chip, which enables automatic whole-cell patch clamp experiments. Planar glass substrates containing a single microaperture produced by ion track etching are used to record currents through ion channels in living mammalian cells.

Falconer et al. (2002) reported high throughput screening for ion channel modulators setting up a Beckman/Sagian core system to fully automate functional fluorescence-based assays that measure ion channel function. Voltage-sensitive fluorescent probes were applied and the activity of channels was measured using Aurora's Voltage/Ion Probe Reader (VIPR). The system provides a platform for fully automated high throughput screening as well as pharmacological characterization of ion channel modulators.

Schroeder et al. (2003) described a high throughput electrophysiology measurement platform consisting of computer-controlled fluid handling, recording electronics, and processing tools capable of voltage clamp whole-cell recordings from thousands of individual cells per day. The system uses a planar, multiwell substrate (a PatchPlate). The system positions one cell into a hole separating two fluid compartments in each well of the substrate. Voltage control and current recordings from the cell membrane are made subsequent to gaining access to the cell interior by applying a permeabilizing agent to the intracellular side.

Willumsen's group recommended ion channel screening with QPatch (Asmild et al. 2003; Kutchisky et al. 2003; Krzywkowski et al. 2004). This system claims to allow fast and accurate electrophysiological characterization of ion channels, e. g., for determination of IC_{50} values for ion channel blockers. The system comprises 16 parallel patch clamp sites, each based on a silicon chip with a micro-etched patch clamp hole. Intra- and extra-cellular fluids are administered by laminar flow through integrated miniature flow channels.

REFERENCES AND FURTHER READING

Asmild M, Oswald N, Krzywkowski FM, Friis S, Jacobsen RB, Reuter D, Taboryski R, Kutchinsky J, Vestergaard RK, Schroder RL, Sorensen CB, Bech M, Korsgaard MP Willumsen NJ (2003) Upscaling and automation of electrophysiology: toward high throughput screening in ion channel drug discovery. Receptor Channels 9:49–58

Bennett PB, Guthrie HRE (2003) Trends in ion channel drug discovery: advances in screening technologies. Trends Biotechnol 21:563–569

Brueggemann A, George M, Klau M, Beckler M, Steimdl J, Behrends JC, Fertig N (2004) Ion channel drug discovery and research. The automated nano-patch-clamp technology. Curr Drug Discover Technol 1:91–96

Brüggemann A, Stoelzle S, George M, Behrends JC, Fertig N (2006) Microchip technology for automated and parallel patch-clamp recording. Small 2:840–646

Cavalié A, Grantyn R, Lux HD (1993) Fabrication of patch clamp pipettes. In: Kettenmann H, Grantyn R (eds) Practical electrophysiological methods. Wiley, New York, pp 235–240
Dietzel ID, Bruns D, Polder HR, Lux HD (1993) Voltage clamp recording. In: Kettenmann H, Grantyn R (eds) Practical electrophysiological methods. Wiley, New York, pp 256–262
Falconer M, Smith F, Surah-Narwal S, Congrave G, Liu Z, Hayter P, Ciaramella G, Keighley W, Haddock P, Waldron G, Sewing A (2002) High-throughput screening for ion channel modulators. J Biomol Screen 7:460–465
Fertig N, Klau M, George M, Blick RH, Behrends JC (2002) Activity of single ion channel proteins detected with a planar microstructure. Appl Physics Lett 81:4865–4867
Hamill OP (1993) Cell-free patch clamp. In: Kettenmann H, Grantyn R (eds) Practical electrophysiological methods. Wiley, New York, pp 284–288
Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
Jackson MB (1993) Cell-attached patch. In: Kettenmann H, Grantyn R (eds) Practical electrophysiological methods. Wiley, New York, pp 279–283
Krzywkowski K, Schroder RL, Ljungstrom T, Kutchinsky J, Friis S, Vestergaard RK, Jacobsen RB, Pedersen S, Helix N, Sorensen CB, Bech M, Willumsen NJ (2004) Automatation of the patch-clamp technique: technical validation through identification and characterization of potassium channel blockers. Biophys J 86:483a
Kutchinsky J, Friis S, Asnild M, Taboryski R, Pedersen S, Vestergaard RK, Jacobsen RB, Krzywkowski K, Schroder RL, Ljungstrom T, Helix N, Sorensen CB, Bech M, Willumsen NJ (2003) Characterization of potassium channel modulators with QPatch automated patch-clamp technology system characteristics and performance. Assay Drug Dev Technol 1:685–693
Liem LK, Simard JM, Song Y, Tewari K (1995) The patch clamp technique. Neurosurgery 36:382–392
Mathes C (2003) Ion channels in drug discovery and development. Drug Discover Today 8:1022–1024
Neher E, Sakmann B (1976) Single-channel currents recorded from membranes of denervated frog muscle fibres. Nature 260:799–802
Sakmann B, Neher E (1995) Single-cell recording. Plenum, New York
Schroeder K, Neagle B, Trezise DJ, Worley J (2003) IonWorks™ HT: A new high-throughput electrophysiology measurement platform. J Biomol Screen 8:50–64
Smith PA (1995) Methods for studying neurotransmitter transduction mechanisms. J Pharmacol Toxicol Meth 33:63–73

A.1.1.24.1 Patch Clamp Technique in Isolated Cardiac Myocytes

PURPOSE AND RATIONALE

The generation of an action potential in heart muscle cells depends on the opening and closing of ion-selective channels in the plasma membrane. The patch clamp technique enables the investigation of drug interactions with ion-channel-forming proteins at the molecular level.

PROCEDURE

Isolated cells from ventricular muscle of rat and guinea pig are prepared as described by Yazawa et al. (1990). Animals are sacrificed by cervical dislocation. Hearts are dissected and mounted on a Langendorff-type apparatus and perfused first with Tyrode solution (in mM: 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 5 HEPES, pH adjusted to 7.4 with NaOH) at 37°C for 3 min at a hydrostatic pressure of 60–70 cmH_2O, then with nominally Ca^{2+}-free Tyrode solution (no Ca^{2+} is added) for 5–7 min, and finally with nominally Ca^{2+}-free Tyrode solution containing 0.12–0.2 mg/ml collagenase (Sigma, type I). After 15–20 min of collagenase treatment, the heart is now soft and is washed with storage solution (in mM: 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 10 HEPES, 0.5 EGTA, pH adjusted to 7.4 with KOH). The ventricles are cut into pieces (about 5mm × 5 mm) and poured into a beaker. The myocytes are dispersed by gently shaking the beaker and filtration through a nylon mesh (365 µm). Then, the myocytes are washed twice by centrifugation at 600–1000 rpm (about 90 *g*) for 5 min and kept at room temperature. The rod shape of the cell and the clear striations of sarcomeres are important criteria for selecting viable cells for the assay. Experiments are performed at 35°C–37°C.

For investigation with the patch clamp technique (Neher and Sakmann 1976; Hamill et al. 1981), the isolated cells are placed into a thermostat-controlled chamber, mounted on the stage of an inverted microscope equipped with differential interference contrast optics. Under optical control (magnification 400×) a glass micropipette, having a tip opening of about 1 µm, is placed onto the cell. The patch pipettes are fabricated from borosilicate glass tubes (outer diameter 1.5 mm, inner diameter 0.9 mm) by means of an electrically heated puller. In order to prevent damage of the cell membrane, the tip of the micropipette is fire polished, by moving a heated platinum wire close to the tip. The patch pipette is filled with either high-NaCl or KCl solution and is mounted on a micro manipulator. A silver chloride wire connects the pipette solution to the head stage of an electronic amplifier. A second silver chloride wire is inserted into the bath and serves a ground electrode.

After establishing contact with the cell membrane, a slight negative pressure is applied to the inside of the patch pipette by means of a syringe. Consequently, a small patch of membrane is slightly pulled into the opening of the micro pipette and close contact

between the glass and membrane is formed, leading to an increase of the electrical input resistance into the giga-ohm range (about 10^{10} Ohm). This high input resistance enables the recording of small electrical currents in the range of picosiemens (10^{-12} S), which flow through channel-forming proteins situated in the membrane patch. The electrical current is driven by applying an electrical potential across the membrane patch, and/or by establishing an appropriated chemical gradient for the respective ion species.

The patch clamp methods allows one to investigate the interaction of drugs with all ion channels involved in the functioning of the heart muscle cell (K^+, Na^+, Ca^{2+} and eventually Cl^- channels). Moreover, the different types of K^+ channels existing in cardiomyocytes can be distinguished by their different single-channel characteristics or by appropriate voltage-pulse protocols in the whole-cell mode.

EVALUATION

Concentration–response curves of drugs which inhibit or activate ion channels can be recorded either at the single channel level or by measuring the whole-cell current. IC_{50} and EC_{50} values (50% inhibition or activation, respectively) can be obtained with both methods.

MODIFICATIONS OF THE METHOD

The patch clamp technique has been used for evaluation of anti-arrhythmic agents (Bennett et al. 1987; Anno and Hondeghem 1990; Gwilt et al. 1991).

Gögelein et al. (1998) used isolated ventricular myocytes from guinea pigs to study a cardioselective inhibitor of the ATP-sensitive potassium channel.

Multiple types of calcium channels have been identified by patch clamp experiments (Tsien et al. 1988).

The effects of potassium channel openers have been measured (Terzic et al. 1994).

Ryttsén et al. (2000) characterized electroporation of single NG108–15 cells with carbon-fiber microelectrodes by patch clamp recordings and fluorescence microscopy.

Monyer and Lambolez (1995) reviewed the molecular biology and physiology at the single cell level, discussing the value of the polymerase chain reaction at the single cell level and the use of patch pipettes for collecting the contents of a single cell on which the reverse transcription is performed.

The patch clamp technique was found to be very versatile in the investigation of ion channels in atrial myocytes, especially from dogs or humans. Cell were obtained from atria either in sinus rhythm or in atrial fibrillation (reviewed in Bosch et al. 1999).

REFERENCES AND FURTHER READING

Anno T, Hondeghem LM (1990) Interaction of flecainide with guinea pig cardiac sodium channels. Circ Res 66:789–803

Bennett PB, Stroobandt R, Kesteloot H, Hondeghem LM (1987) Sodium channel block by a potent, new antiarrhythmic agent, Transcainide, in guinea pig ventricular myocytes. J Cardiovasc Pharmacol 9:661–667

Bosch RF, Zeng X, Grammer JB, Popovic K, Mewis C, Kühlkamp V (1999) Ionic mechanisms of electrical remodeling in human atrial fibrillation. Cardiovasc Res 44:121–131

Gögelein H, Hartung J, Englert HC, Schölkens BA (1998) HMR 1883, a novel cardioselective inhibitor of the ATP-sensitive potassium channel. Part I. Effects on cardiomyocytes, coronary flow and pancreatic β-cells. J Pharmacol Exp Ther 286:1453–1464

Gwilt M, Dalrymple HW, Burges RA, Blackburn KJ, Dickinson RP, Cross PE, Higgins AJ (1991) Electrophysiologic properties of UK-66,914, a novel class III antiarrhythmic agent. J Cardiovasc Pharmacol 17:376–385

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85–100

Monyer H, Lambolez B (1995) Molecular biology and physiology at the single-cell level. Curr Opin Neurobiol 5:382–387

Neher E, Sakmann B (1976) Single-channel currents recorded from membranes of denervated frog muscle fibres. Nature 260:799–802

Pallotta BS (1987) Patch-clamp studies of ion channels. In: Meltzer HY (ed) Psychopharmacology: The third generation of progress. Raven, New York, pp 325–331

Ryttsén F, Farre C, Brennan C, Weber SG, Nolkrantz K, Jardemark K, Chiu DT, Orwar O (2000) Characterization of single-cell electroporation by using patch-clamp and fluorescence microscopy. Biophys J 79:1993–2001

Terzic A, Jahangir A, Kurachi Y (1994) HOE-234, a second generation K^+ channel opener, antagonizes the ATP-dependent gating of cardiac ATP-sensitive K^+ channels. J Pharmacol Exp Ther 268:818–825

Tsien RW, Lipscombe D, Madison DV, Bley RK, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulation. TINS 11:431–438

Yazawa K, Kaibara M, Ohara M, Kameyama M (1990) An improved method for isolating cardiac myocytes useful for patch-clamp studies. Jpn J Physiol 40:157–163

A.1.1.24.2 Voltage Clamp Studies on Sodium Channels

PURPOSE AND RATIONALE

The epithelial Na^+ channel plays an important role in epithelial Na^+ absorption in the distal colon, urinary bladder, salivary and sweat ducts, respiratory tract and, most importantly, in the distal tubules of the kidney (Catterall 1986; Palmer 1992). Regulation of this epithelial Na^+ channel has a major impact on Na^+ balance, blood volume, and blood pressure. Inhibition of epithelial Na^+ channel expression is used for the treatment of hypertension (Endou and Hosoyamada 1995).

Busch et al. (1995) studied the blockade of epithelial Na^+ channels by triamterenes using two-microelectrode voltage clamp experiments in *Xenopus* oocytes expressing the three homologous subunits (α, β, and γ) of the rat epithelial Na^+ channel (rENaC).

PROCEDURE

Xenopus laevis oocytes are injected with the appropriate cRNA encoding for the α, β, and γ-subunits (Canessa et al. 1993) of the rat epithelial Na^+ channel (rENaC). The cRNA for the wild-type α-subunit and its deletion mutant Δ278–273 is always coinjected with an equal amount of β and γ-subunit cRNA (10 ng/oocyte).

Then, 2–8 days after cRNA injection, the two-microelectrode voltage clamp method is used to record currents from *Xenopus* oocytes. Recordings are performed at 22°C using a Geneclamp amplifier (Axon Instruments, Foster City, Calif., USA), and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). The ND 96 solution (control) contains (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.0. In some experiments, Na^+ is replaced by *N*-methyl-D-glucamine (NMDG) solution. The microelectrodes are filled with 3 M KCl solution and have resistances in the range 0.5–0.9 MΩ. Chemicals (e. g., triamterene as standard) are added at concentrations between 0.2 and 100 µM. The amplitude of the induced currents varies considerably, depending on the day of channel expression and the batch of oocytes. The mutant channel induces considerably smaller currents than the wild-type channel. The total Na^+ current amplitude is determined at least once for each experimental day by superfusion with NMDG solution, or with 3 µM or 5 µM amiloride solution at the beginning and at the end of each set of experiments.

EVALUATION

Data are presented as means ± SEM. A paired Student's *t*-test is used. The level of statistical significance is set at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Nawada et al. (1995) studied the effects of a sodium, calcium, and potassium antagonistic agent on the sodium current by the whole cell voltage clamp technique (tip resistance = 5 MΩ[Na]$_i$ and [Na]$_o$ 10 mmol/l at 20°C) in guinea pig isolated ventricular cells.

Sunami and Hiraoka (1996) studied the mechanism of cardiac Na^+ channel block by a charged class I antiarrhythmic agent, in guinea pig ventricular myocytes using patch clamp techniques in the whole-cell, cell-attached and inside-out configurations.

Erdõ et al. (1996) compared the effects of *Vinca* derivatives on voltage-gated Na^+ channels in cultured cells from rat embryonic cerebral cortex. Effects on Na^+ currents were measured by applying voltage steps (20 ms duration) to –10 mV from a holding potential of –70 mV every 20 s. Steady-state inactivation curves were obtained by clamping the membrane at one of a series of 15-s prepulse potentials, followed 1 ms later by a 20-ms test pulse to –10 mV.

Ragsdal et al. (1993) examined the actions of a Na^+ channel blocker in whole-cell voltage clamp recordings from Chinese hamster ovary cells transfected with a cDNA encoding the rat brain type IIA Na^+ channel and from dissociated rat brain neurons.

Taglialatela et al. (1996) studied cloned voltage-dependent Na^+ currents expressed in *Xenopus* oocytes upon injection of the cRNA encoding α-subunits from human and rat brain.

Wang et al. (1997) investigated pharmacological targeting of long QT mutant sodium channels.

Eller et al. (2000) measured the effects of a calcium antagonist on inward Na^+ currents (I_{Na}) in GH3-cells with the whole-cell configuration of the patch clamp technique. I_{Na} was recorded after depolarization from a holding potential of –80 mV to a test potential of +5 mV. Initial "tonic" block (resting state-dependent block) was defined as peak I_{Na} inhibition during the first pulse 2 min after drug application as compared with I_{Na} in the absence of drug. "Use-(frequency-) dependent" block of I_{Na} was measured during trains of 5- or 50-ms test pulses (3 Hz) applied from –80 mV to a test potential of +5 mV after a 2-min equilibrium period in the drug-containing solution. Use-dependent block was expressed as the percentage decrease of peak I_{Na} during the last pulse of the train as compared with I_{Na} during the first pulse.

Khalifa et al. (1999) characterized the effects of an antidepressant agent on the fast inward current (I_{Na}) in isolated guinea pig ventricular myocytes. Currents were recorded in the whole-cell configuration of the patch clamp technique in the presence of Ca^{2+} and K^+ channel blockers.

Haeseler et al. (1999) measured the effects of 4-chloro-*m*-cresol, a preservative added to a wide variety of drugs, on heterologously expressed wild-type, Paramyotonia congenita (R1448H) and hyperkalemic periodic paralysis (M1360V) mutant α-subunits of human muscle sodium channels using whole-cell and inside-out voltage clamp experiments.

Song et al. (2000) studied the effects of *N*-ethyl-maleimide, an alkylating agent to protein sulfhydryl groups, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root neurons using the whole-cell configuration of the patch clamp technique. Rats at the age of 2–6 days were anesthetized with isoflurane and the spinal cord was removed and cut longitudinally. Dorsal root ganglia were plucked from the area between the vertebrae of the spinal column, and incubated in phosphate-buffered saline solution containing 2.5 mg/ml trypsin at 37°C for 30 min. After enzyme treatment, ganglia were rinsed with Dulbecco's Modified Eagle Medium supplemented with 10% horse serum. Single cells were mechanically dissociated by trituration with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips. Cells attached to the coverslips were transferred into a recording chamber on the stage of an inverted microscope. Ionic currents were recorded under voltage clamp conditions by the whole-cell patch clamp technique. The solution in the pipette contained (in mM): CsCl 125, NaF 20, HEPES 5, EGTA 5. The pH was adjusted to 7.2 with CsOH and the osmolarity was 279 mosmol/l on average. The external solution contained (in mM): NaCl 50, choline chloride 90, tetramethylammonium chloride 20, D-glucose 5, HEPES 5, $MgCl_2$ 1, $CaCl_2$ 1. Lanthanum ($LaCl_3$, 10 µM) was used to block calcium channel current. The solution was adjusted to pH 7.4 with tetramethylammonium hydroxide and the osmolarity was 304 mosmol/l on average. An Ag–AgCl pellet/3 M KCl-agar bridge was used for the reference electrode. Membrane currents were recorded using an Axopatch-1D amplifier. Signals were digitized by a 12-bit analog-to-digital interface, filtered with a low-pass Bessel filter at 5 kHz and sampled at 50 kHz using pCLAMP6 software (Axon Instruments) on an IBM-compatible PC. Series resistance was compensated 60%–70%. Capacitative and leakage currents were subtracted by using a P + P/4 procedure (Bezanilla and Armstrong 1977). The liquid junction potential between internal and external solutions was on average –1.7 mV. TTX (100 nM) was used to separate TTX-R sodium currents from TTX-S sodium currents. For the study of TTX-S sodium channels, cells that expressed only TTX-S sodium channels were used. TTX-S sodium channels were completely inactivated within 2 ms when currents were evoked by depolarizing steps to 0 mV, while TTX-R sodium channels persisted for more than 20 ms. The difference in kinetics was used to identify the type of sodium current.

Abriel et al. (2000) described the molecular pharmacology of the sodium channel mutation DI790G linked to long QT syndrome.

Makielski et al. (2003) showed that a ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5AS heart sodium channels.

Viswanathan et al. (2001) studied gating mechanisms for flecainide action in *SNCN5A*-linked arrhythmia syndromes.

REFERENCES AND FURTHER READING

Abriel H, Wehrens XHT, Benhorin J, Kerem B, Kass RS (2000) Molecular pharmacology of the sodium channel mutation DI790G linked to the long-QT- syndrome. Circulation 102:921–925

Bezanilla F, Armstrong CM (1977) Inactivation of the sodium channel: I. Sodium current experiments. J Gen Physiol 70:549–566

Busch AE, Suessbrich H, Kunzelmann K, Hipper A, Greger R, Waldegger S, Mutschler E, Lindemann B, Lang F (1995) Blockade of epithelial Na^+ channels by triamterenes – underlying mechanisms and molecular basis. Pflügers Arch 432:760–766

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC (1994) Amiloride-sensitive N^+ channel is made of three homologous subunits. Nature 367:463–467

Catterall WA (1986) Molecular properties of voltage-sensitive sodium channels. Annu Rev Biochem 55:953–985

Eller P, Berjukov S, Wanner S, Huber I, Hering S, Knaus HG, Toth G, Kimball SD, Striessnig J (2000) High affinity interaction of mibefradil with voltage-gated calcium and sodium channels. Br J Pharmacol 130:699–677

Endou H, Hosoyamada M (1995) Potassium-retaining diuretics: aldosterone antagonists. In: Greger RH, Knauf H, Mutschler E (eds) Handbook of experimental pharmacology, Vol 117. Springer, Berlin Heidelberg New York, pp 335–362

Erdõ SL, Molnár P, Lakics V, Bence JZ, Tömösközi Z (1996) Vincamine and vincanol are potent blockers of voltage-gated Na^+ channels. Eur J Pharmacol 314:69–73

Haeseler G, Leuwer M, Kavan J, Würz A, Dengler R, Piepenbrock S (1999) Voltage-dependent block of normal and mutant muscle sodium channels by 4-Chloro-m-Cresol. Br J Pharmacol 128:1259–1267

Khalifa M, Daleau P, Turgeon J (1999) Mechanism of sodium channel block by venlafaxine in guinea pig ventricular myocytes. J Pharmacol Exp Ther 291:280–284

Makielski JC, Ye B, Valdivia CR, Pagel MD, Pu J, Tester DJ, Ackerman MJ (2003) A ubiquitous splice variant and a common polymorphisms affect heterologous expression of recombinant human SCN5AS heart sodium channels. Circ Res 93:821–828

Nawada T, Tanaka Y, Hisatome I, Sasaki N, Ohtahara A, Kotake H, Mashiba H, Sato R (1995) Mechanism of inhibition of the sodium current by bepridil in guinea-pig isolated ventricular cells. Br J Pharmacol 116:1775–1780

Palmer LG (1992) Epithelial Na channels: function and diversity. Annu Rev Physiol 54:51–66

Ragsdal DS, Numann R, Catterall WA, Scheuer T (1993) Inhibition of Na^+ channels by the novel blocker PD85,639. Mol Pharmacol 43:949–954

Song J-H, Jang Y-Y, Shin Y-K, Lee C-S, Chung S (2000) *N*-Ethyl-maleimide modulation of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root neurons. Brain Res 855:267–273

Sunami A, Hiraoka M (1996) Blockade of cardiac Na^+ channels by a charged class I antiarrhythmic agent, bisaramil: possible interaction of the drug with a pre-open closed state. Eur J Pharmacol 312:245–255

Taglialatela M, Ongini E, Brown AM, di Renzo G, Annunziato L (1996) Felbamate inhibits cloned voltage-dependent Na^+ channels from human and rat brain. Eur J Pharmacol 316:373–377

Viswanathan PV, Bezzina CR, George AL, Jr, Roden DM, Wilde AAM, Balser JR (2001) Gating mechanisms for flecainide action in *SNCN5A*-linked arrhythmia syndromes. Circulation 104:1200–1205

Wang DW, Yazawa K, Makita N, George AL, Jr, Bennett PB (1997) Pharmacological targeting of long QT mutant sodium channels. J Clin Invest 99:1714–1720

A.1.1.24.3 Voltage Clamp Studies on Potassium Channels

PURPOSE AND RATIONALE

Potassium channels represent a very large and diverse collection of membrane proteins which participate in important cellular functions regulating neuronal and cardiac electrical patterns, release of neurotransmitters, muscle contractility, hormone secretion, secretion of fluids, and modulation of signal transduction pathways. The main categories of potassium channels are gated by voltage or an increase of intracellular calcium concentration (Escande and Henry 1993; Kaczorowski and Garcia 1999; Alexander et al. 2001). For ATP-sensitive potassium channels see Sect. K.6.6.12.

The delayed outward potassium current in heart muscle cells of several species is made up of a **rapidly** (I_{Kr}) and a **slowly** (I_{Ks}) activating component (Sanguinetti and Jurkiewicz 1990; Wang et al. 1994; Gintant 1996; Lei and Brown 1996; Carmeliet and Mubagawa 1998). Several potent and selective blockers of the I_{Kr} channel have been shown to prolong the effective refractory period, but have a reverse rate-dependent activity with both normal and elevated extracellular potassium concentrations (Colatsky et al. 1990). Inhibitors of the slow component I_{Ks} were developed in order to circumvent the negative rate dependence of I_{Kr} channel blockers in the effective refractory period (Busch et al. 1996; Suessbrich et al. 1996, 1997; Bosch et al. 1998). Gögelein et al. (2000) studied the effects of a potent inhibitor of I_{Ks} channels in *Xenopus* oocytes and guinea pig ventricular myocytes.

PROCEDURE

Studies in *Xenopus* oocytes are performed with the two-microelectrode voltage clamp method. For isolation of the oocytes, the toads are anesthetized using a 1 g/l solution of 3-aminobenzoic acid ethyl ester and placed on ice. A small incision is made to retrieve sacs of oocytes and is subsequently closed with absorbable surgical suture. On waking up, the toads are placed back into the aquarium. The ovaries are cut up into small pieces and the oocytes are washed in Ca^{2+}-free Or-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) and subsequently digested in Or-2 containing collagenase A (1 mg/ml, Worthington, type II) until follicles are not longer detectable on the oocyte's surface. The oocytes are stored at 18°C in recording solution ND-96 (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.4) with added sodium pyruvate (275 mg/l), theophylline (90 mg/l), and gentamicin (50 mg/l).

For electrophysiological recordings, the two-microelectrode voltage clamp configuration is used to record ion currents from *Xenopus* oocytes. Injection of cRNA is performed according to Methfessel et al. (1986) and Golding (1992). Oocytes are injected individually with cRNA encoding for the human protein minK, guinea pig Kir2.1, human H*erg*, human Kv1.5, mouse Kv1.3, or human HNC2. In the case of minK the functional potassium channel is a heteromultimer composed of the endogenous (*Xenopus*) KvLQT1 and the injected human minK. This heteromultimeric potassium current in then called I_{Ks} (Barhanin et al. 1996; Sanguinetti et al. 1996).

The electrophysiological recordings are performed at room temperature, using a Geneclamp amplifier (Axon Instruments), and MacLab D/A converter. The amplitudes of the recorded currents are measured at the end of the test voltage steps. To amplify the inward potassium current through Kir2.1 and HNC2, the external potassium concentration is raised to 10 mM KCl and the NaCl concentration lowered to 88 mM (ND-88). The microelectrodes are filled with 3 M KCl and have a resistance between 0.5 MΩ and 1 MΩ. During the recordings the oocytes are continuously perfused with ND-96 (or ND-88 in the case of Kir2.1 and HCN2). The test compounds are dissolved in dimethylsulfoxide (DMSO) and added to the buffer ND-96 or ND-88. The current amplitude is determined after 5 min of wash-in time.

For the isolation of *ventricular myocytes*, guinea pigs (weight about 400 g) or Sprague-Dawley rats of either sex are sacrificed by cervical dislocation. The hearts are dissected and perfused retrogradely via the aorta at 37°C: first with nominally Ca^{2+}-free Tyrode solution (in mmol/l): 143 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 10 glucose, 5 HEPES, pH 7.2, then

with Tyrode solution containing 20 mmol/l Ca^{2+} and 3 mg/ml collagenase type CLS II (Biochrom, Berlin Germany). After 5–10 min collagenase treatment the ventricles are cut up into small pieces in the storage solution (in mmol/l): 50 L-glutamic acid monopotassium salt, 40 KCl, 20 taurine, 20 KH_2PO_4, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, pH 7.2. The myocytes are then dispersed by gentle shaking followed by filtration through a nylon mesh (365 μm). The cells are finally washed twice by centrifugation at 90 *g* for 5 min and kept in the storage solution at room temperature.

Whole-cell currents are recorded in the tight-seal whole-cell mode of the patch clamp technique, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes are pulled from borosilicate glass capillaries (wall thickness 0.3 mm, outer diameter 1.5 mm) and their tips are fire-polished. Series resistance is in the range of 1–10 MΩ and 50% compensated by means of the EPC's compensation circuit.

The I_{Ks}, I_{Kr}, and I_{K1} currents in guinea pig ventricular myocytes are investigated. The voltage pulses for recording the current components are as follows: I_{Ks} current: holding potential –80 mV to –50 mV (200 ms) to +60 mV (3 s) to –40 mV (2 s) to –80 mV; I_{Kr} current: holding potential –80 mV to –50 mV (200 ms) to –10 mV (3 s) to –40 mV (2 s) to –80 mV. I_{Kr} is evaluated as the tail current evoked by a voltage pulse from –10 mV to –40 mV; I_{K1}: holding potential –80 mV to –120 mV (200 ms) to –80 mV. In order to suppress the L-type Ca^{2+} current, 5 mmol/l nifedipine is added to the bath solution.

EVALUATION

All average data are presented as means ±SEM. Student's *t*-test is used to determine the significance of paired observations. Differences are considered as significant at $P < 005$.

MODIFICATIONS OF THE METHOD

Using the whole-cell configuration of the patch clamp technique, Grissmer et al. (1994) analyzed the biophysical and pharmacological properties of five cloned voltage-gated K^+ channels stably expressed in mammalian cell lines.

Sanchez-Chapula (1999) studied the block of the transient outward K^+ channel (I_{to}) by disopyramide in isolated rat ventricular myocytes using whole-cell patch clamp techniques.

Using the patch clamp technique, Cao et al. (2001) investigated the effects of a centrally acting muscle relaxant and structurally related compounds on recombinant small-conductance Ca^{2+}-activated K^+ channels (rSK2 channels) in HEK mammalian cells.

Tagliatela et al. (2000) discussed the block of the K^+ channels encoded by the human *ether-á-go-go-related* gene (HERG), termed $K_{V(r)}$, which are the molecular determinants of the rapid component of the cardiac repolarizing current $I_{K(Vr)}$, involved in the cardiotoxic potential and CNS effects of first-generation antihistamines and may be therapeutic targets for antiarrhythmic agents (Vandenberg et al. 2001; Zhou et al. 2005).

Chabbert et al. (2001) investigated the nature and electrophysiological properties of Ca^{2+}-independent depolarization-activated potassium currents in acutely isolated mouse vestibular neurons using the whole-cell configuration of the patch clamp technique. Three types of currents were identified.

Furthermore, Longobardo et al. (1998) studied the effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents stably expressed in *Ltk*$^-$ cells using the whole-cell configuration of the patch clamp technique.

Moreno et al. (2003) studied the effects of a selective angiotensin II type 1 receptor antagonist on cloned potassium channels involved in human cardiac repolarization.

Sanchez-Chapula et al. (2002) investigated the voltage-dependent block of wild-type and mutant HERG K^+ channels by the antimalarial compound chloroquine.

Anson et al. (2004) published molecular and functional characterization of common polymorphism in HERG (KCNH2) potassium channels.

For more information on the evaluation of HERG potassium channels in safety pharmacology see Chap. I.D by Brian D. Guth.

REFERENCES AND FURTHER READING

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS Nomenclature Supplement 2001

Anson BD, Ackerman MJ, Tester DJ, Will ML, Delisle BP, Anderson CL, January CT (2004) Molecular and functional characterization of common polymorphism in HERG (KCNH2) potassium channels. Am J Physiol 286:H2434–H2441

Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, Romey G (1996) KvLQT1 and IsK (minK) proteins associate to form the IKs cardiac potassium current. Nature 384:78–80

Bosch RF, Gaspo R, Busch AE, Lang HJ, Li R-G, Nattel S (1998) Effects of the chromanol 293B, a selective blocker of the slow component of the delayed rectifier K^+ current, on repolarization in human and guinea pig ventricular myocytes. Cardiovasc Res 38:441–450

Busch AE, Suessbrich H, Waldegger S, Sailer E, Greger R, Lang HJ, Lang F, Gibson KJ, Maylie JG (1996) Inhibition of I_{Ks} in guinea pig cardiac myocytes and guinea pig I_{sK} channels by the chromanol 293B. Pflügers Arch 432:1094–1096

Cao Y-J, Dreixler JC, Roizen JD, Roberts MT, Houamed KM (2001) Modulation of recombinant small-conductance Ca^{2+}-activated K^+ channels by the muscle relaxant chlorzoxazone and structurally related compounds. J Pharmacol Exp Ther 296:683–689

Carmeliet A, Mubagawa K (1998) Antiarrhythmic drugs and cardiac ion channels: mechanism of action. Prog Biophys Mol Biol 70:1–71

Chabbert C, Chambard JM, Sans A, Desmadryl G (2001) Three types of depolarization-activated potassium currents in acutely isolated mouse vestibular neurons. J Neurophysiol 85:1017–1026

Colatsky TJ, Follmer CH, Starmer CF (1990) Channel specificity in antiarrhythmic drug action. Mechanism of potassium channel block and its role in suppressing and aggravating cardiac arrhythmias. Circulation 82:2235–2242

Escande D, Henry P (1993) Potassium channels as pharmacological targets in cardiovascular medicine. Eur Heart J 14 [Suppl B]:2–9

Gintant GA (1996) Two components of delayed rectifier current in canine atrium and ventricle. Does I_{Ks} play a role in the reverse rate dependence of class III agents? Circ Res 78:26–37

Gögelein H, Brüggemann A, Gerlach U, Brendel J, Busch AE (2000) Inhibition of I_{Ks} channels by HMR 1556. Naunyn-Schmiedebergs Arch Pharmacol 362:480–488

Golding AL (1992) Maintenance of *Xenopus laevis* and oocyte injection. Methods Enzymol 207:266–279

Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, Karmilowicz MJ, Auperin DD, Chandy KG (1994) Pharmacological characterization of five cloned voltage-gated K^+ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. Mol Pharmacol 45:1227–1234

Kaczorowski GJ, Garcia ML (1999) Pharmacology of voltage-gated and calcium-activates potassium channels. Curr Opin Chem Biol 3:448–458

Lei M, Brown HF (1996) Two components of the delayed rectifier potassium current, IK, in rabbit sinoatrial node cells. Exp Physiol 81:725–741

Longobardo M, Delpón E, Caballero R, Tamargo J, Valenzuela C (1998) Structural determinants of potency and stereoselective block of hKv1.5 channels induced by local anesthetics. Mol Pharmacol 54:162–169

Methfessel C, Witzemann V, Takahashi T, Mishina M, Numa S, Sakmann B (1986) Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor. Pflügers Arch 407:577–588

Moreno I, Caballero R, Ganzález T, Arias C, Valenzuela C, Iriepa I, Gálvez E, Tamargo J, Delpón E (2003) Effects of irbesartan on cloned potassium channels involved in human cardiac repolarization. J Pharmacol Exp Ther 304:862–873

Sanchez-Chapula JA (1999) Mechanisms of transient outward K^+ channel block by disopyramide. J Pharmacol Exp Ther 290:515–523

Sanchez-Chapula JA, Navarro-Polanco RA, Culberson C, Chen J, Sanguinetti MC (2002) Molecular determinants of voltage-dependent human ether-a-go-go related gene (HERG) K^+ channel block. J Biol Chem 277:23587–23595

Sanguinetti MC, Jurkiewicz NK (1990) Two components of cardiac delayed rectifier K^+ currents: differential sensitivity to block by class III antiarrhythmic agents. J Gen Physiol 96:195–215

Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MD (1996) Coassembly of KvLTQ1 and minK (IsK) proteins form cardiac IKs potassium channel. Nature 384:80–83

Suessbrich H, Busch A, Ecke D, Rizzo M, Waldegger S, Lang F, Szabo I, Lang HJ, Kunzelmann K, Greger R, Busch AE (1996) Specific blockade of slowly activating channels by chromanols – impact on the role of I_{sK} channels in epithelia. FEBS Lett 396:271–275

Suessbrich H, Busch AE, Scherz MW (1997) The pharmacology of cloned cardiac potassium channels. Ion Channel Modulators 2:432–439

Tagliatela M, Timmerman H, Annunziato L (2000) Cardiotoxic potential and CNS effects of first-generation antihistamines. Trends Pharmacol Sci 21:52–65

Vandenberg JI, Walker BD, Campbell TC (2001) HERG K^+ channels: friend and foe. Trends Pharmacol Sci 22:240–246

Wang Z, Fermini B, Nattel S (1994) Rapid and slow components of delayed rectifier current in human atrial myocytes. Cardiovasc Res 28:1540–1546

Zhou J, Angelli-Szafran CE, Bradley JA, Chen X, Koci BJ, Volberg WA, Sun Z, Cordes JS (2005) Novel potent human *Ether-à-Go-Go*-related gene (*h*ERG) potassium channel enhancers and their in vitro antiarrhythmic activity. Mol Pharmacol 68:876–884

A.1.1.24.3.1 Studies on Kv1.5 Channel

PURPOSE AND RATIONALE

Treatment of atrial fibrillation/flutter with available potassium channel blockers (Class III antiarrhythmic agents which mainly block the delayed rectifier current I_{kr}) is associated with ventricular proarrhythmia. Prolongation of ventricular repolarization leads to early after-depolarization from which torsades de pointes can evolve. Therefore, blockade of a cardiac current of exclusive relevance in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. The ultrarapid delayed rectifier potassium current (I_{kur}) seems an ideal atrial antiarrhythmic target since it is found to contribute to the action potential in the atrium but not in the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current is the potassium channel Kv1.5, which therefore gained much interest (Li et al. 1996; Longobardo et al. 1998; Perchenet and Clément-Chomienne 2000; Caballero et al. 2000, 2001, 2004; Bachmann et al. 2001; Kobayashi et al. 2001; Matsuda et al. 2001; Choi et al. 2002; Moreno et al. 2003; Choe et al. 2003; Fedida et al. 2003; Godreau et al. 2002, 2003; Peukert et al. 2003, 2004; Plane et al. 2005).

For *in vivo* studies on atrial fibrillation see A.5.0.9, A.5.0.9.1, A.5.0.9.2, A.5.0.9.3.

Gögelein et al. (2004) studied the effects of the antiarrhythmic drug AVE0118 on cardiac ion channels.

PROCEDURE

Molecular Biology and Cell Culture

Human Kv1.5 cDNA was subcloned into the eukaryotic expression vectors pcDNA3.1 and pcDNA3.1/zeo (Invitrogen, Groningen, the Netherlands), cDNA encoding human Kv4.3 long (Kv4.3l; Dilks et al. 1999) was subcloned into pcDNA3.1, and the cDNA encoding human KChIP2 short (KChIP2.2; Decher et al. 2001) was subcloned into pcDNA3.1/zeo expression vector. Chinese hamster ovary (CHO) cells were transfected with either hKv1.5 or hKv4.3 and KChIP2.2 expression constructs. Transfection was carried out using lipofectamine (Life Technologies/Gibco BRL, Karlsruhe, Germany) according to the manufacturer's instructions. To boost Kv1.5 channel expression, CHO cells were consecutively transfected with both Kv1.5 expression constructs. Both hKv1.5 and hKv4.3 + hKChIP2.2 were stably expressed in CHO cells, which were maintained in ISCOVE's medium (Biochrom KG, Berlin, Germany), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 350 µg/ml Zeocin (Invitrogen) and 400 µg/ml G418 (PAA Laboratories). HERG, the potassium channel underlying I_{Kr} currents in human hearts, was cloned and transfected into CHO cells as described previously (Rampe et al. 1997). Cells used for patch clamping were seeded on glass or plastic coverslips 12–36 h before use.

Northern blot analysis of Kv1.5 in the pig heart and cloning of pig Kv1.5

Polyadenylated RNA was isolated from pig cardiac tissues with the Oligotex mRNA purification kit (Qiagen) and 10 µg per tissue was resolved by denaturing formaldehyde electrophoresis and blotted on a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled riboprobe (DIG RNA labeling kit, Roche) encompassing the entire coding sequence of human Kv1.5 and exposed on a Lumi-Imager (Roche). The pig Kv1.5 was cloned by 5′-rapid amplification and 3′-rapid amplification of cDNA ends (RACE) reactions. An adapter ligated, double-stranded cDNA library was prepared from pig heart mRNA with the Marathon cDNA amplification kit (Clontech). The 5′-RACE and 3′-RACE reactions were performed with oligonucleotide primers derived from a partial pig Kv1.5 nucleotide sequence (GenBank accession number AF348084). Overlapping cDNA clones were obtained by repeated reactions and the DNA sequence determined by automated DNA sequencing on both strands (ABI 310, Perkin Elmer). A full-length cDNA clone was established by recombinant PCR. It encodes an open reading frame of 1,083 bp and a protein with 86% overall sequence similarity to the human Kv1.5 protein. The sequence of the pig Kv1.5 cDNA was submitted to GenBank (accession number: AY635585).

For *Xenopus* oocyte expression, cDNAs encoding Kv1.5, Kv4.3, and KChip2.2 were cloned into the oocyte expression vector pSGEM (Villmann et al. 1997), and capped cRNA was synthesized using the T7 mMessage mMachine kit (Ambion, Austin, Tex., USA).

Voltage-clamp experiments in Xenopus Oocytes

Handling and injection of *Xenopus* oocytes were performed according to Bachmann et al. (2001). Adult female *Xenopus laevis* frogs were anesthetized with 3-aminobenzoic acid ethyl ester solution (1 g/l) and intact ovary lobes were removed. The oocytes were defolliculated by treatment with 40 mg collagenase dissolved in 20 ml buffer (in mM: NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5, titrated to pH 7.5 with NaOH) for 120–150 min at 18°C. Oocytes were injected with 50 nl cRNA using a microinjector (World Precision Instruments, Sarasota, Fla., USA). Oocytes were stored under gentle shaking at 18°C in a buffer containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, theophylline 0.5, gentamicin 50 µg/ml, titrated to pH 7.5 with NaOH. They were used for experiments 1–3 days after injection.

Two-electrode voltage clamp recordings were performed at room temperature in a medium containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.5 with NaOH. Microelectrodes were pulled from filament borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). After filling with 3 M KCl, pipettes had a resistance of 0.3–1.3 MΩ. To activate hKv1.5 and hKv4.3 channels, oocytes were clamped from a holding potential of –80 mV to 40 mV for 500 ms. Data were recorded with a Turbo Tec 10CX amplifier (NPI, Tamm, Germany) using an ITC-16 interface (Instrutech Corporation, Long Island, USA) and the Pulse software (HEKA Elektronik, Lambrecht, Germany).

Patch Clamp Experiments with CHO Cells

Cells expressing Kv1.5 or Kv4.3 plus KChIP2.2 were assayed using the standard whole-cell patch clamp technique (Hamill et al. 1981). Cells were mechanically removed from the tissue culture flask and placed in a perfusion chamber with a solution containing (in

mM): NaCl 140, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 1.1, HEPES 10, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM): NaCl 10, KCl 120, EGTA 1, HEPES 10, $MgCl_2$ 1.1 (pH 7.2 with potassium hydroxide, KOH), pipettes had resistances of 2–3 MΩ. Experiments were carried out at 36 ± 1°C. For the recording of hKv1.5, voltage pulses of 450 ms duration were applied from the holding potential of –30 mV to +20 mV at a frequency of 1 Hz. For recording of the hKv4.3+KChIP2.2, the holding potential was –50 mV and test pulses of 200 ms duration were applied to –10 mV at a frequency of 1 Hz. Data were recorded with an EPC-9 patch clamp amplifier (HEKA Elektronik) and the Pulse software (HEKA Elektronik) and stored on a PC for later analysis. Series resistance was in the range of 4–9 MΩ and was compensated by 80% by means of the EPC9's compensation circuit. The experiments were performed under continuous superfusion of the cells with solution heated to 36 ± 1°C.

HERG channel currents were recorded at room temperature using the whole-cell configuration of the patch clamp technique with an Axopatch 200B amplifier (Axon Instruments). Briefly, electrodes (3–6 MΩ resistance) were fashioned from TW150F glass capillary tubes (World Precision Instruments) and filled with pipette solution (in mM: potassium aspartate 120, KCl 20, Na_2ATP 4, HEPES 5, $MgCl_2$ 1, pH 7.2 adjusted with KOH). HERG currents were initiated by a positive voltage pulse (20 mV) followed by a negative pulse (–40 mV) and were recorded for off-line analyses. Once HERG current from a cell perfused with control external solution (in mM: NaCl 130, KCl 5, sodium acetate 2.8, $MgCl_2$ 1, HEPES 10, glucose 10, $CaCl_2$ 1 at pH 7.4 adjusted with NaOH) was stabilized, the cell was perfused with external solution containing the compound at a specific concentration for percentage inhibition. For each concentration from each cell, peak amplitude of the steady-state HERG tail current at –40 mV was measured. The peak amplitude for each concentration was compared with that for the control solution from the same cell and expressed as percent control.

Isolation of Porcine Atrial Myocytes

Male pigs weighing 15–30 kg of the German Landrace were anesthetized with pentobarbital exactly as described previously (Wirth and Knobloch 2001). After a left thoracotomy the lung was retracted, the pericardium incised and the heart was quickly removed and placed in oxygenated nominally Ca^{2+}-free Tyrode solution containing (in mM): NaCl 143, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.25, HEPES 5 and glucose 10, pH adjusted to 7.2 with NaOH. The hearts were then mounted on a Langendorff apparatus and perfused via the left circumflex coronary artery with Tyrode solution (37°C) with constant pressure (80 cmH_2O). All coronary vessels descending to the ventricular walls were ligated, ensuring sufficient perfusion of the left atrium. When the atrium was clear of blood and contraction had ceased (≈5 min), perfusion was continued with the same Tyrode solution, which now contained 0.015 mM $CaCl_2$ and 0.03% collagenase (type CLS II, Biochrom KG, Berlin, Germany), until atrial tissue softened (≈20 min). Thereafter, left atrial tissue was cut into small pieces and mechanically dissociated by trituration. Cells were then washed with storage solution containing (in mM): L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 1, glucose 10, HEPES 10, EGTA 2 (pH 7.2 with KOH) and filtered through a nylon mesh. The isolated cells were kept at room temperature in the storage solution.

Isolation of Guinea Pig Ventricular Myocytes

Ventricular myocytes were isolated by enzymatic digestion according to Gögelein et al. (1998). Dunkin Hardley Pirbright White guinea pigs (weight about 400 g) were sacrificed by cervical dislocation. The hearts were dissected and perfused retrogradely via the aorta at 37°C with the same solutions as used for isolation of pig atrial myocytes.

Electrophysiological Recordings from Cardiac Myocytes

Whole-cell currents were recorded with an EPC-9 patch clamp amplifier (HEKA Elektronik) as described above for CHO cells. A small aliquot of cell-containing solution was placed in a perfusion chamber and after a brief period allowing for cell adhesion to the chamber, the cells were perfused with (in mM): NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.0, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries and heat polished. After filling with (in mM) KCl 130, $MgCl_2$ 1.2, HEPES 10, EGTA 10, K_2ATP 1, GTP 0.1, and phosphocreatine 5 (pH 7.2 with KOH) pipettes had a resistance of 2–3 MΩ. Series resistance was in the range of 6–12 MΩ and was compensated by 60%–70%. Offset voltages generated when the pipette was inserted in NaCl solution (1–5 mV) were zeroed before formation of the seal.

Effects of AVE0118 on the I_{KACh} were recorded from pig left atrial myocytes by applying voltage pulses of 500 ms duration from the holding potential of –80 mV to –100 mV. Carbachol (10 µM) was added

in order to evoke the I_{KACh}. After stabilization of the I_{KACh} (3 min), AVE0118 was added in increasing concentrations in the continuous presence of carbachol. The current was measured at the end of the pulse after 3 min of incubation at each concentration and inhibition of the carbachol-activated current was calculated. In some experiments, AVE0118 was washed out before application of the next higher concentration.

Also the L-type Ca^{2+} current was investigated in pig left atrial cells. In these experiments, KCl in the pipette was replaced by CsCl and voltage pulses of 300 ms duration were applied from the potential of –40 mV to 0 mV. Possible effects of AVE0118 on the currents I_{K1}, I_{Ks}, I_{Kr} and I_{KATP} were investigated in guinea pig ventricular myocytes. I_{K1} currents were recorded by a voltage step from –80 mV to –120 mV lasting for 200 ms. When I_{Ks} and I_{Kr} currents were recorded, 1 µM nisoldipine was added to the bath to block the L-type Ca^{2+} current. I_{Ks} was assessed by voltage pulse to +60 mV for 3 s, starting from –40 mV. I_{Kr} was evaluated as the tail current evoked by a voltage pulse from –10 mV to –40 mV. I_{KATP} was evoked by adding 1 µM rilmakalim (Krause et al. 1995) to the bath and by applying voltage ramps from –130 mV to +80 mV for 500 ms. The rilmakalim-activated current was recorded at the potential 0 mV. All patch clamp experiments were performed under continuous superfusion of the cells with solution heated to 36±1°C.

EVALUATION

All averaged data are presented as the mean ± SEM. The Student's *t*-test was used to determine the significance of paired or unpaired observations. Differences were considered significant at $P < 0.05$. The values for half-maximal inhibition (IC_{50}) and the Hill coefficient were calculated by fitting the data points of the concentration–response curves to the logistic function:

$$f(x) = (a - d)/\left[1 + (x/c)^n\right] + d$$

where a represents the plateau value at low drug concentration and d the plateau value at high drug concentration; c represents the IC_{50} value and n the Hill coefficient. The curve-fitting and the Student's *t*-test were performed with the computer program *Sigma-Plot* 5.0.

REFERENCES AND FURTHER READING

Bachmann A, Gutcher I, Kopp K, Brendel J, Bosch RF, Busch AE, Gögelein H (2001) Characterization of a novel Kv1.5 channel blocker in Xenopus oocytes, CHO cells, and human cardiomyocytes. Naunyn-Schmiedebergs Arch Pharmacol 364:472–478

Caballero R, Delpón E, Valenzuela C, Longobardo M, Tamargo J (2000) Losartan and its metabolite E3174 modify cardiac delayed rectifier K^+ currents. Circulation 101:1199–1205

Caballero R, Delpón E, Valenzuela C, Longobardo M, González T, Tamagro J (2001) Direct effects of candesartan and eprosartan in human cloned potassium channels involved in cardiac repolarization. Mol Pharmacol 59:825–836

Caballero R, Gómez R, Núòez L, Moreno I, Tamargo J, Delpón E (2004) Diltiazem inhibits hKv1.5 and Kv4.3 currents in therapeutic concentrations. Cardiovasc Res 64:457–466

Choe H, Lee YK, Lee YT, Choe H, Ko SH, Joo CU, Kim MH, Kim GS, Eun JS, Kim JH, Chae SW, Kwak YG (2003) Papaverine blocks hKv1.5 channel current und human atrial ultrarapid delayed rectifier K^+ currents. J Pharmacol Exp Ther 304:706–712

Choi BH, Choi JS, Rhie DJ, Yoon SH, Min DS, Jo YH, Kim MS, Hahn SJ (2002) Direct inhibition of the cloned Kv1.5 channel by AG-1478, a tyrosine kinase inhibitor. Am J Physiol 282:C1461–C1468

Decher N, Uyguner O, Scherer CR, Karaman B, Yükselapak M, Busch AE, Steinmeyer K, Wollnik B (2001) hKChIP2 is a functional modifier of hKv4.3 potassium channels: cloning and expression of a short hKChIP2 splice variant. Cardiovasc Res 52:255–264

Dilks D, Ling H-P, Cockett M, Sokol P, Numann R (1999) Cloning and expression of the human Kv4.3 potassium channel. J Neurophysiol 81:1974–1977

Fedida D, Eldstrom J, Hesketh C, Lamorgese M, Castel L, Steele DF, van Wagoner DR (2003) Kv1.5 is an important component of repolarizing K^+ current in canine atrial myocytes. Circ Res 93:744–751

Godreau D, Vranckx R, Hatem SN (2002) Mechanism of action of antiarrhythmic agent bertosamil on hKv1.5 channels and outward current in human atrial myocytes. J Pharmacol Exp Ther 300:612–620

Godreau D, Vranckx R, Maguy A, Goyenvalle C, Hatem SN (2003) Different isoforms of synapse-associated protein, SAP97, are expressed in the heart and have distinct effects on the voltage-gated K^+ channel Kv1.5. J Biol Chem 278:47046–47052

Gögelein H, Hartung J, Englert HC, Schölkens BA (1998) HMR 1883, a novel cardioselective inhibitor of the ATP-sensitive potassium channel. I. Effects on cardiomyocytes, coronary flow and pancreatic β-cells. J Pharmacol Exp Ther 286:1453–1464

Gögelein H, Brendel J, Steinmeyer K, Strübing C, Picard N, Rampe D, Kopp K, Busch AE, Bleich M (2004) Effects of the antiarrhythmic drug AVE0118 on cardiac ion channels. Naunyn-Schmiedebergs Arch Pharmacol 370:183–192

Hamill OP, Marty M, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100

Kobayashi S, Reien Y, Ogura T, Saito T, Masuda Y, Nakaya H (2001) Inhibitory effect of bepridil on hKv1.5 channel current; comparison with amidarone and E-4031. Eur J Pharmacol 430:149–157

Krause E, Englert H, Gögelein H (1995) Adenosine triphosphate-dependent K currents activated by metabolic inhibition in rat ventricular myocytes differ from those elicited by the channel opener rilmakalim. Pflügers Arch 429:625–635

Li GR, Feng J, Wang Z, Fermine B, Nattel S (1996) Adrenergic modulation of ultrarapid rectifier K^+ current in human atrial myocytes. Circ Res 78:903–915

Longobardo M, González T, Navarro-Polanco R, Calballero R, Delpón E, Tamargo J, Snyders DJ, Tamkum MM, Valen-

zuela C (2000) Effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents. Br J Pharmacol 130:391–401
Matsuda T, Masumiya H, Tanaka N, Yamashita T, Tsuruzoe N, Tanaka Y, Tanaka H, Shigenoba K (2001) Inhibition by a novel anti-arrhythmic agent, NIP-142, of cloned human cardiac K^+ channel Kv1.5 current. Life Sci 68:2017–2024
Moreno I, Caballero R, González T, Arias C, Valenzuela C, Iriepa I, Gálvez E, Tamargo J, Delpón E (2003) Effects of irbesartan on cloned potassium channels involved in human cardiac repolarization. J Pharmacol Exp Ther 304:862–873
Perchenet L, Clément-Chomienne O (2000) Characterization of the mibefradil block of the human heart delayed rectifier hKv1.5. J Pharmacol Exp Ther 295:771–778
Peukert S, Brendel J, Pirad B, Bruggemann A, Below P, Kleemann HW, Hemmerle H, Schmidt W (2003) Identification, synthesis, and activity of novel blockers of the voltage-gated potassium channel Kv1.5. J Med Chem 46:486–498
Peukert S, Brendel J, Pirard B, Strübing C, Kleemann HW, Böhme T, Hemmerle H (2004) Pharmacophore-based search, synthesis, and biological evaluation of anthranilic amides as novel blockers of the Kv1.5 channel. Bioorg Med Chem Lett 14 :2823–2827
Plane F, Johnson R, Kerr P, Wiehler W, Thorneloe K, Ishii K, Chen T, Cole W (2005) Heteromultimeric Kv1 channels contribute to myogenic control of arterial diameter. Circ Res 96:216–224
Rampe D, Roy ML, Dennis A, Brown AM (1997) A mechanism for the proarrhythmic effects of cisapride (Propulsid): high affinity blockade of the human cardiac potassium channel HERG. FEBS Lett 417:28–32
Villmann C, Bull L, Hollmann M (1997) Kainate binding proteins possess functional ion channel domains. J Neurosci 17:7634–7643
Wirth KJ, Knobloch K (2001) Differential effects of dofetilide, amiodarone, and class Ic drugs on left and right atrial refractoriness and left atrial vulnerability in pigs. Naunyn-Schmiedebergs Arch Pharmacol 363:166–174

A.1.1.24.4 Voltage Clamp Studies on Calcium Channels

PURPOSE AND RATIONALE

Calcium influx through voltage-gated Ca^{2+} channels mediates a range of cytoplasmatic responses, including muscle contraction, release of neurotransmitters, Ca^{2+} dependent gene transcription and the regulation of neuronal excitability has been reviewed by several authors (Augustine et al. 1987; Bean 1989; Miller 1987; Zamponi 1997; Snutch et al. 2001). In addition to their normal physiological function, Ca^{2+} channels as calcium antagonists are also implicated in a number of human disorders (see also A.4.0.1).

Using patch clamp techniques, the structure and regulation of voltage-gated Ca^{2+} channels has been studied by many authors (Sculptoreanu et al. 1993; Peterson et al. 1997; Catterall 2000).

Berjukow et al. (2000) analyzed the role of the inactivated channel conformation in molecular mechanism of Ca^{2+} channel block by a dihydropyridine derivative in L-type channel constructs and mutants in *Xenopus* oocytes and described the electrophysiological evaluation.

PROCEDURE

Inward barium currents (I_{Ba}) are studied with two microelectrode voltage-clamp of *Xenopus* oocytes 2–7 days after microinjection of approximately equimolar cRNA mixtures of constructs of L-channel mutants. All experiments are carried out at room temperature in a bath solution with the following composition: 40 mM $Ba(OH)_2$, 50 mM NaOH, 5 mM HEPES, 2 mM CsOH (pH adjusted to 7.4 with methanesulfonic acid). Voltage recording and current injecting microelectrodes are filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, 10 mM HEPES (pH 7.4) with resistances of 0.3–2 MΩ. Resting channel block is estimated as peak I_{Ba} inhibition during 100-ms test pulses from -80 to 20 mV at a frequency of 0.033 Hz until steady state is reached. The dose response curves of I_{Ba} inhibition were fitted using the Hill equation:

$$\frac{I_{\text{Ba, drug}}}{I_{\text{Ba, control}}}(\%) = \frac{100 - A}{1 + \left(\frac{C}{\text{IC}_{50}}\right)^{nH}} + A$$

where IC_{50} is the concentration at which I_{Ba} inhibition is half-maximal, C is the applied drug concentration, A is the fraction of I_{Ba} that is not blocked, and nH is the Hill coefficient.

Recovery from inactivation is studied at a holding potential of -80 mV after depolarizing Ca^{2+} channels during a 3-s prepulse to 20 mV by applying 30-ms test pulses (to 20 mV) at various time intervals after the conditioning prepulse. Peak I_{Ba} values are normalized to the peak current measured during the prepulse, and the time course of I_{Ba} recovery from inactivation is fitted to a mono- or biexponential function

$$I_{\text{Ba, recovery}} = A \times \exp\left(\frac{-t}{\tau_{\text{fast}}}\right) + B \times \exp\left(\frac{-t}{\tau_{\text{slow}}}\right) + C$$

Voltage dependence of inactivation under quasi-steady state conditions is measured using a multi step protocol to account for run-down (less than 10%). A control test pulse (50 ms to 20 mV) is followed by a 1.5-s step to -100 mV followed by a 30-s conditioning step, a 4-ms step to -100 mV, and a subsequent test pulse to 20 mV (corresponding to the peak potential of the I-V curves).

Inactivation during the 30 s conditioning pulse is calculated as follows.

$$I_{\text{Ba, inactivation}} = \frac{I_{\text{Ba, test}}(20\,\text{mV})}{I_{\text{Ba, control}}(20\,\text{mV})}$$

The pulse sequence is applied every 3 min from a holding potential of -100 mV. Inactivation curves are drawn according to the following Boltzmann equation.

$$I_{\mathrm{Ba,inactivation}} = I_{\mathrm{SS}} + (1 - I_{\mathrm{SS}}) \left(1 + \exp \left(\frac{V - V_{0.5}}{k} \right) \right)$$

where V is the membrane potential, $V_{0.5}$ is the midpoint voltage, k is the slope factor, and I_{SS} is the fraction of non inactivating current.

Steady state inactivation of the mutate channels at -80 mV is estimated by shifting the membrane holding potential from -80 to -100 mV. Subsequent monitoring of the corresponding changes in I_{Ba} amplitudes until steady state reveals the fraction of Ca^{2+} channels in the inactivated state at -80 mV. Steady state inactivation of different L-type channel constructs at -30 mV is estimated by fitting time course of current inactivation to a biexponential function.

The I_{Ba} inactivation time constants are estimated by fitting the I_{Ba} decay to a mono- or biexponential function.

EVALUATION

Data are given as the means ± SE. Statistical significance is calculated according to Student's unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Besides *Xenopus* oocytes (Waard and Campell 1995; Hering et al. 1997; Kraus et al. 1998), several other cell types and constructs, such as CHO cells (Sculptoreanu et al. 1993; Stephens et al. 1997), HEK293 (human embryonic kidney) cells (Lacinová et al. 1999), tsA-201 cells, a subclone of HEK293, (Peterson et al. 1997; McHugh et al. 2000), cardiac myocytes from rats (Scamps et al. 1990; Tohse et al. 1992; Gomez et al. 1994) and rabbits (Xu et al. 2000), isolated atrial myocytes from failing and non-failing human hearts (Cheng et al. 1996), skeletal muscle myotubes from mice and rabbits, (Johnson et al. 1994), myocytes of guinea pig mesentery artery (Morita et al. 1999), dendrites from rat pyramidal and olfactory bulb neurons (Markram and Sakmann 1994; Stuart and Spruston 1995; Koester and Sakmann 1998; Margie et al. 2001), rat amygdala neurons (Foehring and Srcoggs 1994; Young et al. 2001) were used to study the function of calcium channels.

Using the whole-cell variation of the patch-clamp technique, Yang et al. (2000) studied cellular T-type and L-type calcium channel currents in mouse neuroblastoma N1E115 cells. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 3–4 days. After mechanical agitation, 3×10^4 cells were replanted in 35-mm tissue culture dishes containing 4 ml of bath solution. After cell attachment, the dish was mounted on the stage of an inverted phase-contrast microscope for Ca^{2+} channel current recording. These cells expressed predominantly T channel currents. In experiments where L channels were specifically sought, the cells were grown and maintained at confluence for 3–4 weeks under the same culture conditions with the addition of 2% dimethylsulfoxide (Narahash et al. 1987). Three to 5 days before use, the cells were replanted with the same medium. These cells expressed predominantly L channel currents. A small number of these cells also expressed T channel currents. Hence, cells were selected so that at a holding potential of –40 mV, the T channel component was very small and the inward current measured was conducted predominantly by L channels.

By using whole-cell and perforated patch-clamp techniques, Wu et al. (2000) showed that mifrabidile, a non-dihydropyridine compound, has an inhibitory effect on both T- and L-type Ca^{2+} currents in pancreatic β-cells.

REFERENCES AND FURTHER READING

Augustine GJ, Charlton MP, Smith RJ (1987) Calcium action in synaptic transmitter release. Ann Rev Neurosci 10:633–693

Bean BP, Classes of calcium channels in vertebrate cells. Annu Rev Physiol 51:376–384

Berjukom S, Marksteiner R, Gapp F, Sinneger MJ, Hering S (2000) Molecular mechanism of calcium channel block by israpidine. Role of a drug-induced inactivated channel configuration. J Biol Chem 275:22114–22120

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Ann Rev Cell Dev Biol 16:521–555

Cheng T-H, Lee F-Y, Wei J, Lin C-I (1996) Comparison of calcium-current in isolated atrial myocytes from failing and nonfailing human hearts. Mol Cell Biochem 157:157–162

Gomez JP, Potreau D, Branka JE, Raymond G (1994) Developmental changes in Ca^{2+} currents from newborn rat cardiomyocytes in primary culture. Pflügers Arch 428:214–249

Foehring RC, Srcoggs RS (1994) Multiple high-threshold calcium currents in acutely isolated rat amygdaloid pyramidal cells. J Neurophysiol 71:433–436

Hering S, Aczél S, Klaus RL, Berjukow S, Striessnig J, Timin EN (1997) Molecular mechanism of use-dependent calcium channel block by phenylalkylamines: role of inactivation. Proc Natl Acad Sci USA 94:13323–13328

Hockerman GH, Peterson BZ, Sharp E, Tanada TN, Scheuer T, Catterall (1997) Constriction of a high-affinity receptor site for dihydropyridine agonists and antagonists by single amino acid substitution in a non-L-type Ca^{2+} channel. Proc Natl Aced Sci USA 94:14906–14911

Johnson BD, Scheuer T, Catterall WA (1994) Voltage-dependent potentiation of L-type Ca^{2+} channels in skeletal muscles cells requires anchored cAMP-dependent protein kinase. Proc Natl Acad Sci 91:11492–11406

Koester HJ, Sakmann B (1998) Calcium dynamics in single spines during coincident pre- and postsynaptic activity depend on relative timing of back-propagation action potentials and subthreshold excitatory potentials. Proc Natl Acad Sci USA 95:9596–9601

Kraus RL, Hering S, Grabner M, Ostler D, Striessnig J (1998) Molecular mechanisms of diltiazem interaction with L-type Ca^{2+} channels. J Biol Chem 273:27205–27212

Lacinová L, An HR, Xia J, Ito H, Klugbauer N, Triggle E, Hofmann F, Kass RS (1999) Distinction in the molecular determinants of charged and neutral dihydropyridine block of L-type calcium channels. J Pharmacol Exp Ther 289:1472–1479

Lacinová L, Klugbauer N, Hofmann F (2000) State- and isoform-dependent interaction of israpidine with the α_{1C} L-type calcium channel. Pflügers Arch Eur J Physiol 440:50–60

Margrie TW, Sakmann B, Urban NN (2001) Action potential propagation in mitral cell lateral dendrites is decremental and controls recurrent and lateral inhibition in the mammalian olfactory bulb. Proc Natl Acad Sci USA 98:319–324

Markram H, Sakmann B (1994) Calcium transients in dendrites of neocortical neurons evoked by single subthreshold excitatory postsynaptic potentials via low-voltage-activated calcium channels. Proc Natl Acad Sci USA 91:5207–5211

McHugh D, Sharp EM, Scheuer T, Catterall WA (2000) Inhibition of L-type calcium channels by protein kinase C phophorylation of two sites in the N-terminal domain. Proc Natl Acad Sci USA 97:12334–12338

Miller RJ (1987) Multiple calcium channels and neuronal function. Science 235:46–52

Morita H, Cousins H, Inoue H, Ito Y, Inoue R (1999) Predominant distribution of nifedipine-insensitive, high voltage-activated Ca^{2+} channels in the terminal mesenteric artery of guinea pig. Circ Res 85:596–605

Narahash T, Tsunoo A, Yoshii M (1987) Characterization of two types of calcium channels in mouse neuroblastoma cells. J Physiol 38:231–249

Peterson BZ, Johnson BD, Hockerman GH, Acheson M, Scheuer T, Catterall WA (1997), Analysis of the dihydropyridine receptor site of L-type calcium channels by alanine-scanning mutagenesis. J Biol Chem 272:18752–18758

Scamps F, Mayoux E, Charlemagne D, Vassort G (1990) Calcium current in single cells isolated from normal and hypertrophied rat heart. Circ Res 67:199–208

Sculptoreanu A, Rotman E, Takahasi M, Scheuer T, Catterall WA (1993) Voltage-dependent potentiation of the activity of cardiac L-type calcium channel $\alpha 1$ subunits due to phophorylation by cAMP-dependent protein kinase. Proc Natl Acad Sci USA 90:10135–10139

Snutch TP, Sutton KG, Zamponi GW (2001) Voltage-dependent calcium channels – beyond diydropyridine antagonists. Curr Opin Pharmacol 1:11–16

Stephens GJ, Page KM, Burley JR, Berrow NS, Dolphin AC (1997) Functional expression of brain cloned $\alpha 1$E calcium channels in COS-7 cells. Pflügers Arch 433:525–532

Stuart G, Spruston N (1995) Probing dendritic function with patch pipettes. Curr Opin Neurobiol 5:389–394

Tohse N, Masuda H, Sperelakis N (1992) Novel isoform of Ca^{2+} channel in rat fetal cardiomyocytes. J Physiol (Lond.) 451:295–306

Waard MD, Campell KP (1995) Subunit regulation of the neuronal α_{1A} Ca^{2+} channel expressed in *Xenopus* oocytes. J Physiol (Lond) 485:619–634

Wu S, Zhang M, Vest PA, Bhattacharjee A, Liu L, Li M (2000) A mifrabidile metabolite is a potent intracellular blocker of L-type Ca^{2+} currents in pancreatic β-cells. J Pharmacol Exp Ther 292:939–943

Xu X, Rials SJ, Wu Y, Liu T, Marinchak RA, Kowey PR (2000) Effects of captopril treatment of renovascular hypertension on β-adrenergic modulation of L-type Ca^{2+} current. J Pharmacol Exp Ther 292:196–200

Yang JC, Shan J, Ng KF, Pang P (2000) Morphine and methadone have different effects on calcium channel currents in neuroblastoma cells. Brain Res 870:199–203

Young C, Huang Y-C, Lin C-H, Shen Y-Z, Gean P-W (2001) Selective enhancement of L-type calcium currents by corticotropin in acutely isolated rat amygdala neurons. Mol Pharmacol 59:604–611

Zamponi GW (1997) Antagonist sites of voltage-dependent calcium channels. Drug Dev Res 42:131–143

A.1.1.24.5
Patch Clamp Studies on Chloride Channels

PURPOSE AND RATIONALE

Cl^- channels are a large, ubiquitous and highly diverse group of ion channels involved in many physiological key processes including: regulation of electrical excitability; muscle contraction; secretion; and sensory signal transduction. Cl^- channels belong to several distinct families characterized in detail: voltage-gated Cl^- channels, the cAMP-regulated channel CFTR (cystic fibrosis transmembrane conductance regulator), ligand-gated Cl^- channels that open upon binding to the neurotransmitters GABA or glycine, and Cl^- channels that are regulated by the cytosolic Ca^{2+} concentration (Jentsch and Günther 1997; Frings et al. 2000).

Cliff and Frizel (1990) studied the cAMP- and Ca^{2+}-activated secretory Cl^- conductances in the Cl^- secreting colonic tumor epithelial cell line T84 using the whole-cell voltage-clamp technique.

PROCEDURE

T84 cells are used 1–3 days after plating on collagen-coating coverslips. The cells are maintained at 37°C. At this temperature, the responsiveness of the cells to secretagogues, particularly to cAMP-dependent agonists, is improved. Increases in Cl^- and K^+ conductances are the major electrical events during stimulation of Cl^- secretion. Accordingly, bath-pipette ion gradients are chosen so that transmembrane Cl^- and K^+ currents can be monitored independently at clamp voltages equal to the reversal potentials of these ions. The pipette solution is: 115 MM KCl, 25 mM *N*-methyl-D-glucamine (NMDG) glutamate, 0.5 mM

EGTA, 0.19 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 0.05 mM Na_3GPT, 5 mM HEPES, pH 7.2. The bath solution is: 115 mM NaCl, 40 mM NMDG glutamate, 5 mM potassium glutamate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.2. Bath Na^+ and Cl^- concentrations are reduced by substituting NMDG chloride or sodium glutamate for NaCl. When Na^+ and K^+ free solutions are used, Na^+ and K^+ are replaced by $NMDG^+$, and Cl^- is reduced by replacing Cl^- by glutamate.

During whole-cell recording, the membrane potential is clamped alternately to three different voltages, each for 500-msec duration. Computer-controlled voltage-clamp protocols are used to generate current-voltage (I-V) relations when the transmembrane currents are relatively stable by stepping the clamp voltage between −100 and +100 mV at 20 mV intervals.

Test drugs (e. g., 8-(4-chlorophenylthio) adenosine 3′,5′-cyclic monophosphate, A23187, forskolin, or ionomycin) are solubilized in stock solutions (ethanol of DMSO) and diluted.

EVALUATION

Instantaneous relations are constructed from currents recorded 6 msec after a voltage step.

MODIFICATIONS OF THE METHOD

Maertens et al. (2000) used the whole-cell patch-clamp technique to study the effect of an antimalarial drug on the volume-regulated anion channel (VRAC) in cultured bovine pulmonary artery endothelial cells. They also examined the effects on other Cl^- channels, i. e., the Ca^{2+} activated Cl^- channel and the cystic fibrosis transmembrane conductance regulator to assess the specificity for VRAC.

Pusch et al. (2000) characterized chloride channels belonging to the ClC family. Chiral clofibric acid derivates were tested on the human ClC-1 channel, a skeletal muscle chloride channel, after heterologous expression in *Xenopus laevis* oocytes by means of two microelectrode voltage clamp recordings.

REFERENCES AND FURTHER READING

Frings S, Reuter D, Kleene SJ (2000) Neuronal Ca^{2+} activated Cl^- channels – homing in on an elusive channel species. Progr Neurobiol 60:247–289

Jentsch TJ, Günther W (1997) Chloride channels: an emerging molecular picture. BioAssays 19:117–126

Maertens C, Wie L, Droogmans G, Nilius B (2000) Inhibition of volume-regulated and calcium-activated chloride channels by the antimalarial mefloquine. J Pharmacol Exp Ther 295:29–36

Pusch M, Liantonio A, Bertorello L, Accardi A, de Lucca A, Pierno S, Tortorella V, Camerino DC (2000) Pharmacological characterization of chloride channels belonging to the ClC family by the use of chiral clofibric acid derivates. Mol Pharmacol 58:498–507

A.1.1.25 Inhibition of Hyperpolarization-Activated Channels

PURPOSE AND RATIONALE

The hyperpolarization-activated cation currents (termed I_f, I_h, or I_q) play a key role in the initiation of cardiac and neuronal pacemaker depolarizations. Unlike most voltage-gated channels, they are activated by hyperpolarizing voltage steps to potentials negative to –60 mV, near the resting potential of most cells. This property earned them the designation of I_f for "funny" or I_q for "queer." The funny current, or pacemaker (I_f) current, was first described in cardiac pacemaker cells of the mammalian sino-atrial node as a current that slowly activates on hyperpolarization at voltages in the diastolic voltage range, and contributes to the generation of cardiac rhythmic activity and to its control by sympathetic and parasympathetic innervations (DiFrancesco et al. 1986; Accili et al. 1997, 2002; Robinson and Siegelbaum 2003}; Baruscotti et al. 2005). In sino-atrial cells, f-channels are modulated by cAMP independently of phosphorylation, through a mechanism involving direct interaction of cAMP with the intracellular side of the channels (DiFrancesco and Tortora 1991; Bois et al. 1996). A significant advancement in the study of molecular properties of pacemaker channels was achieved when a new family of channels was cloned, the HCN (hyperpolarization-activated, cyclic nucleotide gated) channels (Ishii et al. 1999; Kaupp and Seifert 2001; Biel et al. 2002; Macri et al. 2002). The HCN family is related to the cyclic nucleotide-gated channel and *eag* potassium channel family and belongs to the superfamily of voltage-gated cation channels. HCN channels are characterized by six membrane-spanning segments (S1–S6) including voltage-sensing (S4) and pore (between S5 and S6) regions. In the C-terminal region they contain a consensus sequence for binding of cyclic nucleotides. In the heart, neurotransmitter-induced control of cardiac rhythm is mediated by I_f through its second-messenger cAMP, whose synthesis is stimulated and inhibited by β-adrenoceptor and muscarinic agonists, respectively.

Inhibition of the I_f channel was recommended for induction of bradycardia and treatment of coronary disease (Thollon et al. 1994, 1997; Simon et al. 1995; Bois et al. 1996; Deplon et al. 1996; Acilli et al. 1997;

Rocchetti et al. 1999; Monnet et al. 2001, 2004; Bucchi et al. 2002; Cerbai et al. 2003; Rigg et al. 2003; Vilaine et al. 2003; Albaladejo et al. 2004; Colin et al. 2004; DiFrancesco and Camm 2004; Moreno et al. 2004; Mulder et al. 2004; Vilaine 2004; Chatelier et al. 2005; Leoni et al. 2005; Romanelli et al. 2005; Schipke et al. 2006).

Romanelli et al. (2005) reported the design, synthesis and preliminary biological evaluation of zatebradine analogs as potential blockers of hyperpolarization-activated current and Chatelier et al. (2005) described that a calmodulin antagonist directly inhibits f-type current in rabbit sino-atrial cells.

PROCEDURE

Sino-atrial Cell Isolation

Sino-atrial node myocytes of the rabbit were isolated (DiFrancesco et al. 1986). Cells were allowed to settle in Petri dishes, and were superfused with normal Tyrode solution containing (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, D-glucose 5.5, Hepes–NaOH 5; pH 7.4.

Electrophysiology

In macro-patch experiments the temperature was kept at 27°C–28°C and the patch pipette solution contained (mM): NaCl 70, KCl 70, $CaCl_2$ 1.8, $MgCl_2$ 1, $BaCl_2$ 1, $MnCl_2$ 2, Hepes–KOH 5; pH 7.4. The control solution perfusing the intracellular side of the membrane patches contained (mM): potassium aspartate 130, NaCl 10, $CaCl_2$ 2, EGTA 5, Hepes–KOH 10; pH 7.2, pCa = 7. In some experiments, the calcium concentration of the bath solution was reduced to 0.1 nM according to the calculation of Fabiato and Fabiato (1979) and the correction of Tsien and Rink (1980).

Macro-patches containing hundreds of f-channels were formed using a large-tipped pipette (0.5 to 2 MΩ) (DiFrancesco and Tortora 1991). The test compound or calmodulin (Calbiochem) was dissolved in either distilled water and ethanol (50/50) or distilled water, respectively, divided into aliquots, and stored at −20°C until use. Ethanol was added to control solutions at the same concentration used in test solutions (lower than 0.1%).

EVALUATION

The time course of macro-patch I_f under the influence of the modifying compounds was recorded by applying hyperpolarizing steps of 3 s duration at a frequency of 1/15 Hz. At steady-state, the voltage dependence of I_f was described by the equation: $I_f(E) = g_f(E) \cdot (E - vE_f) = g_{fmax} \cdot y_\infty(E) \cdot (E - E_f)$, where g_f is the conductance, g_{fmax} the fully activated conductance, y_∞ (E) the steady-state activation parameter and E_f the reversal potential (DiFrancesco and Noble 1985). Steady-state current/voltage (I/V) curves were measured by applying 1-min-long hyperpolarizing voltage ramps with a rate of −115 mV/min from a holding potential of −35 mV. Conductance–voltage (g_f/E) relations were then obtained from the above equation as ratios between steady-state I/V curves (i_f/E) and $E-E_f$, where E_f was set to −12.24 mV (DiFrancesco and Mangoni 1994). Conductance curves were fitted by Boltzmann function: $g_f(E) = g_{fmax} \cdot y_\infty(E) = g_{fmax} \cdot 1/[1 + \exp(E - E_{1/2})/p]$ where $E_{1/2}$ is the half-maximal voltage of activation and p is the inverse-slope factor. This allowed estimation of the shifts of the voltage dependence of conductance (i. e., of the activation parameter y_∞) measured as changes in $E_{1/2}$. Shifts of the I_f activation curve caused by cAMP were also determined by a quicker method not requiring measurement of the conductance–voltage relation (Accili and DiFrancesco 1996). Shifts were obtained by applying hyperpolarizing steps from −35 mV to near the mid-point of the I_f activation curve and adjusting the holding potential (−35 mV in the control solution) until the cAMP-induced change in I_f was compensated and the control I_f magnitude fully restored. Since the compensation involved a change of the test voltage (from E to $E + s_m$, where s_m is the measured displacement of the holding potential in mV), a correction was introduced to obtain the shift of the activation curve (s, mV), according to the relation: $s = s_m \cdot [+ (y_\infty / (dy_\infty / dE)] / (E - E_f)$.

When comparing different sets of data, statistical analysis was performed with either the Student's t-test or analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant. Statistical data were given as mean ± SEM values.

REFERENCES AND FURTHER READING

Albaladejo P, Challande P, Kakou A, Benetos A, Labat C, Louis H, Safar ME, Lacolley P (2004) Selective reduction of heart rate by ivabradine: effect on the visco-elastic arterial properties in rats.. J Hypertens 22:1739–1745

Accili EA, DiFrancesco D (1996) Inhibition of the hyperpolarization-activated current (if) of rabbit SA node myocytes by niflumic acid. Pflügers Arch 431:757–762

Accili EA, Robinson RB, DiFrancesco D (1997) Properties and modulation of I_f in newborn versus adult SA node. Am J Physiol 272:H1549–H1552

Accili EA, Proenza C, Baruscotti M, DiFrancesco D (2002) From funny current to HCN channels: 20 years of excitation. News Physiol Sci 17:32–37

Baruscotti M, Bucchi A, DiFrancesco D (2005) Physiology and pharmacology of the cardiac pacemaker ("funny") current. Pharmacol Ther 107:59–79

Biel M, Schneider A, Wahl C (2002) Cardiac HCN channels: structure, function, and modulation. Trends Cardiovasc Med 12:206–2134
Bois P, Bescond J, Renaudon B, Lenfant J (1996) Mode of action of bradycardic agent, S-16257, on ionic currents of rabbit sinoatrial node cells. Br J Pharmacol 118:1051–1057
Bucchi A, Baruscotti M, DiFrancesco D (2002) Current-dependent block of rabbit sino-atrial node I_f channels by Ivabradine. J Gen Physiol 120:1–13
Cerbai E, de Paoli P, Sartiani L, Lonardo G, Mugelli A (2003) Treatment with Irbesartan counteracts the functional remodeling of ventricular myocytes from hypertensive rats. J Cardiovasc Pharmacol 41:804–812
Chatelier A, Renaudon B, Bescond J, El Chemaly A, Demion M, Bois P (2005) Calmodulin antagonist W7 directly inhibits f-type current in rabbit sino-atrial cells. Eur J Pharmacol 521:29–33
Colin P, Ghaleb B, Monnet X, Hittinger L, Berdeaux A (2004) Effect of graded heart rate reduction with Ivabradine on myocardial oxygen consumption and diastolic time in exercising dogs. J Pharmacol Exp Ther 308:236–240
Deplon E, Valenzuela C, Perez O, Franqueza L, Gay P, Snyders DJ, Tamargo J (1996) Mechanisms of block of human cloned potassium channel by the enantiomers of a new bradycardic agent: S-16257–2 and S-1620–2. Br J Pharmacol 117:1293–1301
DiFrancesco D, Ferroni A, Mazzanti M, Tromba C (1986), Properties of the hyperpolarizing-activated current (i_f) in cells isolated from the rabbit sino-atrial node. J Physiol (Lond) 377:61–88
DiFrancesco P, Tortora D (1991) Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 351:145–147
DiFrancesco D, Mangoni M (1994) Modulation of single hyperpolarization-activated channels i_f by cAMP in the rabbit sino-atrial node. J Physiol (Lond) 474:473–482
DiFrancesco D, Camm JA (2004) Heart rate lowering by specific and selective I_f current inhibition with Ivabradine. Drugs 64:1757–1765
Fabiato A, Fabiato F (1979) Calcium programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J Physiol (Lond) 75:463–505
Ishii TM, Takano M, Xie LH, Noma A, Ohmori H (1999) Molecular characterization of the hyperpolarization-activated cation channel in rabbit heart sinoatrial node. J Biol Chem 274:12835–12839
Kaupp UB, Seifert R (2001) Molecular diversity of pacemaker channels. Annu Rev Physiol 63:235–257
Leoni AL, Marionneau C, Demolombe S, Le Bouter S, Mangoni ME, Escande D, Charpentier F (2005) Chronic heart rate reduction remodels ion channel transcripts in the mouse sinoatrial node but not in the ventricle. Physiol Genomics 24:4–12
Macri V, Proenza C, Agranovich E, Angoli D, Accili EA (2002) Separable gating mechanisms in a mammalian pacemaker channel. J Biochem Chem 277:35939–35946
Monnet X, Ghaleb B, Colin P, de Curzon OP, Guidicelli JF, Berdeaux A (2001) Effects of heart rate reduction with Ivabradine on exercise-induced myocardial ischemia and stunning. J Pharmacol Exp Med 299:1133–1139
Monnet X, Colin P, Ghaleb B, Hittinger L, Giudicelli JF, Berdeaux (2004) Heart rate reduction during exercise-induced myocardial ischemia and stunning. Eur Heart J 25:579–586
Moreno AP (2004) Biophysical Properties of homomeric and heteromultimeric channels formed by cardiac connexins. Cardiovasc. Res. 62:276–286
Mulder P, Barbier S, Chagraoui A, Richard V, Henry JP, Lallemand F, Renet S, Lerebours G, Mahlberg-Gaudin F, Thuillez C (2004) Long-term heart rate reduction induced by the selective If current inhibitor Ivabradine improves left ventricular function and intrinsic myocardial structure in congestive heart failure. Circulation 109:1674–1679
Rigg L, Mattick PAD, Heath BM, Terrar DA (2003) Modulation of the hyperpolarization-activated current (I_f) by calcium and calmodulin in the guinea-pig sino-atrial node. Cardiovasc Res 57:497–504
Robinson RB, Siegelbaum SA (2003) Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 65:453–480
Rocchetti M, Armato A, Cavalieri B, Micheletti M, Zaza A (1999) Lidocaine inhibition of the hyperpolarization-activated current in sinoatrial myocytes. J Cardiovasc Pharmacol 34:434–439
Romanelli MN, Cerbai E, Dei S, Guandalini L, Martelli C, Martini E, Scapecchi S, Teodori E, Mugelli A (2005) Design, synthesis and preliminary biological evaluation of zatebradine analogues as potential blockers of hyperpolarization-activated current. Bioorg Med Chem 13:1211–1220
Schipke JD, Büter I, Hohlfeld T, Schmitz-Spanke S, Gams E (2006) Selektive I_f-Kanal-Hemmung: eine Alternative in der Behandlung der koronaren Herzkrankheit? Herz 31:55–74
Simon L, Ghaleb B, Puybasset L, Giudicelli JF, Berdeaux (1995) Coronary and hemodynamic effects of S 16257, a new bradycardic agent, in resting and exercising conscious dogs. J Pharmacol Exp Ther 275:659–665
Thollon C, Cambarrat C, Vian J, Prost JF, Peglion JL, Vilaine JP (1994) Electrophysiological effects of S 16257, a novel sino-atrial node modulator, on rabbit and guinea-pig cardiac preparations: comparison with UL-FS 49. Br J Pharmacol 112:37–42
Thollon C, Bidouard JP, Cambarrat C, Lesage L, Reure H, Delescluse I, Vian J, Peglion JL, Vilaine JP (1997) Stereospecific in vitro and in vivo effects of the new sinus node inhibitor (+)-S 16257. Eur J Pharmacol 339:43–51
Tsien RW, Rink J (1980) Neutral carrier ion-selective microelectrodes for measurements of intracellular free calcium. Biochim Biophys Acta 599:623–638
Vilaine JP, Bidouard JP, Lesage L, Reure H, Péglion JL (2003) Anti-ischemic effects of Ivabradine, a selective heart rate-reducing agent, in exercise-induced myocardial ischemia in pigs. J Cardiovasc Pharmacol 42:688–696
Vilaine JP (2004) Selection et caracterisation pharmacologique de Procorolan, un inhibiteur selectif du courant pacemaker If. Therapie 59:495–505

A.1.1.26 Measurement of Cytosolic Calcium with Fluorescent Indicators

PURPOSE AND RATIONALE

Intracellular free Ca-concentration can be measured in cultured endothelial cells with a fluorometric methods (Tsien et al. 1982; Grynkiewicz et al. 1985; Lückhoff et al. 1988; Busse and Lamontagne 1991; Hock et al. 1991).

PROCEDURE

Cultured endothelial cells from the pig are seeded on quartz coverslips and grown to confluence. The cells

are loaded with the fluorescent probe indo-1 by incubation with 2 µmol indo-1/AM and 0.025% Pluronic F-127, a non-ionic detergent. Thereafter, the coverslips are washed and transferred to cuvettes, filled with HEPES buffer.

EVALUATION

Fluorescence is recorded in a temperature controlled (37°C) spectrofluorophotometer (excitating wavelength 350 nm, emission wavelength simultaneously measured at 400 and 450 nm).

MODIFICATIONS OF THE METHOD

Lee et al. (1987) measured cytosolic calcium transients from the beating rabbit heart using indo-1 AM as indicator.

Yangisawa et al. (1989) measured intracellular Ca^{2+} concentrations in coronary arterial smooth muscle of dogs with fura-2.

Makujina et al. (1995) measured intracellular calcium by fura-2 fluorescence simultaneously with tension in everted rings of porcine coronary artery denuded of endothelium.

Hayashi and Miyata (1994) described the properties of the commonly used fluorescent indicators for intracellular calcium: Fura-2, Indo-1, and Fluo-3.

Monteith et al. (1994) studied the Ca^{2+} pump-mediated efflux in vascular in spontaneously hypertensive rats.

REFERENCES AND FURTHER READING

Busse R, Lamontagne D (1991) Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. Naunyn-Schmiedeberg's Arch Pharmacol 344:26–129

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{+2} indicators with improved fluorescence properties. J Biol Chem 260:3440–3450

Hayashi H, Miyata H (1994) Fluorescence imaging of intracellular Ca^{2+}. J Pharmacol Toxicol Meth 31:1–10

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J, Schölkens BA (1991) Hoe 140 a new potent and long acting bradykinin antagonist: *in vitro* studies. Br J Pharmacol 102:769–773

Lee HC, Smith N, Mohabir R, Clusin WT (1987) Cytosolic calcium transients from the beating mammalian heart. Proc Natl Acad Sci USA 84:7793–7797

Lückhoff A, Pohl U, Mülsch A, Busse R (1988) Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. Br J Pharmacol 95:189–196

Makujina SR, Abebe W, Ali S, Mustafa SJ (1995) Simultaneous measurement of intracellular calcium and tension in vascular smooth muscle: validation of the everted ring preparation. J Pharmacol Toxicol Meth 34:157–163

Monteith GR, Chen S, Roufogalis BD (1994) Measurement of Ca^{2+} pump-mediated efflux in hypertension. J Pharmacol Toxicol Meth 31:117–124

Tsien RY, Pozzan T, Rink TJ (1982) Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. J Cell Biol 94:325–334

Wiemer G, Popp R, Schölkens BA, Gögelein H (1994) Enhancement of cytosolic calcium, prostaglandin and nitric oxide by bradykinin and the ACE inhibitor ramiprilat in porcine brain capillary endothelial cells. Brain Res 638:261–266

Yanagisawa T, Kawada M, Taira N (1989) Nitroglycerine relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2. Br J Pharmacol 98:469–482

A.1.1.27 Measurement of Contractile Force of Isolated Cardiac Myocytes

PURPOSE AND RATIONALE

Eschenhagen et al. (1997) developed a method for culturing embryonic cardiomyocytes in a collagen matrix to produce a coherently contracting 3-dimensional model heart tissue that allows direct measurement of isometric contractile force.

PROCEDURE

Ventricles from 9–11 day incubated chicken embryos (Cavanaugh 1955) are minced in Dulbecco's minimal essential medium (DMEM), washed once with 0.25% trypsin/0.1% EDTA in phosphate buffered saline (PBS), pH 7.45, and then digested in fresh trypsin/EDTA for 15 min at 37°C. The supernatant is discarded and the pellet is subjected to digestion with 0.1% collagenase (144 U/mg) in PBS, pH 7.45, for 30 min at 37°C. This supernatant is discarded and the pellet digested further with several cycles of collagenase for 10–20 min each until the pellet is completely digested. DNase I (40 µl, 1 mg/ml in PBS) is added between cycles depending on the presence of viscous DNA. The isolated cells are kept in Petri dishes in DMEM supplemented with 15% heat-inactivated fetal calf serum in the CO_2 incubator. After completion of the digestion, the cells are incubated for another 30–60 min in the CO_2 incubator (preplating). The cell suspension is centrifuged at 250 rpm (12 *g*). The pellet is resuspended in 10 ml culture medium (DMEM, 10% inactivated horse serum, 2% chicken embryo extract (Gibco BRL), 2 mmol/l glutamine, 10 µg/ml streptomycin, and 100 U/ml penicillin G, recentrifuged at 250 rpm, and finally resuspended in culture medium at $2–3 \times 10^6$ cells per ml.

For casting cardiomyocyte-populated collagen gels, strips of Velcro are glued with silicone rubber to glass

tubes (13 mm length, 3 mm outer diameter, 2 mm inner diameter). Pairs of Velcro-coated tubes, kept at a fixed distance by a stainless steel wire spacer, are placed in rectangular wells ($15 \times 17 \times 4$ mm) cut into a layer of silicone rubber in a 100 mm polymethylenepentene Petri dish. This assembly is autoclaved before use. For each gel, 1 ml of an ice-cold collagen/cell mixture is poured into each well between the Velcro-coated glass tubes. This mixture has the same composition as the culture medium and contains in addition to 1 mg neutralized collagen I from rat tail (Upstate Biotechnology, Inc.), 1×10^6 cardiomyocytes, the acetic acid in the collagen solution, and the NaOH to neutralize it. The mixture is allowed to gel at 37°C for 60 min before culture medium is added to the dish. Medium changes are performed after overnight and then every other day.

After 6–11 days in culture, the gels are removed from the culture dish, the spacers are withdrawn, and one of the glass tubes is mounted on a fixed electrode; the other tube is connected by an inelastic silk string to an isometric force transducer attached to a Wekagraph thermal array recorder (Föhr Instruments, Heidelberg, Germany). The preparation is adjusted to its original (spacer) length before it is immersed in a convential water bath filled with modified Tyrode's solution maintained at 35°C and continuously gassed with 95% O_2 and 5% CO_2.

After a 30–60 min equilibration period without pacing, force and frequency reaches a stable value. Gels are then electrically stimulated with rectangular pulses (10 ms, 20–40 V) at a standard frequency of 1.5 Hz. Preload is stepwise adjusted to L_{max}, the length at which the preparation develops maximal force. Cumulative doses of inotropic compounds, e. g. isoprenaline or forskoline, are added. All gels are exposed to a concentration-response curve for calcium (1.8–12.6 mmol/l) and one or two additional inotropic stimuli.

EVALUATION

All values are presented as arithmetic means ± SEM. Student's *t*-test for paired observations is used to compare force of contraction, resting tension, or beating frequency before and after other interventions.

MODIFICATIONS OF THE METHOD

Ferrara et al. (1997) studied the role of Gi-proteins and β-adrenoceptors in the age-related decline of contraction in guinea-pig ventricular myocytes. The isolated myocytes were placed in Krebs-Henseleit solution in a Perspex chamber on the stage of a Zeiss IM inverted microscope and superfused with Krebs-Henseleit solution containing 1 mmol/l Ca^{2+} at 2 ml/min and 32°C. Cells were selected using the following criteria: rod shaped, without sarcolemmal blebs, no spontaneous contractions, stable baseline contraction to electrical stimulation at 0.5 Hz and sarcomere length not shorter than 1.67 µm. The image of the cells was displayed on a TV monitor and the length change measured with a video motion detector. Contraction amplitude and velocity was expressed as change in sarcomere length, calculated from the change in length of the myocyte and its original sarcomere length.

Using a similar technique, Harding et al. (1988) studied contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium, and Harding et al. (1992) isolated ventricular myocytes from failing and non-failing human heart.

REFERENCES AND FURTHER READING

Cavanaugh MW (1955) Growth of chick heart cells in monolayer culture. J Exp Zool 128:573–581

Eschenhagen T, Fink C, Remmers U, Scholz H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL (1997) Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model. FASEB J 11:683–694

Ferrara N, Böhm M, Zolk O, O'Gara P, Harding SE (1997) The role of Gi-proteins and β-adrenoceptors in the age-related decline of contraction in guinea-pig ventricular myocytes. J Mol Cell Cardiol 29:439–448

Harding SE, Vescovo G, Kirby M, Jones SM, Gurden J, Poole-Wilson PA (1988) Contractile responses of isolated rat and rabbit myocytes to isoproterenol and calcium. J Mol Cell Cardiol 20:635–647

Harding SE, Jones SM, O'Gara P, del Monte F, Vescovo G, Poole-Wilson PA (1992) Isolated ventricular myocytes from failing and non-failing human heart; the relation of age and clinical status of patients to isoproterenol response. J Mol Cell Cardiol 24:549–564

A.1.1.28 Adrenomedullin

A.1.1.28.1 General Considerations

Adrenomedullin is a 52-amino acid peptide originally discovered in human adrenal pheochromocytoma by monitoring the elevating activity of platelet cAMP (Kitamura et al. 1993). Molecular cloning of rat adrenomedullin was reported by Sakata et al. (1993). The genomic structure of human adrenomedullin gene was reported by Ishimitsu et al. (1994). Adrenomedullin and **proadrenomedullin N-terminal 20 peptide (PAMP)** which are both hypotensive and bronchodilating, are derived from **preproadrenomedullin** (Kanazawa et al. 1995; Iwasaki et al.

1996; Shimosawa and Fujita 1996; Hinson et al. 1998; Samson 1998; Autelitano and Tang 1999; Jimenez et al. 1999; Lopez et al. 1999; Tajima et al. 1999).

Adrenomedullin is found ubiquitously in tissues and organs, especially in cardiovascular tissues and in the kidney, lung, brain and endocrine glands (Wimalawansa 1996; Van Rossum et al. 1997; Eto et al. 1999; Jougasaki and Burnett 2000; Kitamuro et al. 2000). The main biological effect is vasodilatation (Ishiyama et al. 1993; Nikitenko et al. 2002). A hypotensive effect has been found in rats (Khan 1997), rabbits (Fukuhara et al. 1995), and man (Lainchbury et al. 1997). Adrenomedullin belongs to the calcitonin gene-related peptide/calcitonin peptide family as it shares approximately 25% homology with calcitonin gene-related peptide (Kitamura et al. 1993). Several pharmacological studies are related to the vasodilating effect of adrenomedullin, e. g., in mouse aorta (Ashton et al. 2000), in the mesenteric vascular bed (see A.8.2.5), (Santiago et al. 1995), in the hind limb vascular bed (see A.8.2.1), (Santiago et al. 1994; Champion et al. 1996, 1997), in the pulmonary vascular bed (see A.8.2.6), (DeWitt et al. 1994; Lippton et al. 1994; Heaton et al. 1995; Nossaman et al. 1995), on cerebral blood flow in dogs (Baskaya et al. 1995) and in cats (Takao et al. (1999), on renal hemodynamics in dogs (see A.8.2.3) (Ebara et al. 1994; Yukawa 1998), or on vasodilation in perfused rat kidneys (Hayakawa et al. 1999). Intravenous infusion of adrenomedullin exerted diuresis and natriuresis without major changes in blood pressure and produced beneficial hemodynamic and renal vasodilator effects in rats with compensated heart failure (Vari et al. 1996; Nagaya et al. 1999). In isolated perfused, paced rat heart preparations, adrenomedullin showed a dose-dependent inotropic effect (Szokodi et al. 1998). Pulmonary vasodilator responses and vasorelaxant effects in isolated pulmonary artery rings were found by Gumusel et al. (1998). Adrenomedullin is a growth-promoting factor for cultured vascular smooth muscle cells (Iwasaki et al. 1998) and fibroblasts (Isumi et al. 1998).

Willenbrock et al. (1999) showed a beneficial effect of adrenomedullin on renal function in rats with aortocaval shunt.

Adrenomedullin inhibits gastric secretion in rats with chronic gastric fistula (see J.3.1.3), (Rossowski et al. 1997) and inhibits reserpine-induced gastric lesions in rats (Clementi et al. 1998). Tsuchida et al. (1999) found an inhibition of cholecystokinin-stimulated amylase secretion by adrenomedullin in rat pancreatic acini.

Rademaker et al. (2003) discussed the role of adrenomedullin in the pathophysiology of heart failure.

Lewis et al. (1998) described a specific and sensitive radioimmunoassay for human adrenomedullin.

Ohta et al. (1999) developed an one-step direct assay for adrenomedullin with monoclonal antibodies.

N-terminal fragments of adrenomedullin show vasopressor activities (Watanabe et al. 1996).

Adrenotensin, an other adrenomedullin gene product, contract in an endothelium-dependent manner pulmonary blood vessels (Gumusel et al. 1996).

REFERENCES AND FURTHER READING

Ashton D, Hieble P, Gout B, Aiyar N (2000) Vasodilatory effect of adrenomedullin in mouse aorta. Pharmacology 61:101–105

Autelitano DJ, Tang F (1999) Coexpression of preproadrenomedullin with a putative adrenomedullin receptor gene in vascular smooth muscle. Clin Sci 96:493–498

Baskaya MK, Suzuki Y, Anzai M, Seki Y, Saito K, Takayasu M, Shibuya M, Sugita K (1995) Effects of adrenomedullin, calcitonin gene-related peptide, and amylin on cerebral circulation in dogs. J Cerebr Blood Flow Metab 15:827–834

Champion HC, Duperier CD, Fitzgerald WE, Lambert DG, Murphy WA, Coy DH, Kadowitz PJ (1996) [Mpr^{14}]-rADM(14–50), a novel analog of adrenomedullin, possesses potent vasodilator activity in the hindlimb vascular bed of the cat. Life Sci 59: PL1–7

Champion HC, Akers DL, Santiago JA, Lambert DG, McNamara DB, Kadowitz PJ (1997) Analysis of the responses to human synthetic adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat. Mol Cell Biochem 176:5–11

Clementi G, Caruso A, Cutuli VCM, de Bernardis E, Prato A, Mangano NG, Amico-Roxas M (1998) Effect of centrally or peripherally injected adrenomedullin on reserpine-induced gastric lesions. Eur J Pharmacol 360:51–54

DeWitt BJ, Cheng DY, Caminiti GN, Nossaman BD, Coy DH, Murphy WA, Kadowitz PJ (1994) Comparison of responses to adrenomedullin and calcitonin gene-related peptide in the pulmonary vascular bed of the cat. Eur J Pharmacol 257:303–306

Ebara T, Miura K, Okumura M, Matsuura T, Kim S, Yukimura T, Iwao H (1994) Effect of adrenomedullin on renal hemodynamics and function in dogs. Eur J Pharmacol 263:69–73

Eto T, Kitamura K, Kato J (1999) Biological and clinical roles of adrenomedullin in circulation control and vascular diseases. Clin Exp Pharmacol Physiol 26:371–380

Fukuhara M, Tsuchihashi T, Abe I, Fujishima M (1995) Cardiovascular and neurohormonal effects of intravenous adrenomedullin in conscious rabbits. Am J Physiol 269, Regul Integr Comp Physiol 38:R1289–1293

Gumusel B, Chang JK, Hao Q, Hyman A, Lippton H (1996) Adrenotensin: An adrenomedullin gene product contracts pulmonary blood vessels. Peptides 17:461–465

Gumusel B, Hao Q, Hyman AL, Kadowitz PJ, Champion HC, Chang JK, Mehta JL, Lippton H (1998) Analyses of responses to adrenomedullin-(-3.52) in the pulmonary vascular bed of rats. Am J Physiol 274; Heart Circ Physiol 43:H1255–H1263

Hayakawa H, Hirata Y, Kakoki M, Suzuki Y, Sugimoto T, Omata M (1999) Role of nitric oxide-cAMP pathway in

adrenomedullin-induced vasodilation in the rat. Hypertension 33:689–693

Heaton J, Lin B, Chang J-K, Steinberg S, Hyman A, Lippton H (1995) Pulmonary vasodilation to adrenomedullin: A novel peptide in humans. Am J Physiol 268, Heart Circ Physiol 37:H2211–H2215

Hinson JP, Hagi-Pavli E, Thomson LM, Kapas S (1998) Pro-adrenomedullin N-terminal 20 peptide (PAMP) receptors and signal transduction in the rat adrenal gland. Life Sci 62:439–443

Ishimitsu T, Kojima M, Kangawa K, Hino J, Matsuoka H, Kitamura K, Eto T, Matsuo H (1994) Genomic structure of human adrenomedullin gene. Biochem Biophys Res Commun 203:631–639

Ishiyama Y, Kitamura K, Ichiki Y, Nakamura S, Kida O, Kangawa E, Eto T (1993) Hemodynamic effects of a novel hypotensive peptide, human adrenomedullin, in rats. Eur J Pharmacol 241:271–273

Isumi Y, Minamino N, Katafuchi T, Yoshioka M, Tsujii T, Kangawa K, Matsuo H (1998) Adrenomedullin production in fibroblasts: Its possible function as a growth regulator of Swiss 3T3 cells. Endocrinology 139:2552–2563

Iwasaki H, Hirata Y, Iwashina M, Sato K, Marumo F (1996) Specific binding sites for proadrenomedullin N-terminal 20 peptide (PAMP) in the rat brain. Endocrinology 137:3045–3050

Iwasaki H, Eguchi S, Shichiri M, Marumo F, Hirata Y (1998) Adrenomedullin as a novel growth-promoting factor for cultured vascular smooth muscle cells: Role of tyrosine kinase-mediated mitogen-activated protein kinase activation. Endocrinology 139:3432–3441

Jimenez N, Calvo A, Martinez A, Rosell D, Cuttitta F, Montuenga LM (1999) Expression of adrenomedullin and proadrenomedullin N-terminal 20 peptide in human and rat prostate. J Histochem Cytochem 47:1167–1177

Jougasaki M, Burnett JC Jr (2000) Adrenomedullin: Potential in physiology and pathophysiology. Life Sci 66:855–872

Kanazawa H, Kawaguchi T, Fujii T, Kudoh S, Hirata K, Kurihara N, Takeda T (1995) Comparison of bronchodilator responses to adrenomedullin and proadrenomedullin N-terminal 20 peptide. Life Sci 57: PL241-PL245

Khan AI, Kato J, Kitamura K, Kanagawa K, Eto T (1997) Hypotensive effect of chronically infused adrenomedullin in conscious Wistar–Kyoto and spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 24:139–142

Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 192:553–560

Kitamuro T, Takahashi K, Nakayama M, Murakami O, Hida W, Shirato K, Shibahara S (2000) Induction of adrenomedullin during hypoxia in cultures human glioblastoma cells. J Neurochem 75:1826–1833

Lainchbury JG, Cooper GJS, Coy DH, Jiang N-Y, Lewis LK, Yandle TG, Richards AM, Nicholls MG (1997) Adrenomedullin: A hypotensive hormone in man. Clin Sci 92:467–472

Lewis LK, Smith MW, Yandle TG, Richards AM, Nicholls MG (1998) Adrenomedullin (I-52) measured in human plasma by radioimmunoassay: Plasma concentration, adsorption, and storage. Clin Chem 44:571–577

Lippton H, Chang J-K, Hao Q, Summer W, Hyman AL (1994) Adrenomedullin dilates the pulmonary vascular bed *in vivo*. J Appl Physiol 76:2154–2156

Lopez J, Cuesta N, Martinez A, Montuenga L, Cuttitta F (1999) Proadrenomedullin N-terminal 20 peptide (PAMP) immunoreactivity in vertebrate juxtaglomerular cells identified by both light and electron microscopy. Gen Comp Endocrinol 116:192–203

Nagaya H, Nishikimi T, Horio T, Yoshihara F, Kanazawa A, Matsuo H, Kangawa K (1999) Cardiovascular and renal effects of adrenomedullin in rats with heart failure. Am J Physiol 276; Regul Integr Comp Physiol 45:R213–R218

Nikitenko LL, Smith DM, Hague S, Wilson CR, Bicknell R, Rees MCP (2002) Adrenomedullin and the microvasculature. Trends Pharmacol Sci 23:101–103

Nossaman BD, Feng CJ, Cheng DY, Dewitt BJ, Coy DH, Murphy WA, Kadowitz PJ (1995) Comparative effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat. Life Sci 56:63–66

Ohta H, Tsuji T, Asai S, Sasakura K, Teraoka H, Kitamura K, Kangawa K (1999) One-step direct assay for mature-type adrenomedullin with monoclonal antibodies. Clin Chem 45:244–251

Rademaker MT, Cameron VA, Charles CJ, Lainchbury JG, Nicholls MG, Richards AM (2003) Adrenomedullin and heart failure. Regul Pept 112:51–60

Rossowski WJ, Jing N-Y, Coy DH (1997) Adrenomedullin, amylin, calcitonin-gene related peptide and their fragments are potent inhibitors of gastric secretion in rats. Eur J Pharmacol 336:51–63

Sakata J, Shimokubo T, Kitamura K, Nakamura S, Kangawa K, Matsuo H, Eto T (1993) Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. Biochem Biophys Res Commun 195:921–927

Samson WK (1998) Proadrenomedullin-derived peptides. Front Neuroendocrinol 19:100–127

Santiago JA, Garrison E, Ventura VL, Coy DH, Bitar K, Murphy WA, McNamara DB, Kadowitz PJ (1994) Synthetic human adrenomedullin and adrenomedullin 15–52 have potent short-lived vasodilator activity in the hindlimb vascular bed of the cat. Life Sci 55: PL85–PL90

Santiago JA, Garrison E, Purnell WL, Smith RE, Champion HC, Coy DH, Murphy WA, Kadowitz PJ (1995) Comparison of responses to adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat. Eur J Pharmacol 272:115–118

Shimosawa T, Fujita T (1996) Hypotensive effect of a newly identified peptide, proadrenomedullin N-terminal 20 peptide. Hypertension 28:325–329

Szokodi T, Kinnunen P, Tavi P, Weckstrom M, Toth M, Ruskoaho H (1998) Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. Circulation 97:1062–1070

Tajima A, Osamura RY, Takekoshi S, Itoh Y, Sanno N, Mine T, Fujita T (1999) Distribution of adrenomedullin (AM), proadrenomedullin N-terminal 20 peptide, and AM mRNA in the rat gastric mucosa by immunocytochemistry and *in situ* hybridization. Histochem Cell Biol 112:139–146

Takao M, Tomita M, Tanahashi N, Kobari M, Fukuuchi Y (1999) Transient vasodilatory effects of adrenomedullin on cerebral parenchymal microvessels in cats. Neurosci Lett 268:147–150

Tsuchida T, Ohnishi H, Tanaka Y, Mine T, Fujita T (1999) Inhibition of stimulated amylase secretion by adrenomedullin in rat pancreatic acini. Endocrinology 140:865–870

Van Rossum D, Hanisch UK, Quirion R (1997) Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev 21:649–678

Vari RC, Adkins SD, Samson WK (1996) Renal effects of adrenomedullin in the rat. Proc Soc Exp Biol Med 211:178–183

Watanabe TX, Itahara Y, Inui T, Yoshizawa-Kumagaye K, Nakajima K, Sakakibara S (1996) Vasopressor activities of N-terminal fragments of adrenomedullin in anesthetized rats. Biochem Biophys Res Commun 219:59–63

Willenbrock R, Pagel I, Krause EG, Scheuermann M, Dietz R (1999) Acute hemodynamic and renal effects of adrenomedullin in rats with aortocaval shunt. Eur J Pharmacol 369:195–203

Wimalawansa SJ (1996) Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocrine Rev 17:533–585

Yukawa H (1998) Effect of adrenomedullin on systemic and regional hemodynamics in dogs. Teikyo Med J 25:381–388

A.1.1.28.2 Receptor Binding of Adrenomedullin

PURPOSE AND RATIONALE

Muff et al. (1995), Poyer (1997) reviewed the binding characteristics of the structurally related hormones calcitonin, calcitonin gene-related peptide, amylin, and adrenomedullin. Vine et al. (1996) compared *in vitro* binding of adrenomedullin, calcitonin gene-related peptide and amylin.

Specific adrenomedullin binding sites were described in human brain (Sone et al. 1997), in the rat spinal cord (Owji et al. 1996), in cultured brain cells (Zimmermann et al. 1996), and in cultured rat mesangial cells (Osajima et al. 1996).

PROCEDURE

Human brain is obtained at autopsy. For preparation of membranes, tissues are homogenized in ice-cold 50 mM HEPES buffer, pH 7.6; containing 0.25 M sucrose, 10 µg/ml soybean trypsin inhibitor, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml antipain, 0.1 mg/ml benzamidine, 0.1 mg/ml bacitracin, and 30 µg/ml aprotinin. The homogenates are centrifuged at 1500 *g* for 20 min at 4°C. The pellets are resuspended in 10 vol of the above buffer without sucrose and centrifuged at 100,000 *g* for 1 h at 4°C. The final pellets are resuspended to a concentration of 2–10 mg protein/ml, aliquoted, and stored at –80°C.

For the receptor binding assay, brain membranes (100 µg protein) are incubated at 4°C in 0.5 ml binding buffer (20 mM HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM NaCl, 4 mM KCl, 1 mM EDTA, and 0.3% BSA) containing 0.3 nM ^{125}I- human adrenomedullin in siliconized microcentrifuge tubes. Pellets are washed with 0.5 ml binding buffer at 4°C and counted in a γ-counter.

EVALUATION

Nonspecific binding is determined in the presence of 200 nM unlabeled human adrenomedullin. Specific binding is defined as total binding minus nonspecific binding. Data are calculated as mean ± SEM.

MODIFICATIONS OF THE METHOD

Eguchi et al. (1994) studied the binding of human adrenomedullin and analogs and the adenylate cyclase activity in cultured rat vascular smooth muscle cells.

Zimmermann et al. (1995) showed that adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured human neuroblastoma SK-N-MC cells.

Moody et al. (1997) investigated the binding affinity of adrenomedullin in C6 glioma cells.

Findings of Belloni et al. (1998) suggested the existence of different receptor subtypes for adrenomedullin in the human adrenal cortex.

Mazzocchi et al. (1999) found abundant [^{125}I]-adrenomedullin binding sites in both zona glomerulosa and adrenal medulla in the rat adrenal gland.

REFERENCES AND FURTHER READING

Belloni AS, Meneghelli V, Champion HC, Murray WA, Coy DH, Kadowitz PJ, Nussdorfer GG (1998) Autoradiographic evidence that zona glomerulosa and capsular vessels of the human adrenal cortex are provided with different subtypes of adrenomedullin receptors. Peptides 19:1581–1584

Eguchi S, Hirata Y, Iwasaki H, Sato K, Watanabe TX, Inui T, Nakajima K, Sakakibara S, Marumo F (1994) Structure-activity relationship of adrenomedullin a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. Endocrinology 135:2454–2458

Mazzocchi G, Albertin G, Andreis PG, Neri G, Malendowicz LK, Champion HC, Bahceliouglu M, Kadowitz PJ, Nussdorfer GG (1999) Distribution, functional role, and signaling mechanisms of adrenomedullin receptors in the rat adrenal gland. Peptides 20:1479–1487

Moody TW, Miller MJ, Martinez A, Unsworth E, Cuttitta F (1997) Adrenomedullin binds with high affinity, elevates cyclic AMP, and stimulates c-fos mRNA in C6 glioma cells. Peptides 18:1111–1115

Muff R, Born W, Fischer JA (1995) Receptors for calcitonin, calcitonin gene related peptide, amylin, and adrenomedullin. Can J Physiol Pharmacol 73:963–967

Osajima A, Uezono Y, Tamura M, Kitamura K, Mutoh Y, Ueta Y, Kangawa K, Kawamura M, Tanenao E, Yamashita H, Izumi F, Tagasugi M, Kuroiwa H (1996) Adrenomedullin-sensitive receptors are preferentially expressed in cultured rat mesangial cells. Eur J Pharmacol 315:319–325

Owji AA, Gardiner JV, Upton PD, Mahmoodi M, Ghatei MA, Bloom SR, Smith DM (1996) Characterization and molecular identification of adrenomedullin binding sites in the spinal cord: A comparison with calcitonin gene-related peptide receptors. J Neurochem 67:2172–2179

Poyner DR (1997) Molecular pharmacology of receptors for calcitonin-gene-related peptide, amylin and adrenomedullin. Biochem Soc Transact 25:1032–1036

Sone M, Takahashi K, Satoh F, Murakami O, Totsune K, Ohneda M, Sasano H, Ito H, Mouri T (1997) Specific adrenomedullin binding sites in the human brain. Peptides 18:1125–1129

Vine W, Beaumont K, Gedulin B, Pittner R, Moore CX, Rink TJ, Young AA (1996) Comparison of the *in vitro* and *in vivo* pharmacology of adrenomedullin, calcitonin gene-related peptide and amylin in rats. Eur J Pharmacol 314:115–121

Zimmermann U, Fischer JA, Muff R (1995) Adrenomedullin and calcitonin gene-related peptide interact with the same receptor in cultured human neuroblastoma SK-N-MC cells. Peptides 16:421–424

Zimmermann U, Fischer JA, Frei K, Fischer AH, Reinscheid RK, Muff R (1996) Identification of adrenomedullin receptors in cultured rat astrocytes and in neuroblastoma × glioma hybrid cells (NG108–15). Brain Res 724:238–245

A.1.1.29 Atrial Natriuretic Factor (ANF)

A.1.1.29.1 General Considerations

PURPOSE AND RATIONALE

The atria of mammalian hearts synthesize and secrete peptides with potent natriuretic and vasoactive properties known as ANF = atrial natriuretic factor (de Bold et al. 1981). The atrial natriuretic peptide hormonal system consists of a 126-amino acids prohormone synthesized within myocytes of the heart and stored in storage granules within the heart before release into circulation (Kangawa and Matsuo 1984; Oikawa et al. 1984; Vesely 1992). This hormonal system contains several peptides from the 126-amino acid-prohormone with blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties (Martin et al. 1990; Vesely et al. 1994). Thus, peptides consisting of amino acid 1 to 30 (LANP = long-acting natriuretic peptide), 31 to 67 (vessel dilator), 79 to 98 (kaliuretic peptide) and 99 to 126 (ANF) each have blood pressure lowering, natriuretic, diuretic and/or kaliuretic properties both in humans and in animals. Human and rat atria predominantly secrete a peptide of 28 amino acid residues, ANF-(99–126), which represents the C-terminus of a precursor sequence of 126 amino acid residues. In addition, vessel dilator and LANP circulate as distinct entities after having been proteolytically cleaved from the rest of the amino terminus by proteases (Ackerman et al. 1997).

Plasma immunoreactive ANF-(99–126) concentration increases in normal rats after volume expansion, while infusion of the peptide lowers blood pressure in several animal models of hypertension.

An international standard for atrial natriuretic factor was established by an international collaborative study (Poole et al. 1988). Human ANF-(99–126) was synthesized, highly purified and distributed to several laboratories, who performed radioimmunoassays, radioreceptor assays and an *in vitro* assay using the vasorelaxant activity in precontracted rat aortic strips.

The C-type natriuretic peptide is a 22-amino acid peptide that was initially identified in the central nervous system (Ogawa et al. 1992; Barr et al. 1996; Amin et al. 1996). The distribution of C-type natriuretic peptide, which has structural homology with atrial and brain natriuretic peptides and also similar activities, is wide and includes the endothelium, myocardium, gastrointestinal and genitourinary tracts. **Brain natriuretic peptide** has been described as a novel cardiac hormone (Nakao et al. 1991). Yasue et al. (1994) studied the localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. N-terminal pro-brain natriuretic peptide became a diagnostic screening tool to differentiate between patients with normal and left ventricular systolic function (Bay et al. 2003; Gardner et al. 2003).

Jiao and Baertschi (1993) reviewed the neural control of the endocrine rat heart. Stimulation of cardiac sympathetic nerves potently stimulates ANF secretion.

ANF inhibits proliferation in non-myocardial cells and is anti-hypertrophic in cardiomyocytes. Silberbach et al. (1999) reported that activation of an extracellular signal-regulated protein kinase is required for the anti-hypertrophic effect of atrial natriuretic factor in neonatal rat ventricular myocytes.

REFERENCES AND FURTHER READING

Ackerman BH, Overton RM, McCormick MT, Schocken DD, Vesely DL (1997) Disposition of vessel dilator and long-acting natriuretic peptide in healthy humans after one-hour infusion. J Pharm Exp Ther 282:603–608

Amin J, Carretero OA, Ito S (1996) Mechanism of action of atrial natriuretic factor and C-type natriuretic peptide. Hypertension 27:684–687

Barr CS, Rhodes P, Struthers AD (1996) C-type natriuretic peptide. Peptides 17:1243–1251

Bay M, Kirk V, Parner J, Hassager C, Nielsen H, Krogsgaard K, Trawinski J, Boesgaard S, Aldershvile J (2003) NT-proBNP: a new diagnostic screening tool to differentiate between patients with normal and left ventricular systolic function. Heart 89:150–154

De Bold AJ, Borenstein HB, Veress AT, Sonnenberg HA (1981) A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sci 28:89–94

Gardner RS, Özlalp F, Murday AJ, Robb SD, McDonagh TA (2003) N-terminal pro-brain natriuretic peptide. A new gold standard in predicting mortality in patients with advanced heart failure. Eur Heart J 24:1735–1743

Jiao J-H, Baertschi AJ (1993) Neural control of the endocrine rat heart. Proc Natl Acad Sci 90:7799–7803

Kangawa K, Matsuo H (1984) Purification and complete amino acid sequence of a α-human atrial natriuretic polypeptide (α-hANP) Biochem Biophys Res Commun 118:131–138

Martin DR, Pevahouse JB, Trigg DJ, Vesely DL, Buerkert JE (1990) Three peptides from the ANF prohormone NH_2-terminus are natriuretic and/or kaliuretic. Am J Physiol 258:F1401–F1408

Nakao K, Mukoyama M, Hosoda K, Suga S, Ogawa Y, Saito Y, Shirakai G, Arai H, Jougasaki M, Imura H (1991) Biosynthesis, secretion, and receptor selectivity of human brain natriuretic peptide. CanJ Physiol Pharmacol 69:1500–1506

Ogawa Y, Nakao K, Nakagawa O, Komatsu Y, Hosoda K, Suga S, Arai H, Nagata K, Yoshida N, Imura H (1992) Human C-type natriuretic peptide. Characterization of the gene and peptide. Hypertension 19:809–813

Oikawa S, Imai M, Ueno A (1984) Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. Nature 309:724–726

Poole S, Gaines Das RE, Dzau VJ (1988) The international standard for atrial natriuretic factor. Calibration by an international collaborative study. Hypertension 12:629–634

Silberbach M, Gorenc T, Hershberger RE, Stork PJS, Steyger PS, Roberts CT Jr (1999) Extracellular signal-regulated protein kinase activation is required for the anti-hypertrophic effect of atrial natriuretic factor in neonatal ventricular myocytes. J Biol Chem 274:24858–24864

Vesely DL (1992) Atrial Natriuretic Hormones. Prentice Hall: Englewood Cliffs, NJ

Vesely DL, Douglass MA, Dietz JR, Gower WR Jr, McCormick MD, Rodriguez-Paz G, Schocken DD (1994) Three peptides from the atrial natriuretic factor prohormone amino terminus lower blood pressure and produce a diuresis, natriuresis, and/or kaliuresis in humans. Circulation 90:1129–1140

Yasue H, Yoshimura M, Sumida H, Kikuta H, Kugiyama K, Jougasaki M, Ogawa H, Okumura K, Mukoyama M, Nakao K (1994) Localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. Circulation 90:195–203

A.1.1.29.2 Bioassay for ANF

PURPOSE AND RATIONALE

Matsui et al. (1987) described a rapid bioassay for quantification of atrial natriuretic polypeptides in rats with continuous recording of the conductivity of the urine, urine flow and blood pressure.

PROCEDURE

Male Sprague Dawley rats weighing 180–240 g are anesthetized with 60 mg/kg i.p. pentobarbital sodium. Anesthesia is maintained by injection of supplemental doses of pentobarbital sodium. After tracheotomy, catheters are placed into the left jugular vein and the right carotid artery for injection of samples or infusion of 10% mannitol in 0.9% saline and for blood pressure recording. Through a small suprapubic incision the bladder is cannulated for collection of urine, and the cannula is connected to a device for continuous measurement of urine conductivity, by which the electrolyte concentration is estimated. Urine flow rate is recorded using a drop counter, and urine samples are collected in a plastic tube.

After completion of surgery, 0.6–0.8 ml of 10% mannitol in 0.9% saline is administered into the jugular vein and is continuously infused at a rate of 4.0 ml/h by a syringe pump. Following an equilibration of 45–60 min, the bioassay is started when the urine flow is increased to 50–75 µl/min. All test samples with a volume of 100 µl are directly injected into the jugular vein followed by a wash injection of ~30 µl of saline. Mean arterial blood pressure, urine conductivity, and urine flow rate are simultaneously recorded.

For dose-response curves, serial dilutions of human ANF (α-hANP) and of test substance are prepared. Vehicle and various doses of α-hANP or test substance are injected in a randomized sequence. Immediately after the injection, urine is collected for 10 min. Urine volumes are determined by weighing, and urinary sodium and potassium concentrations are measured by flame photometry.

EVALUATION

Linear-regression analyses by the method of least squares are used for evaluating dose-response relationship. One-way analysis of variance for repeated measures and the Newman-Keuls test are used to detect statistical differences.

MODIFICATIONS OF THE METHOD

Petersen et al. (1988) determined atrial content and plasma levels of atrial natriuretic peptides in rats with chronic renal failure. The natriuretic activity in the bioassay was estimated as the increase in Na excretion in urine samples from the control period to the maximal natriuretic response.

Allen and Gellai (1987) measured cardioinhibitory effects of atrial peptide in conscious chronically instrumented rats. The hemodynamic and renal excretory responses were measured with and without replacement of urinary fluid losses.

Thibault et al. (1984) characterized the biological activities of atrial natriuretic factor-related peptides *in vivo* by a natriuretic bioassay and *in vitro* by relaxation of contracted intestinal smooth muscle (chick rectum).

Schiller et al. (1986) tested synthetic analogs of atrial natriuretic peptide in the rabbit aorta assay and in a bioassay monitoring suppression of aldosterone secretion from bovine zona glomerulosa cells.

Dlouha and McBroom (1986) measured diuretic and natriuretic activity of atrial extracts of taurine-treated normal and cardiomyopathic hamsters by urine flow and Na^+ excretion in the rat bioassay.

St.-Louis and Schiffrin (1988) measured vasorelaxant effects of different atrial natriuretic peptides on rat aortic and mesenteric artery rings and compared the results with the potency of the same peptides to displace ^{125}I-labeled ANP on membrane preparations of aorta and of mesenteric vein bed.

Kohse et al. (1992) described a bioassay for quantitative determination of natriuretic peptides in human biological samples using bovine aortic and bovine kidney epithelial cultured cells. The amount of cyclic AMP produced by these cells was measured by radioimmunoassay.

Kecskemeti et al. (1996) studied the effects of atrial natriuretic peptide (ANP) on action potential characteristics in various (human, rabbit, guinea-pig) atrial and guinea pig ventricular papillary muscles. The data suggested that ANP inhibits the slow inward Ca^{2+} channel activity and facilitates the K^+ channel activity.

Salt-sensitive hypertension was found in ANP knockout mice (Melo et al. 1998).

REFERENCES AND FURTHER READING

Allen DE, Gellai M (1987) Cardioinhibitory effect of atrial peptide in conscious rats. Am J Physiol 252 (Regul Integr Comp Physiol 21): R610–616

Dlouha H, McBroom (1986) Atrial natriuretic factor in taurine-treated normal and cardiomyopathic hamsters. Proc Soc Exp Biol Med 181:411–415

Kecskemeti V, Pacher P, Pankucsi C, Nanasi P (1996) Comparative study of electrophysiological effects of atrial natriuretic peptide. Mol Cell Biochem 160/161:53–59

Kohse KP, Feifel K, Wisser H (1992) Quantitative determination of natriuretic peptides in human biological samples with a bioassay using cultured cells. Eur J Clin Chem Clin Biochem 30:837–845

Matsui K, Kimura T, Ota K, Shoji M, Inoue M, Iitake K, Yoshinaga K (1987) A rapid bioassay for quantification of atrial natriuretic polypeptides. Am J Physiol 252 (Regul Integr Comp Physiol 21): R1009–R1014

Melo LG, Veress AT, Chong CK, Pang SC, Flynn TG, Sonnenberg H (1998) Salt-sensitive hypertension in ANP knockout mice: potential role of abnormal plasma renin activity. Am J Physiol 274 (Regul Intergr Comp Physiol 43): R255–R261

Petersen JS, Bech OM, Steiness E, Kirstein D, Korsgaard N, Baanrup U, Christensen S (1988) Atrial content and plasma levels of atrial natriuretic peptides in rats with chronic renal failure. Scand J Clin Lab Invest 48:431–439

Schiller PW, Bellini F, Dionne G, Maziak LA, Garcia L, DeLéan A, Cantin M (1986) Synthesis and activity profiles of atrial natriuretic peptide (ANP) analogs with reduced ring size. Biochem Biophys Res Commun 138:880–886

St.-Louis JU, Schiffrin EL (1988) Vasorelaxant effects of and receptors for atrial natriuretic peptides in the mesenteric artery and aorta of the rat. Can J Physiol Pharmacol 66:951–956

Thibault G, Garcia R, Carrier F, Seidah NG, Lazure C, Chrétien M, Cantin M, Genest J (1984) Structure-activity relationships of atrial natriuretic factor (ANF). I. Natriuretic activity and relaxation of intestinal smooth muscle. Biochem Biophys Res Commun 125:938–046

A.1.1.29.3 Receptor Binding of ANF

PURPOSE AND RATIONALE

Schiffrin et al. (1985) described receptors for atrial natriuretic factor in the rat.

PROCEDURE

Synthetic ANF-(99–126) is iodinated with ^{125}I by a modification (Gutskowska et al. 1984) of the chloramine T method (Greenwood and Hunter 1963). Separation of radiolabeled ANF from free iodine is achieved by immunoaffinity chromatography followed by C-18 reverse phase high pressure liquid chromatography.

For preparation of membranes, Sprague Dawley rats weighing 300 g are sacrificed by decapitation. The atria, ventricles, renal arteries, mesentery, the mesentery vascular bed, and adrenals are processed for binding studies. Adrenal capsules are separated by manual compression. The tissues are immersed in 0.25 M sucrose solution, finely minced with scissors, and homogenized in a Polytron (setting 8, 10 s twice). The homogenate is centrifuged at 1550 *g* for 10 min at 4°C; the supernatant is decanted and recentrifuged. The final supernatant is filtered through a cheesecloth, then centrifuged at 10,400 *g* for 30 min. The pellet is resuspended in a 0.05 M Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM phenyl methyl sulfonyl fluoride, 0.1% bacitracin, and 1 μM aprotinin. Proteins are measured by the Coomassie blue method (Spector 1978). Next, bovine serum is added at a concentration of 0.2%, and the membranes are diluted to a protein concentration of 0.25 to 1 mg/ml in the Tris-buffer containing 0.2% albumin.

The ^{125}I-AFN binding assay uses 30–50 pM of labeled ANF and 10^{-13} to 10^{-6} unlabeled ANF in competition experiments. In saturation experiments, increasing concentrations of ^{125}I-AFN (6–200 pM) are used, and nonspecific binding is determined by incubation in the presence of 1 μM unlabeled ANF for each point of the saturation curves. Incubation is done with 25–100 μg of receptor protein per tube, at 4°C for 60 min. All assays are performed in duplicate. Separation of bound and free radioactivity is achieved by rapid filtration through polyethylenimine-treated Whatman GF/C filters soaked with the assay buffer. The filters are washed twice with 3 ml of 0.9% NaCl,

then are allowed to dry and are counted in a gamma counter.

EVALUATION

Binding data are analyzed by computer assisted nonlinear regression analysis using the LIGAND program (Munson and Rodbard 1980). The inhibition constant K_i is calculated according the Cheng and Prussoff equation.

MODIFICATIONS OF THE METHOD

Misono (2000) found that the binding of atrial natriuretic factor to its receptor is dependent on chloride concentration.

REFERENCES AND FURTHER READING

Greenwood FC, Hunter WM (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 89:114–123

Gutkowska J, Thibault G, Januszewicz P, Cantin M, Genest J (1984) Direct radioimmunoassay of atrial natriuretic factor. Biochem Biophys Res Commun 122:593–601

Misono KS (2000) Atrial natriuretic factor binding to its receptor is dependent on chloride concentration. A possible feedback-control mechanisms in renal salt regulation. Circ Res 86:1135

Munson PJ, Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239

Schiffrin EL, Chartier L, Thibault G, St-Louis J, Cantin M, Genest J (1985) Vascular and adrenal receptors for atrial natriuretic factor in the rat. Circ Res 56:801–807

Spector T (1978) Refinement of the Coomassie blue method of protein quantitation. Anal Biochem 86:142–146

A.1.1.29.4 ANF Gene Expression

PURPOSE AND RATIONALE

Production of atrial natriuretic factor and brain natriuretic peptide can be measured by gene expression using total RNA extraction and Northern blot analysis and the quantitative competitive reverse transcription polymerase chain reaction (Hama et al. 1995; Ogawa et al. 1996, 1997, 1998, 1999).

PROCEDURE

Extraction of Plasma and Tissue Samples

Plasma samples are acidified by adding 100 µl/ml of 1 mol/l HCl and passed through Sep-Pak C_{18} cartridges (Millipore) that are pre-wetted with 5 ml of 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) and 10 ml of 0.1% TFA. The cartridges with the absorbed peptides are washed with 20 ml of 0.1% TFA and eluted with 3 ml of 60% acetonitrile in 0.1% TFA. Tissue samples are homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/l acetic acid, and 1% NaCl and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatants are then extracted with the use of Sep-Pak C_{18} cartridges by elution with 80% acetonitrile in 0.1% TFA. The eluates from tissue or plasma are freeze-dried and processed for RIA.

Total RNA Extraction and Northern Blot Analysis

Atrial and ventricular tissue samples from individual rats are extracted using Trizol (GIBCO BRL). Total RNA from the atrium (10 µg) and ventricle (20 µg) are electrophoretically separated in an agarose-formaldehyde gel followed by blotting to nylon membranes (Hybond N+, Amersham) overnight. Membranes are prehybridized in 2.5 × Denhardt's solution, 5 × SSC, 50% formamide, 25 mmol/l KH_2PO_4, pH 6.4, 0.2% SDS, and 0.2 mg/mL herring sssDNA for 3 h at 42°C for cDNA probes, or prehybridized in 5 × Denhardt's solution, 6 × SSC, 50 mmol/l NaH_2PO_4, 0.5% SDS, and 0.2 mg/ml herring sssDNA for 3 h at 5°C below the calculated T_m for oligonucleotide probes. Hybridization is then carried out for 16 h at the same temperature and the same solution as the prehybridization condition except for the presence of the radiolabeled probes. Five cDNA probes and two oligonucleotide probes are used. The cDNA probes used are as follows: (1) a 900-bp *Eco*RI/*Hin*dIII fragment containing the full-length rat ANF cDNA, (2) a 595-bp *Sal*I fragment containing full-length rat BNP cDNA, (3) a 5-kb *Eco*RI/*Sal*I fragment of the mouse 28S rRNA cDNA probe, (4) a 2-kb *Bam*HI/*Bgl*II fragment of the mouse PGK gene cDNA, and (5) rat α_1-III collagen cDNA containing 1300 bp of the 3′ noncoding and coding regions. The two oligonucleotide probes are 39 and 24 base fragments specific for unique regions in the 3′ untranslated regions of the rat α-MHC and β-MHC genes. The α-sequence is 5′GGGATAGCAACAGCGAGGCTCT TTCTGCTGGACAGGTTA-3′(T_m = 60°C),and the β-sequence is 5′-CTCCAGGTCTCAGGGCTTCACA GG-3′ (T_m = 52°C).

The cDNAs are labeled with 5′-[α^{32}P]dCTP (3000 Ci/mmol, Amersham) using the Megaprime DNA labeling system (Amersham). The oligonucleotides are labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) using a 5′-end-labeling kit (Amersham). At the end of hybridization, the membranes are washed twice at 42°C with 2 × SSC and 1% SDS and twice at 55°C with 1 × SSC and 0.1% SDS for the cDNA probes or are washed once at 30°C with 5 × SSC and 0.1% SDS and twice at the same temperature as the hybridization with 1 × SSC and 0.1% SDS for

the oligonucleotide probes. Before additional probing, bound counts are completely stripped from the membranes by washing twice in 10 mmol/l sodium citrate, pH 6.8, 0.25% SDS for 10 min at 100°C. Autoradiographs are scanned with an Ultrascan XL laser densitometer (LKB Produckter) and LKB 2400 Gelscan XL software package. The scanning values of ANF, BNP, collagen-III, and α-MHC and β-MHC mRNAs are normalized to 28S ribosomal RNA or PGK mRNA as internal controls to correct for differences in the amount of RNA applied and transfer efficiency.

Plasma and cardiac tissue concentrations of immunoreactive ANF and BNP are determined by RIA with anti-rat $ANF_{99–126}$ and anti-rat $BNP_{64–95}$ sera, respectively, from Peninsula Laboratories.

Quantitative competitive reverse transcription polymerase chain reaction

RNA samples are reverse transcribed with Super Script II RNase H 2 Reverse Transcriptase and oligo(dT)12–18 primer with the use of a reverse transcription kit (GIBCO BRL). An aliquot of the cDNA product is used for PCR amplification with ANF primers. A dilution series of total RNA (5 mg) aliquots is prepared for each sample. Each dilution is spiked with competitor RNA. After the PCR, aliquots (5 ml) of the PCR product are electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Photographs are taken with Polaroid 55 film, and the negatives are scanned with the use of an Ultrascan XL laser densitometer and Gelscan XL 2000 software package. The ratio of the density of the competitor RNA to the target RNA is plotted against the amount of the competitor RNA added to each reaction.

EVALUATION

All results are expressed as mean $\pm$ SEM. A level of $P < 0.5$ is considered significant. ANOVA is performed to determine statistical differences among multiple groups. When significance is obtained by ANOVA, Fisher's least squares difference post hoc analysis is used to determine pairwise differences.

MODIFICATIONS OF THE METHOD

Ramirez et al. (1997) reported that the nuclear δ_B isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates atrial natriuretic gene expression in cultured neonatal rat ventricular myocytes.

Thuerauf et al. (1998) found that the p38 mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element and discussed the potential role for the transcription factor ATF6.

Kakita et al. (1999) studied p300 protein as a coactivator of the transcription factor GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene.

Bianciotti and de Bold (2000) investigated the effect of selective ET_A receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension in rats.

REFERENCES AND FURTHER READING

Bianciotti LG, de Bold AJ (2000) Effect of selective ET_A receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension. Am J Physiol 279 (Heart Circ Physiol):H93–H101

Hama N, Itoh H, Shirakami G, Nakagawa O, Suga S-I, Ogawa Y, Masuda I, Nakanishi K, Yoshimasa T, Hashimoto Y, Yamaguchi M, Hori R, Yasue H, Nakao K (1995) Rapid ventricular induction of brain natriuretic peptide gene expression in experimental acute myocardial infarction. Circulation 92:1558–1564

Kakita T, Hasegawa K, Morimoto T, Kaburagi S, Wada H, Sasayama S (1999) p300 Protein as a coactivator of GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene. J Biol Chem 274:34096–34102

Ogawa T, Linz W, Stevenson M, Bruneau BG, Kuroski de Bold ML, Chen J-H, MD, Eid H, Schölkens BA, de Bold AJ (1996) Evidence for load-dependent and load-independent determinants of cardiac natriuretic peptide production. Circulation 93:2059–2067

Ogawa T, Bruneau BG, Yokota N, Kuroski de Bold ML, de Bold AJ (1997) Tissue-specific regulation of renal and cardiac atrial natriuretic gene expression in desoxycorticosterone acetate-salt rats. Hypertension 30:1342–1347

Ogawa T, Linz W, Schölkens BA, de Bold AJ (1998) Regulation of aortic atrial natriuretic factor and angiotensinogen in experimental hypertension. J Cardiovasc Pharmacol 32:1001–1008

Ogawa T, Linz W, Schölkens BA, de Bold AJ (1999) Variable renal atrial natriuretic factor gene expression in hypertension. Hypertension 33:1342–1347

Ramirez MT, Zhao X-L, Schulman H, Brown JH (1997) The nuclear δ_B isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates atrial natriuretic gene expression in ventricular myocytes. J Biol Chem 272:31203–31208

Thuerauf DJ, Arnold ND, Zechner D, Hanford DS, DeMartin KM, McDonough PM, Prywes R, Glembotski CC (1998) p38 Mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element. A potential role for the transcription factor ATF6. J Biol Chem 273:20636–20643

A.1.1.29.5 Radioimmunoassay for ANF

PURPOSE AND RATIONALE

Gutkowska et al. (1984) developed a direct radioimmunoassay of atrial natriuretic factor (ANF). The

method uses a synthetic 26-amino-acid fragment (8–33 ANF) of the native peptide.

PROCEDURE

Because 8–33 ANF is a small molecule, it is necessary to covalently conjugate the peptide to a larger protein (bovine thyroglobulin) for immunization. To 50 mg thyroglobulin dissolved in 2 ml distilled water, pH 7.4, 30 mg CDI [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl] is added in 1 ml distilled water, pH 7.4. Then 5 mg 8–33 ANF in water is added dropwise while stirring. The solution is kept overnight at 4°C, then another 30 mg CDI is added and the mixture is kept for 2 h at room temperature with constant stirring. The cloudy mixture is dialyzed for 24 h at 4°C against 0.9% saline. The dialyzed material is then fractionated and stored at –70°C.

For immunization, 100 µg of the ANF-thyroglobulin complex are suspended in 1 ml saline, thoroughly mixed with 1 ml complete Freund's adjuvant and injected into the shaved backs of New Zealand white rabbits. Each animal receives also 0.5 ml *Bordetella pertussis* vaccine subcutaneously with the primary immunization. The animals are reimmunized at monthly intervals with 100 µg of antigen in incomplete Freund's adjuvant and bled by ear artery 10 days after the booster injection.

For iodination, 5 µg ANF in 5 µl 0.01 M ammonium acetate, pH 5.0 is introduced in a 1.5 ml Eppendorf vial followed by the addition of 1 mCi Na ^{125}I in a volume of 25 µl. Chloramine T 10 µg/10 µl is added to the reaction vial and 30 s later sodium metabisulfide (20 µg/10 µl) is added. Each addition is followed by mixing. Purification of the iodinated tracer is achieved by HPLC on a µBondapax C_{18} column, eluted with a linear gradient of 20 to 50% acetonitrile with 0.1% trifluoroacetic acid with a slope of 0.5%/min and a flow rate of 1 ml/min.

The radioimmunoassay procedure is performed in polystyrene tubes at 4°C by mixing 100 µl of standard or sample, 100 µl of antiserum diluted 1:4000, 100 µl of ^{125}I-ANF and 300 µl of the same buffer containing 1% BSA. After incubation for 24 h at 4°C the free from antigen-bound ^{125}I-ANF is separated by dextran-coated charcoal. One ml of dextran-charcoal suspension is added to each tube. After 5 s agitation the tubes are centrifuged at 4000 rpm at 4°C for 10 min. The supernatant is decanted and the radioactivity counted in a gamma counter.

EVALUATION

Dose-response curves are prepared and Scatchard analysis is performed.

MODIFICATIONS OF THE METHOD

Radioimmunoassays were also developed for long-acting natriuretic peptide and vessel dilator (Vesely et al. 1994; Winters et al. 1989).

REFERENCES AND FURTHER READING

Gutkowska J, Thibault G, Januszewicz P, Cantin M, Genest J (1984) Direct radioimmunoassay of atrial natriuretic factor. Biochem Biophys Res Commun 122:593–601

Vesely DL, Douglass MA, Dietz JR, Giordano AT, McCormick MT, Rodriguez-Paz G, Schocken DD (1994) Negative feedback of atrial natriuretic peptides. J Clin Endocrinol Metab 78:1128–1134

Winters CJ, Sallman AL, Baker BJ, Meadows J, Rico DM, Vesely DL (1989) The N-terminus and a 4000 molecular weight peptide from the mid portion of the N-terminus of the atrial natriuretic factor prohormone each circulate in humans and increase in congestive heart failure. Circulation 80:438–449

A.1.1.30 Urotensin II

A.1.1.30.1 General Considerations

PURPOSE AND RATIONALE

Urotensin II is a cyclic peptide originally isolated from the urophysis, the hormone storage-secretion organ of the caudal neurosecretory system of teleost fishes, such as *Gillichthys mirabilis* (Pearson et al. 1980; Maguire and Davenport 2002).

Several structural forms of urotensin II have been reported in different species (Grieco et al. 2004). These peptides show smooth muscle contracting activity. Itoh et al. (1987) reported contraction of major artery segments of rat, especially of the thoracic aorta, by urotensin II.

Human urotensin II is an 11-amino-acid peptide that retains the cyclic portion typical of fish urotensin II. It has been found in vascular and cardiac tissues and is a very potent constrictor of certain human isolated arteries and veins as well as of several vessels of other species (Douglas et al. 2000; Douglas 2003).

The potency of urotensin II as a vasoconstrictor is an order of magnitude greater than that of endothelin-1, making human urotensin II the most potent vasoconstrictor identified so far.

Human urotensin II is also a potent endothelium-dependent relaxant in rat precontracted arteries (Katano

et al. 2000) and in human small pulmonary and systemic abdominal resistance arteries (Stirrat et al. 2001).

Bottrill et al. (2000) found that human urotensin II contracted endothelium-intact rat isolated left anterior descending coronary arteries. The contractile response was significantly enhanced by removal of the endothelium. However, human urotensin II caused concentration-dependent relaxation of 5-HT-precontracted arteries, which was abolished by *N*-nitro-L-arginine methyl ester (L-NAME) or removal of the endothelium.

Using merino ewes as experimental animals, Watson et al. (2003) found that urotensin II acts centrally to increase epinephrine and adrenocorticotrophic hormone (ACTH) release and causes potent inotropic and chronotropic actions.

Coy et al. (2002) investigated structural requirements at the N-terminus of urotensin II octapeptides.

Carotenuto et al. (2004) investigated the active conformation of urotensin II by CD spectroscopy and NMR analysis in SDS micelles.

Watson and May (2004) reviewed the role of urotensin II in central and peripheral cardiovascular control.

REFERENCES AND FURTHER READING

Bottrill FE, Douglas FE, Hiley CR, White R (2000) Human urotensin II is an endothelium-dependent vasodilator in rat small arteries. Br J Pharmacol 130:1865–1870

Carotenuto A, Grieco P, Campiglia P, Novellino E, Rovero P (2004) Unravelling the active conformation of urotensin II. J Med Chem 47:1652–1661

Coy DH, Rossowski WJ, Cheng BL, Taylor JE (2002) Structural requirements at the N-terminus of urotensin II octapeptides. Peptides 23:2259–2264

Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar NV, Ohlstein EH, Wilette RN (2000) Differential vasoconstrictor activity of human urotensin II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. Br J Pharmacol 131:1262–1274

Douglas SA (2003) Human urotensin-II as a novel cardiovascular target: "heart" of the matter or simply a fishy "tail"? Curr Opin Pharmacol 3:159–167

Grieco P, Rovero P, Novellino E (2004) Recent structure-activity studies of the peptide hormone urotensin II, a potent vasoconstrictor. Curr Med Chem 11:969–979

Itoh H, Itoh Y, Rivier J, Lederis K (1987) Contraction of major artery segments of rat by fish neuropeptide urotensin II. Am J Physiol 252(2Pt2):R361–R366

Katano Y, Ishihata A, Aita T, Ogaki T, Horie T (2000) Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors, on rat coronary arteries. Eur J Pharmacol 402:209–211

Magzire JJ, Davenport AP (2002) Is urotensin-II the new endothelin? Br J Pharmacol 137:579–588

Pearson D, Shively JE, Clark BR, Geschwing II, Barkley M, Nishioka RS, Bern HA (1980) Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. Proc Natl Acad Sci USA 77:5021–5024

Stirrat A, Gallagher M, Douglas SA, Ohlstein EH, Berry C, Kirk A, Richardson M, MacLean MR (2001) Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries. Am J Physiol 280:H925–H928

Watson AMD, Lambert GW, Smith KJ, May CN (2003) Urotensin II acts centrally to increase epinephrine and ACTH release and cause potent inotropic and chronotropic actions. Hypertension 42:373–379

Watson AM, May CN (2004) Urotensin II, a novel peptide in central and peripheral cardiovascular control. Peptides 25:1759–1766

A.1.1.30.2 Rat Thoracic Aorta Bioassay for Urotensin II

PURPOSE AND RATIONALE

Most studies on urotensin analogs, agonists and antagonists used the isolated thoracic aorta of rats as a pharmacological model (Itoh et al. 1988; Nothacker et al. 1999; Sauzeau et al. 2001; Camarda et al. 2002a; Grieco et al. 2002a, 2002b; Rossowski et al. 2002). Several urotensin II antagonists have been tested in this model (Behm et al. 2002; Herold et al. 2003).

Patacchine et al. (2003) tested urantide, an ultrapotent urotensin II antagonist peptide, in the rat aorta.

PROCEDURE

Male albino rats (Wistar strain, 275–350 g) are decapitated under ether anesthesia. The thoracic aorta is cleared of surrounding tissue and excised from the aortic arch to the diaphragm. From each vessel, a helically cut strip is prepared, and then it is cut into two parallel strips. The endothelium is removed by gently rubbing the vessel intimal surface with a cotton-tip applicator; the effectiveness of this maneuver is assessed by the loss of relaxation response to acetylcholine (1 μM) in preparations precontracted with noradrenaline (1 μM). All preparations are placed in 5-ml organ baths filled with oxygenated normal Krebs–Henseleit solution. Motor activity of the strips is recorded isotonically (load 5 mN). A cumulative concentration–response curve to hU-II is constructed on one of the two strips, which serves as control. The other strip receives the antagonist peptide under examination and, after a 30-min incubation period, hU-II is administered cumulatively. Maximal contractile responses of preparations to hU-II are obtained by administration of KCl (80 mM) at the end of the cumulative curves.

EVALUATION

Antagonist activity is expressed in terms of pK_B (negative logarithm of the antagonist dissociation constant) and, assuming a slope of –1.0, is estimated as the mean

of the individual values obtained with the equation: pK_B = log[dose ratio–1]–log[antagonist concentration] (Kenakin 1997). Competitive antagonism is checked by the Schild plot method: a plot with linear regression line and slope not significantly different from unity is considered as proof of simple reversible competition (Kenakin 1997).

MODIFICATIONS OF THE METHOD

Gibson et al. (1988) studied the influence of urotensin II on calcium flux in rat aorta. Urotensin II caused an increase in uptake of ^{45}Ca by segments of rat aorta. This increase was abolished by calcium channel blocking drugs.

Douglas et al. (2000) found differential vasoconstrictor activity of human urotensin II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey depending on species and anatomical localization.

Camarda et al. (2002b) studied the effects of human urotensin II in isolated vessels (aorta, large arteries, veins) of various species (rats, guinea pigs, rabbits, pigs, human) and compared them with other vasoactive agents (noradrenaline, angiotensin II, endothelin I).

Watanabe et al. (2001) found a synergistic effect of urotensin II with serotonin on rabbit vascular smooth muscle cell proliferation.

Tamura et al. (2003) examined the effects of urotensin II on activation of extracellular signal-regulated kinase and focal adhesion kinase in cultured vascular smooth muscle cells.

Tzanidis et al. (2003) studied direct actions of urotensin II on the heart in a rat model of heart failure after myocardial infarction and the implications for cardiac fibrosis and hypertrophy.

Matsushita et al. (2003) showed that urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line LLCPK1.

Behm et al. (2004) investigated the role of urotensin II in the etiology of essential hypertension. Intravenous injection in anesthetized cats induced an increase in systemic blood pressure and peripheral vascular resistance.

REFERENCES AND FURTHER READING

Behm DJ, Herold CL, Ohlstein EH, Knight SD, Dhanak D, Douglas SA (2002) Pharmacological characterization of SB-710411 (Cpa-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Cpa-amide), a novel peptidic urotensin-II receptor antagonist. Br J Pharmacol 137:449–458

Behm DJ, Doe CPA, Johns DG, Maniscalco K, Stankus GP, Wibberley A, Willette RN, Douglas SA (2004) Urotensin-II: a novel systemic hypertensive factor in the cat. Naunyn Schmiedebergs Arch Pharmacol 369:274–280

Camarda V, Guerrini R, Kostenis E, Rizzi A, Calo G, Hattenberger A, Zucchini M, Salvadori S, Reguli D (2002a) A new ligand for the urotensin II receptor. Br J Pharmacol 137:311–314

Camarda V, Rizzi A, Calò G, Gendron G, Perron SI, Kostenis E, Zamboni P, Mascoli F, Regoli D (2002b) Effects of human urotensin II in isolated vessels of various species: comparison with other vasoactive agents. Naunyn-Schmiedebergs Arch Pharmacol 365:141–149

Douglas SA, Ashton DJ, Sauermelch CF, Coatney RW, Ohlstein DH, Ruffolo MR, Ohlstein EH, Aiyar NV, Willette RN (2000) Human urotensin II is a potent vasoactive peptide: pharmacological characterization in the rat, mouse, dog and primate. Cardiovasc Pharmacol 36/Suppl1:S163–166

Gibson A, Conyers S, Bern HA (1988) Influence of urotensin II on calcium flux in rat aorta. J Pharm Pharmacol 40:893–985

Grieco P, Carotenuto A, Patacchini R, Maggi CA, Novellino E, Rovero P (2002a) Design, synthesis, conformational analysis and biological studies of urotensin-II lactam analogues. Bioorg Med Chem 10:3731–3739

Grieco P, Carotenuto A, Campiglia P, Zampelli E, Patacchini R, Maggi CA, Novellino E, Rovero P (2002b) A new potent urotensin II receptor peptide agonist containing a pen residue at the disulfide bridge. J Med Chem 45:4391–4394

Herold CL, Behm DJ, Buckley PT, Foley JJ, Wixted WE, Sarau HM, Douglas SA (2003) The neuromedin B receptor antagonist, BIM-23127, is a potent antagonist at human and rat urotensin-II receptors. Br. J Pharmacol 139:203–207

Itoh H, McMaster D, Lederis K (1988) Functional receptors for fish neuropeptide urotensin II in major rat arteries. Eur J Pharmacol 149:61–66

Kenakin P (1997) Pharmacologic analysis of drug – receptor interaction, 3rd edn. Lippincott-Raven, Philadelphia, Pa.

Matsushita M, Shichiri M, Fukai N, Ozawa N, Yoshimoto T, Takasu N, Hirata Y (2003) Urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line, LLCPK1. Endocrinology 144:1825–1831

Nothacker HP, Wang Z, McNeil AM, Saito Y, Merten S, O'Dowd B, Duckles SP, Civelli O (1999) Identification of the natural ligand of an G-protein-coupled receptor involved in the regulation of vasoconstriction. Nature Cell Biol 1:383–385

Patacchini R, Santicioli P, Giuliani S, Grieco P, Novellino E, Rovero P, Maggi CA (2003) Urantide: an ultrapotent urotensin II antagonist peptide in the rat aorta. Br J Pharmacol 140:1155–1158

Rossowski WJ, Cheng BL, Taylor JE, Datta R, Coy DH (2002) Human urotensin II-induced aorta ring contractions are mediated by protein kinase C, tyrosine kinases and Rho-kinase: inhibition by somatostatin receptor antagonists. Eur J Pharmacol 438:159–170

Sauzeau V, Le Mellionnec E, Bertoglio J, Scalbert E, Pacaud P, Loirand G (2001) Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-Kinase. Circ Res 88:1102–1104

Tamura K, Okazaki M, Tamura M, Isozumi K, Tasaki H, Nakashima Y (2003) Urotensin II-induced activation of extracellular signal-regulated kinase in cultured vascular smooth muscle cells: involvement of cell adhesion-mediated integrin signaling. Life Sci 72:1049–1060

Tzanidis A, Hannan RD, Thomas WG, Onan D, Autelitano DJ, See F, Kelly DJ, Gilbert RE, Krum H (2003) Direct actions of urotensin II on the heart. Implications for cardiac fibrosis and hypertrophy. Circ Res 93:246–253

Watanabe T, Pakala R, Katagiri T, Benedict CR (2001) Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation. J Hypertens 19:2191–2196

A.1.1.30.3 Intracellular Calcium Mobilization Assay

PURPOSE AND RATIONALE

Human urotensin II induces concentration-dependent increases in intracellular calcium in HEK-293 cells expressing human GPR14 (Ames et al. 1999). Herold et al. (2003) used this assay to test a synthetic antagonist at human and rat urotensin II receptors.

PROCEDURE

Cell Culture

HEK293 cells stably expressing the hUT or rUT receptors are generated and propagated as described previously (Ames et al. 1999).

Intracellular Calcium ($Ca^{2+}{}_i$) Mobilization Assay

hUT-HEK293 cells or rUT-HEK293 cells are seeded in blackwalled, clear-bottomed 96-well Biocoat plates (Beckton-Dickinson, Bedford, Mass., USA, Herold et al. 2004) at a density of 45,000 cells/well, grown in the incubator at 37°C for 18–24 h, and prepared for $Ca^{2+}{}_i$ measurements (Ames et al. 1999). Plates are placed into the Fluorometric Imaging Plate Reader (Molecular Devices, Sunnyvale, Calif., USA) where cells, loaded with Fluo 3 (Molecular Probes, Eugene, Ore., USA), are exposed to excitation (488 nm) from a 6-W argon laser. Fluorescence is monitored at 566 nm emission for all 96 wells simultaneously, and data are read every 1 s for 1 min and then every 3 s thereafter. Agonist is added after 10 s and concentration–response curves are obtained by calculating the maximal fluorescence counts above background after the addition of each concentration of agonist. For antagonist studies, BIM-23127 (Bachem, King of Prussia, Pa., USA) is added 10 min prior to the addition of hU-II (California Peptide Research, Napa, Calif., USA).

EVALUATION

Concentration–response curves are analyzed by nonlinear regression using GraphPad Prism 3.0 software (GraphPad, San Diego, Calif., USA).

MODIFICATIONS OF THE METHOD

Flohr et al. (2002) performed structure–activity relationship studies on urotensin II by investigating peptide analogs and their ability to mobilize calcium in GPR14-transfected CHO cells.

Camarda et al. (2002) evaluated a new ligand for the urotensin II receptor ([Orn^8]U-II) in calcium functional assays performed on HEK293 cells expressing the recombinant rat and human UT receptor.

Croston et al. (2002) used a functional mammalian cell-based R-SAT assay (ACADIA Pharmaceuticals, San Diego, Calif., USA) to identify non-peptide agonists of the CRP14/urotensin II receptor in high-throughput screening. According to Shapiro et al. (2002) NIH-3T3 cells were grown in 96-well tissue culture plates to 70%–80% confluence in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin/Gln. Cells were transfected for 12–16 h with plasmid DNAs using Superfect Reagent (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocols. R-SATs were performed with 0.5–50 ng/well receptor and 20 ng/well β-galactosidase plasmid DNA. After overnight transfection, medium was replaced with serum-free Dulbecco's modified Eagle's medium containing 2% cyto-sf3 (Kemp Biotechnologies, Frederick, Md., USA) and 1% penicillin/streptomycin/Gln and varying concentrations of drug. Cells were grown in a humidified atmosphere with 5% ambient CO_2 for 4–6 days. Medium was removed from the plates, and β-galactosidase activity was measured by the addition of *o*-nitrophenyl β-D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40 detergent). The resulting colorimetric reaction was measured using a spectrophotometric plate reader (Titertek, Huntsville, Ala., USA) at 420 nM. For HTS, NIH-3T3 cells transiently transfected with the urotensin II receptor expression vector and plasmid were frozen and for the assay thawed, plated and exposed to drug.

REFERENCES AND FURTHER READING

Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, Louden CS, Foley JJ, Sauermelch CF, Coatney RW, Ao Z, Dias J, Holmes SD, Stadel JM, Martin JD, Liu WS, Glover GI, Wilson S, McNulty DE, Ellis CE, Elshourbagy NA, Shabon U, Trill JJ, Hay DWP, Ohlstein EN, Bergsma DJ, Douglas SA (1999) Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. Nature 401:282–286

Camarda V, Guerrini R, Kostenis E, Rizzi A, Calo G, Hattenberger A, Zucchini M, Salvadori S, Reguli D (2002) A new ligand for the urotensin II receptor. Br J Pharmacol 137:311–314

Croston GE, Olsson R, Currier EA, Burstein ES, Weiner D, Nash N, Severance D, Allenmark SG, Thunberg L, Ma JN, Mohell N, O'Dowd B, Brann MR, Hacksell U (2002) Discovery of the first nonpeptide agonist of the CRP14/urotensin II receptor: 3-(4-chlorophenyl)-3-(2-(dimethylamino)ethyl)isochroman-1-one (AC-7954). J Med Chem 45:4950–4953

Flohr S, Kurz M, Kostenis E, Brkovic A, Fournier A, Klabunde T (2002) Identification of nonpeptide urotensin II receptor antagonists by virtual screening on a pharmacophore model derived from structure-activity relationships and nuclear magnetic resonance studies on urotensin II. J Med Chem 45:1799–1805

Herold CL, Behm DJ, Buckley PT, Foley JJ, Wixted WE, Sarau HM, Douglas SA (2003) The neuromedin B receptor antagonist, BIM-23127, is a potent antagonist at human and rat urotensin-II receptors. Br J Pharmacol 139:203–207

Shapiro DA, Kristiansen K, Weinert DM, Kroeze WK, Roth BL (2002) Evidence for a model of agonist-induced activation of 5-hydroxytryptamine 2A serotonin receptors that involves the interruption of a strong ionic interaction between helices 3 and 6. J Biol Chem 277:11441–11449

A.1.1.30.4 Receptor Binding of Urotensin II

PURPOSE AND RATIONALE

The G-protein-coupled receptor GPR14/SENR was described by Marchese et al. (1995) and Tal et al. (1995). Liu et al. (1999) and Mori et al. (1999) identified urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. It was renamed as urotensin II (UT) receptor by UPHAR (Douglas and Ohlstein 2000). Human, rat, mouse and monkey receptors have been cloned. The amino acid sequence identity of the monkey UT receptor is 97% and 77% identical to the human and rat sequences respectively, while the mouse UT receptor is 76% and 93% identical to the human and rat sequences, respectively.

Brkovic et al. (2003) performed functional and binding characterizations of urotensin-II-related peptides in human and rat urotensin II receptor assays.

PROCEDURE

Reagents and Solvents

The following fluorenylmethyloxycarbonyl-protected amino acids were purchased from Chem-Impex International (Wood Dale, Ill., USA): Ala, Cys(Trt), His(Trt), Phe, Trp, Lys-(Boc), Tyr(tBu), Asp(OtBu), Glu(OtBu), Pro, Gln(Trt), Thr(tBu), Arg(Pbf), Orn(Boc), HomoCys(Trt), Cys(Acm), and D-Trp. Biograde TFA was obtained from Halocarbon (River Edge, N.J., USA). Diisopropylethylamine was from Aldrich (Milwaukee, Wis., USA). Wang resin and benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate were purchased from Albatross (Montreal, QC).

Basal ISCOVE medium and fetal calf serum (FCS) were from Biochrom (Berlin, Germany). The GC-melt PCR kit as well as the human and rat genomic DNA were purchased from CLONTECH (Palo Alto, Calif., USA). The pEAK8 mammalian episomal expression vector and the selection marker puromycin were from Edge Biosystems (Gaithersburg, Md., USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, HEPES, LipofectAMINE reagent, and penicillin-streptomycin were from Invitrogen (Carlsbad, Calif., USA). The pCDNA3.1(+) mammalian expression vector was from Invitrogen. The calcium-sensitive fluorescence dye Fluo-4 and Pluronic F-127 were obtained from Molecular Probes (Eugene, Ore., USA). Flashplates PLUS and monoiodinated human [^{125}I-Tyr9]urotensin II for radioligand binding assays were from Perkin Elmer Life Sciences (Boston, Mass., USA). Gentamicin, the transfection reagent FuGene 6, and Complete protease inhibitor were purchased from Roche (Basel, Switzerland). Bacitracin, EDTA-disodium salt, probenecid, $MgCl_2$, NaCl, and sucrose were obtained from Sigma-Aldrich (St. Louis, Mo., USA).

Cloning of Human and Rat Urotensin II Receptor

As the putative human urotensin II receptor sequence is intronless, this protein was cloned from human genomic DNA via PCR. PCR conditions, established to amplify the human GPR14 sequence, were 94°C; 10 min followed by 35 cycles of 94°C, 1 min, 60°C, 1 min, 72°C, and 2 min using the GC-melt kit. Primers designed to amplify the coding sequence contained a *Bam*HI site in the forward and a *Xba*I site in the reverse primer, respectively. The urotensin receptor coding region, flanked by *Bam*HI/*Xba*I sites, was cloned into the pCDNA3.1(+) mammalian expression vector and sequenced in both directions. For generation of stable cell lines, the human U-II receptor coding sequence flanked by a 5′*Eco*RI site and a 3′*Eco*RV site was cloned into the mammalian episomal expression vector pEAK8.

The rat U-II receptor coding sequence flanked by a 5′ *Eco*RI and 3′ *Not*I site was amplified via PCR from rat kidney cDNA and cloned into the mammalian pEAK8 expression vector. Sequences of all urotensin-II-receptor-expressing plasmids were verified by dideoxy sequencing in both directions.

Cell Culture and Transfection

CHO K1-cells were grown in basal ISCOVE medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml), and 25 mg/ml gentamicin at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected with the U-II receptor cDNAs using the LipofectAMINE reagent according to the manufacturer's protocol. After 18–24 h following the

transfection, cells were split into blackwalled 96-well plates at a density of 50,000 cells/well and cultured for an additional 18- to 24-h period before being used in the functional fluorescence imaging plate reader (FLIPR) assay (described below in detail) measuring intracellular Ca^{2+} release upon receptor activation.

FLIPR ASSAY

Cells were loaded in 96-well plates for 1 h (37°C, 5% CO_2) with 100 µl of PBS (without Ca^{2+}, Mg^{2+} and $NaHCO_3$) containing 4 µM of the fluorescent calcium indicator Fluo-4, 0.22% Pluronic F-127 in dimethyl sulfoxide, 2.5 mM probenecid, 1 mM EGTA, and 1% FCS. Cells were then washed three times with PBS (without Ca^{2+}, Mg^{2+} and $NaHCO_3$) containing 1 mM EDTA, 0.5 mM $MgCl_2$, and 2.5 mM probenecid. After the final wash, a 100-µl residual volume remained on the cells. Peptides were aliquoted as 2× solutions in 96-well plates and transferred by the instrument from the ligand plate to the cell plate. Fluorescence was recorded with the fluorometric imaging plate reader FLIPR (Molecular Devices, Sunnyvale, Calif., USA) over a period of 3 min. Fluorescence was recorded simultaneously in all wells at 3-s intervals during the first minute and at 10-s intervals during the last 2 min. Fluorescence data were generated in duplicate and repeated at least three times.

Generation of Stable Human and Rat U-II Receptor Expressing Cell Lines

For the generation of stable cell lines expressing the human and rat urotensin II receptor, HEK293 cells were transfected with human- and rat-pEAK8 constructs using the FuGene 6 transfection reagent according to the supplier's protocol. Two days after transfection, cells were selected in DMEM, supplemented with 10% FCS, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml), and 1 µg/ml puromycin for a period of 4 weeks (37°C, 5% CO_2, 95% relative humidity). Functional activity of the urotensin-II-receptor-expressing cell population was verified with a FLIPR assay recording urotensin-II-mediated intracellular Ca^{2+} release, as described above.

Membrane Preparation and Radioligand Binding Assays

HEK293 cells stably expressing human or rat urotensin II receptors were cultured up to 80% confluency in DMEM, supplemented with 10% FCS, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml), and 1 µg/ml puromycin (37°C, 5% CO_2, 95% relative humidity). Cells were washed once with ice-cold PBS and a second time with PBS containing the protease inhibitor cocktail (Complete). Cells were scraped off and centrifuged gently. The pellet was resuspended in a buffer containing 5 mM HEPES, 1 mM EDTA-disodium salt, and the cocktail of protease inhibitor Complete and then incubated on ice for 15 min. Cells were pelleted again and resuspended with a homogenizer (Unit F8B, Constant cell disruption systems; Honiley, Warwickshire, UK). The supernatant and resuspended pellet were combined and centrifuged at 50,000 *g* (Beckman Avanti J251). The cell membrane pellet was resuspended in a buffer consisting of 20 mM HEPES, 1 mM EDTA-disodium salt, 150 mM NaCl, and 10% sucrose. Membrane aliquots were stored at –80°C. One day before the binding assay, membranes were thawed, pelleted, and resuspended in the assay buffer consisting of 20 mM HEPES, 150 mM NaCl, 1 mM EDTA-disodium salt, 160 µg/ml bacitracin, and Complete protease inhibitor (two tablets per 100 ml).

Membranes were distributed into 96-well wheat-germ-agglutinin Flashplates PLUS, incubated overnight for adsorption, and then the Flashplates PLUS were washed twice with the assay buffer. For equilibrium binding assays, 0.2 nM human [^{125}I-Tyr9]urotensin II (initial specific activity 2200 Ci/mmol, or 81,400 GBq/mmol) was incubated with the indicated amounts of unlabeled competitors for 4 h and radioactivity counted in a 1450 Microbeta Wallac Jet (Wallac, Turku, Finland).

EVALUATION

Data of the FLIPR assay were analyzed by non-linear curve fitting using GraphPad Prism version 3.0 (GraphPad Software, San Diego, Calif., USA).

Non-specific binding was determined in the radioligand binding assays in the presence of 10 µM human U-II and corresponded to 31 ± 2% ($n = 5$, mean ± SEM) of the total binding for membranes expressing the human urotensin II receptor and to 8 ± 1% ($n = 5$, mean ± SEM) of the total binding for membranes expressing the rat urotensin II receptor. Data analysis was performed with GraphPad Prism 3.0.

MODIFICATIONS OF THE METHOD

Flohr et al. (2002) identified non-peptide urotensin II receptor antagonists by virtual screening on a phamacophore model derived from structure–activity relationships and nuclear magnetic resonance studies on urotensin II.

REFERENCES AND FURTHER READING

Brkovic A, Hattenberger A, Kostenis E, Klabunde T, Flohr S, Kurz M, Bourgault S, Fournier A (2003) Functional and binding characterizations of urotensin II-related peptides in human and rat urotensin II- receptor assay. J Pharmacol Exp Ther 306:1200–1209

Douglas SA, Ohlstein EH (2000) Urotensin receptors. In: Girdlestone D (ed) The IUPHAR compendium of receptor characterization and classification. IUPHAR Media, London, pp 365–372

Flohr S, Kurz M, Kostenis E, Brkovic A, Fournier A, Klabunde T (2002) Identification of nonpeptide urotensin II receptor antagonists by virtual screening on a phamacophore model derived from structure-activity relationships and nuclear magnetic resonance studies on urotensin II. J Med Chem 45:1799–1805

Liu Q, Pong SS, Zeng Z, Zhang Q, Howard AD, Williams DL, Jr, Davidoff M, Wang R, Austin CP, McDonald TP, Bai C, George SR, Evans JF, Caskey CT (1999) Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. Biochem Biophys Res Commun 266:174–178

Maguire JJ, Kuc RE, Davenport AP (2000) Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. Br J Pharmacol 131:441–446

Marchese A, Heiber M, Nguyen T, Heng HH, Saldivia VR, Cheng R, Murphy PM, Tsui LC, Shi X, Gregor P (1995) Cloning and chromosomal mapping of three novel genes, GPR9, GPR10 and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors. Genomics 29:335–344

Mori M, Sugo T, Abe M, Shimomura Y, Kurihara M, Kitada C, Kikuchi K, Shintani Y, Kurokawa T, Onda H, Nishimura O, Fujino M (1999) Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). Biochem Biophys Res Commun 265:123–129

Nothacker HP, Wang Z, McNeil AM, Saito Y, Merten S, O'Dowd B, Duckles SP, Civelli O (1999) Identification of the natural ligand of an G-protein-coupled receptor involved in the regulation of vasoconstriction. Nature Cell Biol 1:383–385

Tal M, Ammar DA, Karpuj M, Krizhanovsky V, Naim M, Thompson DA (1995) A novel putative neuropeptide receptor expressed in neural tissue, including sensory epithelia. Biochem Biophys Res Commun 209:752–759

A.1.1.30.5 Urotensin II Gene Expression

PURPOSE AND RATIONALE

The UT receptor is expressed in vascular tissue (Ames et al. 1999; Maguire et al. 2000). A direct link between U-II, the UT receptor and vasoconstriction has to be assumed (Ames et al. 1999; Douglas and Ohlstein 2001). Behm et al. (2003) used homologous recombination in embryonic stem (ES) cells to generate mice lacking the UT receptor coding region to determine the effect(s) of this receptor on vascular reactivity both *in vivo* and *in vitro* and to verify that U-II exerts its effects on vascular smooth muscle tone via an interaction with UT.

PROCEDURE

Targeting the UT Gene and Generation of Mutant Mice

Gene targeting was performed in murine E14.1 ES cells, replacing the single coding exon of the UT receptor locus with a positive selection cassette containing the neomycin phosphotransferase gene (Neo) driven by the phosphoglycerate kinase I (PGK) promoter. 5′- and 3′-homology arms, both of ∼4.0 kb, were cloned from a 129SVJ mouse genomic bacterial artificial chromosome (BAC) library and placed on either side of the positive selection cassette. Homologous recombination in neomycin-resistant ES cells was confirmed by Southern blot of *Bam*HI-digested genomic DNA using an ∼800-bp *Bam*HI/ *Sma*I restriction fragment as the 5′ external probe (which detects 6.5- and 6.0-kb bands at the wild-type and targeted locus, respectively). Approximately 1 in 80 G418-resistant clones had undergone homologous recombination. Homologous recombination at the 3′ end was confirmed in these ES cell clones by Southern blot of *Hind*III-digested genomic DNA using a ∼700-bp *Xmn*I/*Hind*III restriction fragment as the 3′-external probe (which detects 5.5- and 5.0-kb bands at the wild-type and targeted locus, respectively). Three targeted clones were injected into C57B16/J-derived blastocysts. Male chimeras were crossed with C57B16/J females to give N1F0 offspring, which were subsequently intercrossed to generate N1F1 offspring. In addition, N1F0 offspring were successively backcrossed to C57B16/J females to generate N5F0 mice. These were intercrossed to create an N5F1 population.

Genotyping of Study Populations

N1F1 and N5F1 study populations were genotyped by polymerase chain reaction (PCR) and Southern blot of genomic DNA isolated from the hearts of animals used in the studies. Hearts of wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice were cut into small pieces (∼1 mm) and placed into polypropylene tubes. Extraction buffer [2 ml; 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 0.5% SDS, 20 µg ml^{-1} RNase, 100 µg ml^{-1} proteinase K] was added and samples were incubated at 50°C–55°C for 4 h until completely lysed. The mixture was then extracted two times each with phenol, phenol/chloroform and chloroform (Maniatis et al. 1989). Genomic DNA was precipitated by adding 2.5 vols of cold ethanol, washed with 70% v/v ethanol (–20°C) and dissolved in 200 µl of 10 mM Tris-HCl, 1 mM EDTA TE, pH 8.0. Once dissolved, the purity and concentration of the DNA

were measured by spectrophotometry (absorbency at 260 and 280 nm wavelength).

PCR amplification was performed using 50-µl aliquots (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 17 mM $MgCl_2$, 200 nM 2′-deoxynucleotide 5′-triphosphates (dNTPs), 10% v/v dimethylsulfoxide, 1.25U *Taq* DNA polymerase; (Perkin-Elmer, Norwalk, Conn., USA) using 200 ng of genomic DNA as the template and PCR primers specific to the neomycin resistance gene present at the targeted locus (5′-TGA ACA AGA TGG ATT GCA CGC AGG TTC TCC GGC-3′ and 5′-GCC AAG CTC TTC AGC AAT ATC ACG GGT AGC-3′, yielding an ∼700-bp product)) and mouse UT gene-specific primers (5′-CTG GCT GAC CTG CTG TAT CTG CT-3′ and 5′-CAG GGT CAC ACA AAG CAC TCT CA-3′, yielding an ∼900-bp product). A 500-bp mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon was used as the internal control (5′-TGG CCA AGG TCA TCC ATG AC-3′ and 5′-GTC CAC CAC CCT GTT GCT GTA G-3′, yielding an ∼500-bp product). Amplification was performed for 30 cycles at 60°C annealing for 30 s, 72°C extension for 90 s and 94°C denaturing for 30 s. Amplification of a 500-bp (GAPDH)/700-bp doublet alone corresponded to a $UT^{(-/-)}$ genotype and a 500-bp (GAPDH)/900-bp doublet alone corresponded to a $UT^{(+/+)}$ genotype.

Genomic DNA (20 µg) was digested with *Bam*HI and run on an agarose gel (1%). The agarose gel was then treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, followed by neutralization with 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30–40 min. The DNA was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Mass., USA) and probed with cDNA corresponding to the full-length mouse UT receptor open reading frame (ORF). cDNA fragments were labeled with [α-^{32}P] 2′-deoxycytidine 5′-triphosphate (dCTP) using standard random primed methods (T7 QuickPrime; Pharmacia Biotech, Piscataway, N.J., USA). Membranes were prehybridized for 2 h at 42°C and incubated overnight at 42°C with 1×10^9 cpm μg^{-1} denatured radiolabeled probe in standard buffer [50% deionized formamide, 6 × sodium chloride, sodium citrate (SSC), 5 × Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 µg ml^{-1} denatured, fragmented salmon sperm DNA]. Membranes were washed under conditions of low stringency (three 15-min washes in 1 × SSC, 0.1% SDS at 28°C) followed by a high stringency wash in 0.1 × SSC/0.1% SDS for 30 min at 55°C. Hybridization signals were detected by conventional X-ray autoradiography (Hyper film, Amersham Life Science, UK) and phosphor imaging (Storm 860, Molecular Dynamics, Sunnyvale, Calif., USA).

Hemodynamics and Echocardiography

Male wild-type ($UT^{(+/+)}$) and homozygous UT receptor knockout ($UT^{(-/-)}$) mice, anesthetized with 1.5% isoflurane, underwent transthoracic echocardiographic determination of left ventricular end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), cardiac output (CO) and ejection fraction (EF). Further to this, mice were re-anesthetized the following day for hemodynamic evaluation where a fluid-filled catheter was inserted into the left carotid artery for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The catheter was then advanced into the left ventricle (LV) to obtain measurements of left ventricular end-systolic (LVESP) and end-diastolic pressure (LVEDP). At the end of the study, selected organs (right and left kidney, heart, right and left ventricle and lungs) were isolated and wet weights were measured.

Preparation and Utilization of Mouse Isolated Aortae and Mesenteric Arteries

Male (4 months; 27 g) wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice were anesthetized with inhaled isoflurane (5% in O_2) and killed by cervical dislocation. Proximal descending thoracic aortae were isolated and cleaned of adherent tissue. Vessels approximately 3 mm in length were suspended in 10-ml organ baths containing Krebs solution of the following composition (mM): NaCl 112.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, dextrose 11.0. Krebs solution was maintained at 37 ± 1°C and aerated with 95% O_2, 5% CO_2 (pH 7.4). For contraction studies, vessels were denuded of endothelium by rubbing with a fine forceps and indomethacin (10 mM) was added to the buffer. Changes in isometric force were measured under 0.5 *g* optimal resting tension using FT03 force-displacement transducers (Grass Instruments, Quincy, Mass., USA) coupled to Model 7D polygraphs.

The Halpern–Mulvany wire myograph (Model 610M; Danish Myo Technology, Denmark) was used for measurement of isometric force development of endothelium-intact superior mesenteric arteries (optimal resting tension of 0.5 *g*) and data were recorded using a Grass 7400 direct thermal recorder.

Following a 60-min equilibration period, vessels were treated with standard concentrations of KCl (60 mM) and phenylephrine (1 mM) to which subsequent agonist-induced responses were normalized.

Once the contractile response to phenylephrine had plateaued, carbachol (10 μM) was added to the vessels in order to evaluate endothelial integrity.

Cumulative concentration–response curves to phenylephrine (0.1 nM to 10 μM), angiotensin II (0.1 nM to 10 μM), endothelin-1 (0.1 nM to 1 mM) and hU-II (0.01 nM to 3 μM) were obtained for each vessel by adding the spasmogen to the tissue bath in half-log increments. During relaxation studies, vessels were preconstricted with an EC_{80} concentration of phenylephrine and contractile tone was reversed by adding cumulative amounts of carbachol (1 nM to 30 μM) or sodium nitroprusside (0.1 nM to 1 μM). Each response was allowed to plateau before the addition of subsequent agonist concentrations. Vessels were allowed to recover for at least 30 min between subsequent agonist–response curves, and were not exposed to subsequent agonists after treatment with either endothelin-1 or hU-II.

EVALUATION

All values are expressed as mean ± SEM and *n* represents the total number of animals from which the vessels were isolated. Statistical comparisons were made using an unpaired, two-tailed *t*-test or Fisher's exact tests and differences were considered significant when $P < 0.05$. Concentration–response curves were fitted to a logistic equation as previously described (Douglas et al. 1995).

MODIFICATIONS OF THE METHOD

Coulouarn et al. (1998) reported cloning of the cDNA encoding the urotensin II precursor (Prepro-UII cDNA) in frog and human. Intense expression of the urotensin gene in motoneurons of the spinal cord was found indicating important physiological functions of urotensin II.

Elshourbagy et al. (2002) reported molecular and pharmacological characterization of genes encoding urotensin II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. Monkey and mouse preproU-II genes were identified to encode 123 and 125 amino acids. Monkey and mouse UT receptors were 389, and 386 amino acids, respectively. Expression of mouse and monkey U-II/UT receptor mRNA was found also in extravascular tissues, including lung, pancreas, skeletal muscle, kidney and liver.

REFERENCES AND FURTHER READING

Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, Louden CS, Foley JJ, Sauermelch CF, Coatney RW, Ao Z, Dias J, Holmes SD, Stadel JM, Martin JD, Liu WS, Glover GI, Wilson S, McNulty DE, Ellis CE, Elshourbagy NA, Shabon U, Trill JJ, Hay DWP, Ohlstein EN, Bergsma DJ, Douglas SA (1999) Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. Nature 401:282–286

Behm DJ, Harrison SM, Ao Z, Maniscalco K, Pickering SJ, Grau EV, Woods TN, Coatney RM, Doe CPA, Willette RN, Johns DG, Douglas SA (2003) Deletion of the UT receptor gene results in the selective loss of urotensin-II contractile activity in aortae isolated from UT receptor knockout mice. Br J Pharmacol 139:464–472

Coulouarn Y, Lihrmann I, Jegou S, Anouar Y, Tostivint H, Beauvillain JC, Conlon JM, Bern HA, Vaudry H (1998) Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin gene in motoneurons of the spinal cord. Proc Natl Acad Sci USA 95:15803–15808

Douglas SA, Beck GR, Elliott JD, Ohlstein EH (1995) Pharmacological evidence for the presence of three distinct functional endothelin receptor subtypes in the rabbit lateral saphenous vein. Br J Pharmacol 114:1529–1540

Douglas SA, Ohlstein EH (2000) Urotensin receptors. In: Girdlestone D (ed) The IUPHAR compendium of receptor characterization and classification. IUPHAR Media, London, pp 365–372

Elshourbagy NA, Douglas SA, Shabon U, Harrison S, Duddy G, Sechler JL, AO, Z, Maleeff BE, Naselsky D, Disa J, Aiyar NV (2002) Molecular and pharmacological characterization of genes encoding urotensin-II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. Br J Pharmacol 136:9–22

Maguire JJ, Kuc RE, Davenport AP (2000) Orphan-receptor ligand human urotensin II: receptor localization and comparison of vasoconstrictor responses with endothelin-1. Br J Pharmacol 131:441–446

Maniatis T, Fritsch EF, Sambrook J (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor, New York

A.1.1.31 Apelin

A.1.1.31.1 General Considerations

PURPOSE AND RATIONALE

The peptide apelin, consisting of 36 amino acids, is found increasingly to play a role in biology and medicine (Kleinz and Davenport 2005; Masri et al. 2005). The family of apelin peptides is derived from a single gene and activates the 7-transmembrane G-protein-coupled receptor APJ. In the search for an endogenous ligand of the orphan G-protein-coupled receptor APJ, Tatemoto et al. (1998) isolated and characterized endogenous peptide ligands, designated apelin, from bovine stomach extracts. Apelin-13, consisting of 13 amino acids, was more active than apelin-36. APJ was first identified in a human gene by O'Dowd et al. (1993), sharing close identity to the angiotensin receptor. In rats, the greatest expression of APJ mRNA was detected in the lung, suggesting that APJ and its ligand play an important role in the pulmonary system

(Hosoya et al. 2000). Cloning, pharmacological characterization and brain distribution of the rat apelin receptor were reported by De Mota et al. (2000), and the physiological role of apelin and its receptor in rat brain by Reaux et al. (2001). The hypothalamic and hypophyseal distribution of the receptor suggested an involvement of apelin in the control of neuro- and adenohypophyseal hormone release, whereas the presence in the pineal gland and in discrete higher brain structures pointed to possible roles in the regulation of circadian rhythms and of water and food intake behavior. Studying the distribution of apelin-synthesizing neurons in the adult rat brain, Reaux et al. (2002) suggested multiple roles of apelin especially in the central control of ingestive behaviors, pituitary hormone release and circadian rhythms. Studying apelin immunoreactivity in the rat hypothalamus and pituitary, Brailoiu et al. (2002) concluded that apelin may be a signaling peptide released from the hypothalamic-hypophyseal axis. De Mota et al. (2004) found that apelin is a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. Masri et al. (2002) reported that apelin activates extracellular signal-regulated kinases via a system sensitive to pertussis toxin (PTX). Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin were reviewed by Medhurst et al. (2003). Jászberény et al. (2004) studied the behavioral, neuroendocrine, and thermoregulatory actions of apelin-13. Wang et al. (2004) investigated the localization of apelin in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Boucher et al. (2005) found in isolated mouse and human adipocytes that apelin is upregulated by insulin and obesity.

REFERENCES AND FURTHER READING

Boucher J, Masri B, Daviaud D, Gesta S, Guigné C, Mazzucotelli A, Castan-Laurell I, Tack I, Knibiehler B, Carpéné C, Audigier Y, Saulnier-Blache JS, Valet P (2005) Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology 146:1764–1771

Brailoiu GC, Dun SL, Yang J, Ohsawa M, Chang JK, Dun NJ (2002) Apelin-immunoreactivity in the rat hypothalamus and pituitary. Neurosci Lett 327:193–197

De Mota N, Lenkei Z, Llorens-Cortès C (2000) Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. Neuroendocrinology 72:400–407

De Mota N, Reaux-le Goazigo A, el Messari S, Chartrel N, Roesch D, Gujardin C, Kordon C, Vaudry H, Moos F, Llorens-Cortès C (2004) Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. Proc Natl Acad Sci USA 101:10464–10469

Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, Kitada C, Honda S, Kurukawa T, Onda H, Nishimura O, Fujino M (2000) Molecular and functional characterization of APJ. J Biol Chem 275:21061–21067

Jaszberenyi M, Bujdoso E, Telegdy G (2004) Behavioral, neuroendocrine and thermoregulatory actions of apelin-13. Neuroscience 129:811–816

Kleinz MJ, Davenport AP (2005) Emerging roles of apelin in biology and medicine. Pharmacol Ther 107:198–211

Masri B, Knibiehler B, Audigier Y (2005) Apelin signalling: a promising pathway from cloning to pharmacology. Cell Signal 17:415–426

Masri B, Lahlou H, Mazarguil H, Knibiehler B, Audigier Y (2002) Apelin (65–77) activates extracellular signal-regulated kinases via a PTX-sensitive system. Biochem Biophys Res Commun 290:539–545

Medhurst AD, Jennings CA, Murdock P, Darker JG (2003) Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin. J Neurochem 84:1162–1172

O'Dowd BF, Heiber M, Chan A, Heng HHQ, Tsui LC, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T (1993) A human gene that shows identity with the gene encoding the angiotensin receptor in located on chromosome 11. Gene 136:355–360

Reaux A, de Mota N, Skultetyova I, Lenkei Z, el Messari S, Gallatz K, Corvol P, Palkovits M, Llorens-Cortès (2001) Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. J Neurochem 77:1085–1096

Reaux A, Gallatz K, Palkovits M, Llorens-Cortès C (2002) Distribution of apelin-synthesizing neurons in the adult rat brain. Neuroscience 113:653–662

Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada T, Kurukawa T, Onda H, Fujino M (1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 251:471–476

Wang G, Anini Y, Wei W, Qi X, O'Carroll AM, Mochizuki T, Wang HQ, Hellmich MR, Englander EW, Greeley GH Jr (2004) Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Endocrinology 145:1342–1348

A.1.1.31.2 Cardiovascular Actions of Apelin

PURPOSE AND RATIONALE

Most reports concentrate on the functional role of apelin in the cardiovascular system (Tatemoto et al. 2001; Katugampola et al. 2002; Sokodi et al. 2002; Katugampola and Davenport 2003; Chen et al. 2003; Berry et al. 2004; Kleinz and Davenport 2004; Kagiyama et al. 2005; Losano 2005).

Cheng et al. (2003) studied the venous dilator effect of apelin in conscious rats using a method for measurement of body venous tone published by Pang (2000).

Ashley et al. (2005) found that apelin improves cardiac contractility and reduces cardiac loading *in vivo* in mice.

PROCEDURE

Male C57Bl/ 6 mice were used. The following studies were performed: magnetic resonance imaging of the heart, pressure–volume hemodynamics, effects of chronic apelin infusion, assessment of ventricular hypertrophy after sacrifice, and expression of the APJ receptor.

Pressure–volume hemodynamics were assessed using the Aria system (Millar Instruments, Houston, Tex., USA), This measurement platform, specifically designed for small rodents, comprises an ultra-miniature 1.4 F (0.47 mm outer diameter) catheter, which incorporates pressure and conductance sensors, possessing hardware including analog-digital conversion and analysis software. Pressure is measured directly in mmHg, while the conductivity of blood is used to estimate volume and allow construction of pressure–volume relationships in real time.

Male mice aged 8–16 weeks were anesthetized with 1%–2% isoflurane in oxygen. The internal jugular vein was cannulated with PE tubing and a 10% albumin solution infused at 5 µl/min following a bolus of 150 µl over 5 min. After tracheostomy, a 19-gauge cannula was inserted into the trachea and the animal was ventilated at a tidal volume of 200 µl at 100 breaths per minute. Following an incision just dorsal to the xyphoid cartilage, the diaphragm was visualized from below, and after diaphragmatic incision, the left ventricular apex was visualized. The pressure–volume catheter was inserted along the long axis of the left ventricle, from where it was adjusted to obtain rectangular-shaped pressure–volume loops. Baseline loops were recorded following volume replacement, at which point the inferior vena cava was visualized within the chest and occlusion parameters were recorded during and after a 5-s manual occlusion of the vessel. Next, the albumin solution was replaced by one containing 100 nM apelin, which was infused at 5 µl/min for 20 min, following which baseline and occlusion loops were recorded once again.

Signals from the catheter were digitized using the Powerlab system and stored for offline analysis using the PVAN software. This allows analyses of pressure (e. g., end-systolic pressure, end-diastolic pressure) and derivation of pressure–time and volume–time parameters at steady state.

For **magnetic resonance imaging**, male C57Bl/6 mice aged 16 weeks were scanned twice on subsequent days. The animals underwent general anesthesia while breathing spontaneously via a nose cone fitted carefully to minimize escape of anesthetic into the environment. Two percent isoflurane was administered with an oxygen flow rate of 1–2 l/min. Platinum needle ECG leads were inserted subcutaneously. Respiration was monitored by means of a pneumatic pillow sensor positioned against the abdomen. Mouse body temperature was maintained during scanning at 37°C by a flow of heated air thermostatically controlled by a rectal temperature probe. Magnetic resonance images were acquired on a 4.7 T Oxford magnet controlled by a Varian Inova console (Varian, Palo Alto, Calif., USA) using a transmit–receive, quadrature, volume coil with an inner diameter of 3.5 cm. Image acquisition was gated to respiration and to the ECG R wave (SA Instruments, Stony Brook, N.Y., USA). Coronal and sagittal scout images led to the acquisition of multiple contiguous 1-mm-thick, short axis slices orthogonal to the interventricular septum. Nine cine frames were taken at each slice level with the following sequence parameters: TE = 2.8 ms, NEX = 12, FOV = 3 × 3 cm, matrix = 128 × 128, flip angle = 60°. Cine frames were spaced 16 ms apart and acquired through slightly more than one cardiac cycle guaranteeing acquisition of systole and diastole. On the second day of scanning, mice received 300 µg/kg body weight of apelin as an intraperitoneal injection 1 h prior to scanning. A pilot study had previously identified 1 h as an appropriate time within which to identify apelin effects resulting from peritoneal absorption. Planimetry measurements of end-diastolic and end-systolic dimensions were derived offline from short axis views of the left ventricle at the level of the papillary muscles using ImageJ software (National Institutes of Health, Bethesda, Md., USA). Ejection fraction was calculated as [LVEDA-LVESA]/LVEDA, where LVEDA is left ventricular end-diastolic area and LVESA is left ventricular end-systolic area.

EVALUATION

Data were analyzed using Student's *t* statistic (paired) or repeated measures analysis of variance using the post hoc comparison of Fisher.

MODIFICATIONS OF THE METHOD

Katugampola et al. (2001, 2002) described [^{125}I](Pyr^{1})apelin as a radioligand for localizing the APJ orphan receptor in human and rat tissues.

Kasai et al. (2004) found that apelin worked as an angiogenic factor in the retinal endothelial cell line RF/6A.

REFERENCES AND FURTHER READING

Ashley EA, Powers J, Chen M, Kundo R, Finsterbach T, Caffarelli A, Deng A, Eichorn J, Mahajan R, Agrawal R,

Greve J, Robbins R, Patterson AJ, Bernstein D, Quertermous T (2005) The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. Cardiovasc Res 65:73–82

Berry MF, Pirolli TJ, Jayasankar V, Burdick J, Morine KJ, Gardner TJ, Woo YJ (2004) Apelin has in vivo inotropic effects in normal and failing hearts. Circulation 110[suppl II]:II187–II193

Chen MM, Ashley EA, Deng DXF, Tsalenko A, Deng A, Tabibiazar R, Ben-Dor A, Fenster B, Yang E, King JY, Fowler M, Robbins R, Johnson FL, Bruhn L, McDonagh T, Dargie H, Yakhini Z, Tsao PS, Quertermous T (2003) Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. Circulation 108:1432–1439

Cheng X, Cheng XS, Pang CCY (2003) Venous dilator effect of apelin, an endogenous peptide ligand for the orphan APJ receptor, in conscious rats. Eur J Pharmacol 470:171–175

Ishida J, Hashimoto T, Hashimoto Y, Nishiwak S, Iguchi T, Harada S, Sugaya T, Matsuzaki H, Yamamoto R, Shiota N, Okunishi H, Kihara M, Umemura S, Sugiyama F, Yagami KI, Kasuya Y, Mochizuki N, Fukamizu A (2004) Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure *in vivo*. J Biol Chem 279:262674–26279

Kagiyama S, Fukuhara M, Matsumara K, Lin Y, Fujii K, Iida M (2005) Central and peripheral cardiovascular actions of apelin in conscious rats. Regul Peptídes 125:55–59

Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, Hinuma S, Baba A (2004) Apelin is a novel angiogenic factor in retinal endothelial cells. Biochem Biophys Res Commun 325:395–400

Katugampola SD, Maguire JJ, Matthewson SR, Davenport AP (2001) [^{125}I](Pyr^{1})Apelin-13 is a novel radioligand for localizing the APJ orphan receptor in human and rat tissues. Br J Pharmacol 132:1255–1260

Katugampola SD, Maguire JJ, Kuc RE, Wiley KE, Davenport AP (2002) Discovery of recently adopted orphan receptors for apelin, urotensin II, and ghrelin identified using novel radioligands and functional role in the human cardiovascular system. Can J Physiol Pharmacol 80:369–374

Katugampola SD, Davenport AP (2003) Emerging roles for orphan G-protein-coupled receptors in the cardiovascular system. Trend Pharmacol Sci 24:30–35

Kleinz MJ, Davenport AP (2004) Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endothelial cells. Regul Peptides 118:119–125

Losano GA (2005) On the cardiovascular activity of apelin. Cardivasc Res 65:8–9

Pang CCY (2000) Measurement of body venous tone. J Pharmacol Toxicol Meth 44:341–360

Szokodi I, Tavi P, Földes G, Voutilainen-Myllylä S, Ilves M, Tokola H, Pikkarainen S, Piuhola J, Rysä J, Tóth M, Ruskoaho H (2002) Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. Circ Res 91:434–440

Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, Fujimiya M (2001) The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regul Peptides 99:87–92

A.1.1.32 TRP Channels

A.1.1.32.1 General Considerations

The transient receptor potential (TRP[1]) ion channels are named after the role of the channels in *Drosophila* phototransduction (Montell 2001). The mammalian genes are encoded by at least 28 channel subunit genes (Clapham 2003; Moran et al. 2004; Clapham et al. 2005). Six protein families comprise the mammalian TRP superfamily: the classic TRPs (TRPCs), the vanilloid receptor TRPs (TRPVs), the melastatin or long TRPs (TRPMs), the mucolipins (TRPMLs), the polycystins (TRPPs), and ankyrin transmembrane protein 1 (ANKTM1, TRPA1). The TRP channel primary structures predict six transmembrane (TM) domains with a pore domain between the fifth (S5) and sixth (S6) segments and both C and N termini presumably located intracellularly (Vannier et al. 1998).

The TRP superfamily includes >20 related cation channels that play critical roles in processes ranging from sensory physiology to vasorelaxation and male fertility. Defects in TRP channels have been associated with changes in growth control and one TRP-related protein may be a tumor suppressor. Moreover, mutations in a member of the TRP superfamily are a common cause of polycystic kidney disease, while disruption of another is responsible for mucolipidosis, a neurodegenerative disease. TRP proteins are widely expressed in the nervous system, and, in non-excitable cells, TRP-related channels may be the primary mode of Ca^{2+} entry. TRP proteins are cation channels; however, they vary significantly in their selectivity and mode of activation. Nevertheless, members of the TRP superfamily share significant sequence homology and predicted structural similarities.

Based on primary amino acid homology, the conventional TRP proteins can be classified into three subfamilies: TRPC, TRPV, and TRPM (Birnbaumer et al. 2003). Three additional, more distantly related subfamilies have recently been defined: ANKTM1, a Ca^{2+}-permeant, non-selective cation channel, is the only mammalian member of the TRPA branch of TRP proteins. It is activated by noxious cold temperature, pungent mustard oil compounds and may additionally serve as a mechanosensitive transduction channel in auditory hair cells as well as an ionotropic cannabinoid receptor. The three mucolipins, TRPML1, 2, and 3, appear to be ion channels in intracellular vesicles. Mutations in TRPML1 cause mucolipidosis type IV,

a neurodegenerative lysosomal storage disorder, while genetic defects in TRPP2, a member of the TRPP subfamily, are frequently encountered in patients suffering from autosomal dominant polycystic kidney disease.

REFERENCES AND FURTHER READING

Albert AP, Large WA (2003) Store-operated Ca^{2+}-permeable non-selective cation channels in smooth muscle cells. Cell Calcium 33:345–356

Birnbaumer L, Yidirim E, Abramowitz J (2003) A comparison of the genes coding for canonical TRP channels and their M, V and P relatives. Cell Calcium 33:419–432

Clapham DE (2003) TRP channels as cellular sensors. Nature (Lond) 426:517–524

Clapham DE, Julius D, Montell C, Schultz G (2005) International Union of Pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels. Pharamcol Rev 57:427–450

Flockerzi V (2007) An introduction to TRP channels. Handb Exp Pharmacol 179:1–19

Gudermann T, Flockerzi V (2005) TRP channels as new pharmacological targets. Naunyn-Schmiedebergs Arch Pharmacol 371:241–244

Harteneck C (2003) Proteins modulating TRP channel function. Cell Calcium 33:303–310

Kumar B, Dreja K, Shah SS, Cheong A, Xu SZ, Sukumar P, Naylor J, Forte A, Cipollaro M, McHugh D, Kingston PA, Heagerty AM, Munsch CM, Bergdahl A, Hultgårdh-Nilsson A, Gomez MF, Porter KE, Hellstrand P, Beech DJ (2006) Upregulated TRPC1 channel in vascular injury in vivo and its role in human neointimal hyperplasia. Circ Res 98:557–563

Li S, Westwick J, Poll C (2003) Transient receptor potential (TRP) channels as potential drug targets in respiratory disease. Cell Calcium 33:551–558

Montell C (2001) The venerable invertebrate TRP channels. Cell Calcium 31:409–417

Montell C, Birnbaumer L, Flockerzi V (2002) The TRP channels, a remarkable functional family. Cell 108:595–598

Moran MM, Xu H, Clapham DE (2004) TRP ion channels in the nervous system. Curr Opin Neurobiol 14:362–369

Nilius B (2003) From TRPs to SOCs, CCEs, and CRACs. Consensus and controversies. Cell Calcium 33:293–298

Schilling WP (2001) TRP proteins. Novel therapeutic targets for regional blood pressure control? Circ Res 88:256–259

Vannier B, Zhu X, Brown D, Birnbaumer L (1998) The membrane topology of human transient receptor potential 3 as inferred from glycosylation-scanning mutagenesis and epitope immunocytochemistry. J Biol Chem 273:8675–8679

Voets T, Nilius B (2003) The pore of TRP channels: trivial or neglected? Cell Calcium 33:299–302

A.1.1.32.2 TRPC Channels

The TRPC family can be divided into three subgroups by sequence homology as well as functional similarities: C1/C4/C5, C3/C6/C7, and C2. **TRPC1** was the first member of the mammalian TRP family purported to form an ion channel (Zitt et al. 1996). Given the widespread expression of TRPC1 and its ability to coassemble with other TRPC subunits (Xu et al. 1997; Lintschinger et al. 2000; Strübing et al. 2003), TRPC1 might be a component of different heteromeric TRP complexes. The subgroup most closely related to TRPC1 comprises TRPC4 and TRPC5. TRPC4 and TRPC5 are PDZ motif-containing proteins that can form homomeric cation channels that are activated following stimulation of G_q-coupled receptors (Okada et al. 1999) as well as receptor tyrosine kinases (Schaefer et al. 2000).

Xu and Beech (2001) found that TRPC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells.

Vandebrouck et al. (2002) described involvement of TRPC in the abnormal calcium influx observed in dystrophic (*mdx*) mouse skeletal muscle fibers.

Venkatachalam et al. (2003) discussed regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C.

Bergdahl et al. (2003) found that cholestrerol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca^{2+} entry dependent on TRPC1.

Amiri et al. (2003) published FRET-based analysis of TRPC subunit stoichiometry.

Jho et al. (2005) reported that angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^{2+} influx.

Rao and Kaminski (2006) found that induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels.

Takai et al. (2004) distinguished two types of non-selective cation channels by muscarinic stimulation with carbachol in bovine muscle cells.

Kumar et al. (2006) studied upregulated TRPC1 channel in vascular injury *in vivo* and its role in human neointimal hyperplasia.

Less information is available about **TRPC2**, which shares approximately 30% sequence identity with the TRPC3/6/7 subfamily. Full-length TRPC2 mRNA and several N-terminal splice variants have been found in mouse and rat tissue, but TRPC2 seems to be a pseudogene in humans (Vannier et al. 1999; Liman 2003). TRPC2 protein was localized to neuronal micovilli in rat vomeronasal organ (Liman 2003) and in the head of mouse sperm (Jungnickel et al. 2001).

TRPC3, **TRPC6**, and **TRPC7** are 75% identical. When expressed they constitute non-selective cation currents that rectify in both the inward (- voltages) and outward (+ voltages) directions. TRPC3, TRPC6, and TRPC7 are inwardly and outwardly rectifying, have relatively low selectivity for Ca^{2+} over Na^+, and are ac-

tivated by diacylglycerol (DAG) (Hofmann et al. 1999; Okada et al. 1999; Putney et al. 2004). These channels seem to play important roles in vascular and airway smooth muscle (Trebak et al. 2003; Yu et al. 2003; Corteling et al. 2004).

Groschner and Rosker C (2005) described TRPC3 as a versatile transducer molecule that serves integration and diversification of cellular signals.

Graziani et al. (2006) investigated the potential role of membrane cholesterol as a regulator of cellular TRPC3 conductance.

Smyth et al. (2006) found a dissociation of regulated trafficking of TRPC3 channels in the plasma membrane from their activation by phospholipase C.

TRPC4 and TRPD5 are receptor-operated Ca^{2+}-permeable non-selective cation channels (Plant and Schaefer 2003).

Odell et al. (2005) found that epidermal growth factor induces tyrosine phosphorylation, membrane insertion and activation of transient receptor potential channel 4.

Saleh et al. (2006) described that angiotensin II activates two cation channels with distinct TRPC1 and TRCP6 channel properties in rabbit mesenteric artery myocytes.

Shimizu et al. (2006) reported that Ca^{2+}-calmodulin-dependent myosin light chain kinase is essential for activation of TRPC5 channels expressed in HEK293 cells.

Shi et al. (2004) described multiple regulation by calcium of murine homologs of transient receptor potential proteins TRP6 and TRP7 expressed in HEK293 cells.

Xu et al. (2006) identified sphingosine-1-phosphate as an activator of the calcium channel TRPC5, which controls vascular smooth muscle activity.

Estacion et al. (2004) studied activation of human TRPC6 channels by receptor stimulation.

Basora et al. (2003) reported that 20-hydroxyeicosatetraenoic acid (20-HETE) activates mouse TRPC6 channels expressed in HEK293 cells.

Maruyama et al. (2006) found that heteromultimeric TRPC6-TRPC7 channels contribute to arginine vasopressin-induced cation current of A7r5 vascular smooth muscle cells.

REFERENCES AND FURTHER READING

Amiri H, Schultz G, Schäfer M (2003) FRET-based analysis of TRPC subunit stoichiometry. Cell Calcium 33:461–470

Basora N, Boulay G, Bilodeau L, Rousseau E, Payett MD (2003) 20-Hydroxyeicosatetraenoic adid (20-HETE) activates mouse TRPC6 channels expressed in HEK293 cells. J Biol Chem 278:31709–31716

Bergdahl A, Gomez MF, Dreja K, Xu SZ, Adner M, Beech DJ, Broman J, Hellstrand P, Swärd K (2003) Cholesterol depletion impairs vascular reactivity to endothelin-1 by reducing store-operated Ca^{2+} entry dependent on TRPC1. Circ Res 93:839–847

Corteling RL, Li S, Giddings J, Westwick J, Poll C, Hall IP (2004) Expression of transient receptor potential C6 and related transient receptor potential family members in human airway smooth muscle and lung tissue. Am J Respir Cell Mol Biol 30:145–154

Estacion M, Li S, Sinkins WG, Gosling M, Bahra P, Poll C, Westwick J, Schilling WP (2004) Activation of human TRPC6 channels by receptor stimulation. J Biol Chem 279:22047–22056

Graziani A, Rosker C, Kohlwein SD, Zhu MX, Romanin C, Sattler W, Groschner K, Poteser M (2006) Cellular cholesterol controls TRPC3 function: evidence for a novel dominant-negative knockdown strategy. Biochem J 396:147–155

Groschner K, Rosker C (2005) TRPC3: a versatile transducer molecule that serves integration and diversification of cellular signals. Naunyn-Schmiedebergs Arch Pharmacol 371:251–256

Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature (Lond) 397:259–263

Jho D, Mehta D, Ahmmed G, Gao XP, Tiruppathi C, Broman M, Malik AB (2005) Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca^{2+} influx. Circ Res 96:1282–1290

Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM (2001) Trp2 regulates entry of Ca^{2+} into mouse sperm triggered by egg ZP3. Nat Cell Biol 3:499–502

Kumar B, Dreja K, Shah SS, Cheong A, Xu SZ, Sukumar P, Naylor J, Forte A, Cipollaro M, McHugh D, Kingston PA, Heagerty AM, Munsch CM, Bergdahl A, Hultgårdh-Nilsson A, Gomez MF, Porter KE, Hellstrand P, Beech DJ (2006) Upregulated TRPC1 channel in vascular injury in vivo and its role in human neointimal hyperplasia. Circ Res 98:557–563

Lee J, Cha SK, Sun TJ, Huang CL (2005) PIP2 activates TRPV5 and releses its inhibition by intracellular Mg^{2+}. J Gen Physiol 126:439–451

Liman ER (2003) Regulation by voltage and adenine nucleotides of a Ca^{2+}-activated cation channel from hamster vomeronasal sensory neurons. J Physiol (Lond) 548:777–787

Lintschinger B, Balzer-Geldsetzer M, Baskaran T, Graier WF, Romanin C, Zhu MX, Groschner K (2000) Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+}-sensitive cation channels. J Biol Chem 275:27799–27805

Maruyama Y, Nakanishi Y, Walsh EJ, Wilson DP, Welsh DG, Cole WC (2006) Heteromultimeric TRPC6-TRPC7 channels contribute to arginine vasopressin-induced cation current of A7r5 vascular smooth muscle cells. Circ Res 98:1520–1527

Odell AF, Scott JL, Van Helden DF (2005) Epidermal growth factor induces tyrosine phosphorylation, membrane insertion and activation of transient receptor potential channel 4. J Biol Chem 280:37974–37987

Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, Imoto K (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca^{2+}-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. J Biol Chem 274:27359–27370

Plant TD, Schaefer M (2003) TRPC4 and TRPD5: receptor-operated Ca^{2+}-permeasble nonselective cation channels. Cell Calcium 33:441–450

Putney JW Jr, Trebak M, Vazquez G, Wedel B, Bird GS (2004) Signalling mechanisms for TRPC3 channels. Novartis Found Symp 258:123–133

Rao GK, Kaminski NE (2006) Induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels. J Leukoc Biol 79:202–213

Saleh SN, Albert AP, Peppiat CM, Large WA (2006) Angiotensin II activates two cation channels with distinct TRPC1 and TRCP6 channel properties in rabbit mesenteric artery myocytes. J Physiol (Lond) 577:479–495

Schaefer M, Plant TD, Obukhov AG, Hofmann T, Gudermann T, Schultz G (2000) Receptor-mediated regulation of the nonselective cation channels TRPC4 and TRPC5. J Biol Chem 275:17517–17526

Shi J, Mori E, Mori Y, Mori M, Li J, Ito Y, Inoue R (2004) Multiple regulation by calcium of murine homologues of transient receptor potential proteins TRP6 and TRP7 expressed in HEK293 cells. J Physiol (Lond) 561:415–437

Shimizu S, Yoshida T, Wakamori M, Ishii M, Okada T, Takahashi M, Seto M, Sakurada K, Kiuchi Y, Mori Y (2006) Ca^{2+}-calmodulin-dependent myosin light chain kinase is essential for activation of TRPC5 channels expressed in HEK293 cells. J Physiol (Lond) 570:219–235

Smyth JT, Lemonnier L, Vazquez G, Bird GS, Putney JW (2006) Dissociation of regulated trafficking of TRPC3 channels in the plasma membrane from their activation by phospholipase C. J Biol Chem 281:11712–11720

Strübing C, Krapivinsky G, Krapivinsky L, Clapham DE (2003) Formation of novel TRPC channels by complex subunit interactions in embryonic brain. J Biol Chem 278:39014–39019

Takai Y, Sugawara R, Ohinata H, Takai A (2004) Two types of non-selective cation channels by muscarinic stimulation with carbachol in bovine muscle cells. J Physiol (Lond) 559(3):899–922

Trebak M, Vazquez G, Bird GS, Putney JW (2003) The TRPC3/6/7 subfamily of cation channels. Cell Calcium 33:451–461

Vandebrouck C, Martin D, Colson-Van Schoor M, Debaix H, Gailly P (2002) Involvement of TRPC in the abnormal calcium influx observed in dystrophic (*mdx*) mouse skeletal muscle fibers. J Cell Biol 158:1089–1096

Vannier B, Peyton M, Boulay G, Brown D, Qin N, Jiang M, Zhu X, Birnbaumer L (1999) Mouse trp2, the homologue of the human trpc2 pseudogene, encodes mTrp2, a store depletion-activated capacitative Ca^{2+} entry channel. Proc Natl Acad Sci USA 96:2060–2064

Venkatachalam K, Zheng F, Gill DL (2003) Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. J Biol Chem 278:29031–29040

Xu XZ, Li HS, Guggino WB, Montell C (1997) Coassembly of TRP and TRPL produces a distinct store-operated conductance. Cell 89:1155–1164

Xu SZ, Beech DJ (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. Circ Res 88:84–87

Xu SZ, Muraki K, Zeng F, Li J, Sukumar P, Shah S, Dedman AM, Flemming OK, McHugh D, Naylor J, Cheong A, Bateson AN, Munsch CM, Porter KLE, Beech DJ (2006) A sphingosine-1-phosphate-activated calcium channel controlling vascular smooth muscle activity. Circ Res 98:1381–1389

Yu Y, Sweeney M, Zhang S, Platoshyn O, Landsberg J, Rothman A, Yuan JX (2003) PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. Am J Physiol 284:C316–C330

Zitt C, Zobel A, Obukhov AG, Harteneck C, Kalkbrenner F, Lückhoff A, Schultz G (1996) Cloning and functional expression of a human Ca^{2+}-permeable cation channel activated by calcium store depletion. Neuron 16:1189–1196

A.1.1.32.3 TRPM Channels

The TRPM subfamily has eight members divided into four groups: M1 (melastatin)/M3, M7 (TRP-PLIK)/M6, M2/M8, and M4/M5 (Hartzeneck 2005; Fonfria et al. 2006). **TRPM1** may be regulated through direct interaction with a cytosolic isoform generated by alternative RNA splicing (Xu et al. 2001). **TRPM2** is a 1503-amino-acid protein that is highly expressed in brain (Nagamine et al. 1998) and present in blood cells.

Perraud et al. (2003) reviwed TRPM2 Ca^{2+}-permeable cation channels.

Hill et al. (2004a, 2004b) reported inhibition of TRPM2 channels by the antifungal agents clotrimazole and econazole as well as by flufenamic acid; Kraft et al. (2006) by *N*-(*p*-amylcinnamoyl)anthranilic acid.

Identified first by sequencing projects, the function of **TRPM3** is poorly understood. The hTRPM3 gene maps to human chromosome 9q-21.12 and encodes a 1555-amino-acid protein.

TRPM4 and **TRPM5** have similar characteristics. TRPM4b, a splice variant of TRPM4, and TRPM5 are Ca^{2+}-activated, voltage-modulated, monovalent-selective cation channels with 25-pS single-channel conductances (Launay et al. 2002; Hofmann et al. 2003; Nilius et al. 2003). Pérez et al. (2003) studied the ion channel TRPM5 in taste receptor cells.

TRPM6 and **TRPM7** comprise a unique subfamily of TRP proteins with both channel and kinase activities. TRPM7, which has 1863 amino acid residues, was identified in a yeast two-hybrid screen as a protein interacting with phospholipase C (PLC) (Runnels et al. 2001; Schmitz et al. 2003). It seems to be ubiquitously expressed. Takezawa et al. (2004) described receptor-mediated regulation of the TPRM7 channel through its endogenous protein kinase domain.

TRPM8 is a 1104-amino-acid protein that does not seem to contain associated enzymatic domains. TRPM8 is a non-selective, voltage-modulated conductance. At colder temperatures (8°C–28°C) or in the presence of menthol, TRPM8 current is activated at a more physiological range of voltages (Brauchi et al. 2004; Voets et al. 2004). This channel is expressed in

small-diameter primary sensory neurons, where it presumably functions as a thermosensor (McKemy et al. 2002; Peier et al. 2002).

REFERENCES AND FURTHER READING

Brauchi S, Orio P, Latorre R (2004) Clues to understanding cold sensation: thermodynamics and electrophysiological analysis of the cold receptor TRPM8. Proc Natl Acad Sci USA 101:15494–15499

Fonfrai E, Murdock PR, Cusdin FS, Benham CD, Kelsell RE, McNulty S (2006) Tissue distribution profiles of the human TRPM cation channel family. J Recept Signal Transduct Res 26:159–178

Hartzeneck C (2005) Function and pharmacology of TRPM cation channels. Naunyn-Schmiedebergs Arch Pharmacol 371:307–314

Hill K, McNuty S, Randall AD (2004a) Inhibition of TRPM2 channels by the antifungal agents clotrimazole and econazole. Naunyn-Schmiedebergs Arch Pharmacol 370:227–237

Hill K, Benham CD, McNulty S, Randall AD (2004b) Flufenamic acid is a pH-dependent antagonist of TRPM2 channels. Neuropharmacol ogy 47:450–460

Hofmann T, Chubanov V, Gudermann T, Montell C (2003) TRPM5 is a voltage-modulated and Ca^{2+}-activated monovalent selective cation channel. Curr Biol 13:1153–1158

Kraft R, Grimm C, Frenzel H, Harteneck C (2006) Inhibition of TRPM2 cation channels by N-(p-amylcinnamoyl) anthranilic acid. Br J Pharmacol 148:264–273

Launay P, Fleig A, Perraud AL, Scharenberg AM, Penner R, Kinet JP (2002) TRPM4 is a Ca^{2+}-activated nonselective cation channel mediating cell membrane depolarization. Cell 109:397–407

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature (Lond) 416:52–58

Nagamine K, Kudoh J, Minoshima S, Kawasaki K, Asakawa S, Ito F, Shimizu N (1998) Molecular cloning of a novel putative Ca^{2+} channel protein (TRPC7) highly expressed in brain. Genomics 54:124–131

Nilius B, Prenen J, Droogmans G, Voets T, Vennekens R, Freichel M, Wissenbach U, Flockerzi V (2003) Voltage dependence of the Ca^{2+}-activated cation channel TRPM4. J Biol Chem 278:30813–30820

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002) A TRP channel that senses cold stimuli and menthol. Cell 108:705–715

Pérez CA, Margolskee RF, Kinnamon SC, Ogura T (2003) Making sense with TRP channels: store-operated calcium entry and the ion channel Trpm5 in taste receptor cells. Cell Calcium 33:541–549

Perraud AL, Schmitz C, Scharenberg AM (2003) TRPM2 C^{a2+} permeable cation channels. From gene to biological function. Cell Calcium 33:519–531

Runnels LW, Yue L, Clapham DE (2002) The TRPM7 channel is inactivated by PIP_2 hydrolysis. Nat Cell Biol 4:329–336

Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. Cell 114:191–200

Takezawa R, Schmitz C, Demeuse P, Scharenberg AM, Penner R, Fleig A (2004) Receptor-mediated regulation of the TPRM7 channel through its endogenous protein kinase domain. Proc Natl Acad Sci USA 101:6009–6014

Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. Nature (Lond) 430:748–754

Xu XZ, Moebius F, Gill DL, Montell C (2001) Regulationn of melastatin, a TRP-realted protein, through interaction with a cytoplasmic isoform. Proc Natl Acad Sci U S A. 98(19):10692–7

A.1.1.32.4 TRPV Channels

The TRPV subfamily is named after the first mammalian member of the subfamily, vanilloid receptor 1 (Caterina et al. 1997; Benham et al. 2002; Montell et al. 2002; Clapham 2003; Mutai and Heller 2003). TRPV5 and TRPV6 are highly Ca^{2+}-selective TRP channels that mediate trans-epithelial Ca^{2+} transport in kidney and intestine (Hoenderop et al. 2003).

The TRPV channel subfamily has six members divided into two groups: V1/V2/V3/V4 and V5/V6. The vanilloid receptor, TRPV1, is the best understood ion channel in this class (Caterina et al. 1997; Caterina and Julius 2001).

TRPV channels as temperature sensors were revived by Benham et al. (2003).

The expressed **TRPV1** capsaicin receptor is a heat/proton/lipid/voltage-modulated Ca^{2+}-permeant (P_{Ca}/P_{Na} 10) ion channel. A more voltag-gating-centric explanation is that at warmer temperatures (> 37°C) or in the presence of capsaicin, TRPV1 current is activated by a more physiological range of voltages (Brauchi et al. 2004). TRPV1 is desensitized by internal Ca^{2+}; it is not activated by store depletion. TRPV1, V2, and V3 are activated by the synthetic compound 2-aminoethoxydiphenylborate (2-APB) (Chung et al. 2004; Hu et al. 2004). Endogenous cannabinoid receptor ligands, such as anandamide, are potential TRPV1 agonists.

The vanilloid receptor-like channel **TRPV2** is 50% identical to TRPV1, but is insensitive to capsaicin (Caterina et al. 1999). Like TRPV1 it is more permeable to Ca^{2+} than to Na^+ ($P_{Ca}/P_{Na} = 3:1$). It has been proposed to mediate high-threshold noxious heat sensation, perhaps in the lightly myelinated A nociceptors, but its presence in non-sensory tissue suggests other functions as well.

TRPV3 is expressed widely but most strikingly in skin. Increasing temperature from 22 to 40°C in mammalian cells transfected with hTRPV3 elevates intracellular calcium by activating a non-selective cationic conductance (P_{Ca}/P_{Na} 10:1) (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002). As in sensory neurons, the current is steeply dependent on temperature, sen-

sitizes with repeated heating, and displays a striking hysteresis on heating and cooling, but the extent of expression in sensory neurons is controversial. Based on these properties, TRPV3 is thermosensitive in the physiological range of temperatures between TRPM8 and TRPV1 and may play a role in pain.

TRPV4 is 40% identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na} = 6$), which, like TRPV1, displays a gently outwardly rectifying *I–V* relation. TRPV4 is 40% identical to TRPV1 and TRPV2 (Liedtke et al. 2000; Strotmann et al. 2000). When expressed in mammalian cells it comprises a moderately selective cation channel ($P_{Ca}/P_{Na} = 6$), which, like TRPV1, displays a gently outwardly rectifying *I–V* relation.

TRPV5 and TRPV6 comprise a separate subfamily of TRPVs with only 30% identity with TRPV1. The expressed channels strongly inwardly rectify and are the most Ca^{2+}-selective ($P_{Ca}/P_{Na} > 100$) (Nilius et al. 2000; Vennekens et al. 2000; Yue et al. 2001; Den Dekker et al. 2003) of all TRP channels. These properties are consistent with proposed mechanisms for Ca^{2+}-selective channels in which negatively charged glutamic or aspartic acid residues provide a binding site for divalents within the pore. Intra- and extracellular [Ca^{2+}] (Yue et al. 2001; Hirnet et al. 2003; Bödding and Flockerzi 2004) and calmodulin (Lambers et al. 2004) regulate TRPV6 activity. The localization of TRPV5 and TRPV6 to the proximal small intestine and collecting duct of the kidney, along with mouse knockout data, suggests that this family is important in calcium uptake via epithelial cells (Hoenderop et al. 2005).

Lee et al. (2005) reported that PIP_2 activates TRPV5 and releases its inhibition by intracellular Mg^{2+}.

REFERENCES AND FURTHER READING

Benham CD, Davis JB, Randall AD (2002) Vanilloid and TRP channels: a family of lipid-gated cation channels. Neuropharmacology 42:873–888

Benham CD, Gunthorpe MJ, Davis JB (2003) TRPV channels as temperature sensors. Cell Calcium 33:479–487

Bödding M, Flockerzi V (2004) Ca^{2+} dependence of the Ca^{2+}-selective TRPV6 channel. J Biol Chem 279:36546–36552

Brauchi S, Orio P, Latorre R (2004) Clues to understanding cold sensation: thermodynamics and electrophysiological analysis of the cold receptor TRPM8. Proc Natl Acad Sci USA 101:15494–15499

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature (Lond) 389:816–824

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature (Lond) 398:436–441

Caterina MJ, Julius D (2001) The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 24:487–517

Chung MK, Lee H, Mizuno A, Suzuki M, Caterina MJ (2004) 2-Aminoethoxydiphenyl borate activates and sensitizes the heat-gated ion channel TRPV3. J Neurosci 24:5177–5182

Clapham DE (2003) TRP channels as cellular sensors. Nature 426:517–524

Den Dekker E, Hoenderop JGJ, Nilius B, Bindels RJM (2003) The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. Cell Calcium 33:497–507

Hirnet D, Olaussson J, Fecher-Trost C, Bödding M, Nastainczyk W, Wissenbach U, Flockerzi V, Freichel M (2003) The TRPV6 gene, cDNA,and protein. Cell Calcium 33:509–518

Hoenderop JG, Voets T, Hoefs S, Weidema F, Prenen J, Nilius B, Bidels RJ (2003) Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. EMBO J 22:776–785

Hoenderop JG, Nilius B, Bindels RJ (2005) Calcium absorption across epithelia. Physiol Rev 85:373–422

Hu HZ, Gu Q, Wang C, Colton CK, Tang J, Kinoshita-Kawada M, Lee LY, Wood JD, Zhu MX (2004) 2-Aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2 and TRPV3. J Biol Chem 279:35741–35748

Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ (2004) Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. J Biol Chem 279:28855–28861

Lee J, Cha SK, Sun TJ, Huang CL (2005) PIP_2 activates TRPV5 and releases its inhibition by intracellular Mg^{2+}. J Gen Physiol 126:439–451

Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 103:525–535

Montell C, Birnbaumer L, Flockerzi V, Bindels RJ, Bruford EA, Caterina MJ, Clapham DE, Harteneck C, Heller S, Julius D (2002) A unified nomenclature for the superfamily of TRP cation channels. Mol Cell 92:229–231

Mutai M, Heller S (2003) Vertebrate and invertebrate TRPV-like mechanoreceptors. Cell Calcium 33:471–478

Nilius B, Vennekens R, Prenen J, Hoenderop JG, Bindels RJ, Droogmans G (2000) Whole-cell and single channel monovalent cation currents through the novel rabbit epithelial Ca^{2+} channel ECaC. J Physiol (Lond) 527 (Pt 2):239–248

Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A (2002) A heat-sensitive TRP channel expressed in keratinocytes. Science 296:2046–2049

Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L et al (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. Nature (Lond) 418:186–190

Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol 2:695–702

Vennekens R, Hoenderop GJ, Prenen J, Stuiver M, Willems PHGM, Droogmans G, Nilius B, Bindels RJM (2000) Permeation and gating properties of the novel epithelial Ca^{2+} channel. J Biol Chem 275:3963–3969

Xu H, Ramsey I, Kotecha SA, Moran M, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y et al (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature (Lond) 418:181–186

Yue L, Peng JB, Hediger MA, Clapham DE (2001) CaT1 manifests the pore properties of the calcium-release-activated calcium channel. Nature (Lond) 410:705–709

A.1.1.32.5 TRPV1 Receptor Assay

PURPOSE AND RATIONALE

The vanilloid receptor-1 (TRPV1) is a non-selective cation channel, predominantly expressed by peripherally sensory neurons, which is known to play a key role in the detection of noxious painful stimuli, such as capsaicin and heat. Smart et al. (2000, 2001) found that the endogenous lipid anandamide is a full agonist at the human vanilloid receptor and characterized human vanilloid VR1 pharmacology using FLIPR. With these methods, Gunthorpe et al. (2004) identified and characterized a potent and selective vanilloid receptor (VR1/TRPV1) antagonist.

PROCEDURE

Cloning and Expression of VR1 Receptors in HEK293 Cells

Human VR1 cDNA was identified using the published rat VR1 sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double-strand sequenced, and stably expressed in HEK293 cells. Rat VR1 cDNA was amplified from rat DRG cDNA and similarly expressed in HEK293 cells.

Cell Culture

hVR1-HEK293 cells were grown as monolayers in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% fetal calf serum, and 0.2 mM L-glutamine, and maintained under 95%/5% O_2/CO_2 at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper et al. (1990).

Electrophysiological Studies

Cells were plated and cultured on glass coverslips at 26,000 cells cm^{-2} and whole-cell voltage clamp recordings were performed at room temperature (20–24°C), using standard methods. The extracellular solution consisted of (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 30, HEPES-NaOH 25, pH 7.3. For anandamide application this solution was supplemented with 0.2% lipid-free bovine serum albumin. Patch pipettes of resistance 2–5 MΩ were fabricated on a Sutter Instruments P-87 electrode puller and were filled with the following solution (mM): CsCl 140, $MgCl_2$ 4, EGTA 10, HEPES-CsOH 10, pH 7.3. All recordings were made from single, well isolated, phase-bright cells. Currents were recorded at a holding potential of –70 mV using an Axopatch 200B amplifier. Data acquisition and analysis were performed using the pClamp7 software suite. Drug applications were affected with an automated fast-switching solution exchange device (Warner Instruments SF-77B; time for solution exchange ≈30 ms).

Measurements of $[Ca^{2+}]_i$ Using the FLIPR

hVR1-HEK293 cells were seeded into black-walled clear-base 96-well plates (Costar, UK) at a density of 25,000 cells per well in MEM, supplemented as above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator Fluo-3AM (4 μM; Teflabs, Austin, Tex., USA) at 25°C for 120 min. The cells were washed four times with, and finally cultured in, Tyrode's medium containing 0.2% BSA, before being incubated for 30 min at 25°C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, UK) to monitor cell fluorescence ($\lambda_{EX} = 488$ nM, $\lambda_{EM} = 540$ nM) (Sullivan et al. 1999) before and after the addition of various agonists.

EVALUATION

Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± SEM unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software, Calif., USA). pK_B values were generated from IC_{50} curves for the antagonist vs a fixed EC_{80} concentration of agonist using the Cheng–Prusoff equation.

MODIFICATIONS OF THE METHOD

Rao and Kaminski (2006) reported that induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels.

REFERENCES AND FURTHER READING

Gunthorpe MJ, Benham CD, Randall A, Davis JB (2002) The diversity in the vanilloid (TRPV) receptor family of ion channels. Trends Pharmacol Sci 23:191

Gunthorpe MJ, Rami HK, Jerman JC, Smart D, Gill CH, Soffin EM, Hanna SL, Lappin SC, Egerton J, Smith GD, Worby A, Howett L, Owen D, Nasir S, Davies CH, Thompson M, Wyman PA, Randall AD, Davis JB (2004) Identification and characterization of SB-366971, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. Neuropharmacology 46:133–149

Rao GK, Kaminski NE (2006) Induction of intracellular elevation by Δ^9-tetrahydrocannabinol in T cells involves TRPC1 channels. J Leukoc Biol 79:202–213

Skaper SD, Facci L, Milani D, Leon A, Toffano G (1990) Culture and use of primary and clonal neural cells. In: Conn PM (ed) Methods in neurosciences, Vol 2. Academic Press, New York, pp 17–33

Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, Davus JB (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br J Pharmacol 129:227–230

Smart D, Jerman JC, Gunthorpe MJ, Brough SJ, Ranson J, Cairns W, Hayes PD, Randall AD, Davis JH (2001) Characterisation using PLIPR of human vanilloid receptor pharmacology. Eur J Pharmacol 417:51–58

Sullivan E, Tucker EM, Dale IL (1999) Measurement of $[Ca^{2+}]_i$, usuing the fluorometric imaging plate reader (FLIPR). In: Lambert DG (ed) Calcium signaling protocols. Humana Press, New Jersey, pp 125–136

A.1.2 Studies in Isolated Organs

A.1.2.1 α-Sympatholytic Activity in Isolated Vascular Smooth Muscle

PURPOSE AND RATIONALE

Noradrenaline and other sympathomimetic drugs increase vascular smooth muscle tone by stimulation of α-adrenergic receptors. Contractions can be antagonized by α-adrenergic receptor blocking agents such as phentolamine. Drugs can be tested for their capacity of reducing vascular smooth muscle contractions induced by the adrenergic receptor-activating agent noradrenaline. Moreover, effects of peptides, such as bradykinin, can be tested with strips of aorta or pulmonary artery.

PROCEDURE

As donor animals Pirbright White guinea pigs of either sex weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 200–300 g are used. The vessels to be tested are the thoracic aorta or the arteria pulmonalis. The animals are sacrificed by stunning and exsanguination. The pulmonary artery or the thoracic aorta is quickly removed and cut into helical strips of 1–2 mm width and 15–20 mm length. The strips are mounted in an organ bath with a preload of 1 g. Krebs-Henseleit buffer solution containing 11.5 M glucose is maintained at 37°C and oxygenated with 95% O_2, 5% CO_2. Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Experimental Course

Following an equilibration period of 60 min, contractions are induced by repeated administrations of (–)noradrenaline HCl in concentrations of 2×10^{-6} M for testing the contractions of the pulmonary artery and in concentrations of 2×10^{-8} M for testing the contractions of the aorta. After obtaining a stable plateau of identically sized contractions, cumulative doses of the test compound are added into the organ bath. Consecutive concentrations are given when the response of the previous dose has reached a plateau.

Controls at the end of the experiment: If a compound does not show vasorelaxing activity at any dose, the sensitivity of the preparation is tested by adding phentolamine (1×10^{-7} M).

If a compound shows vasorelaxing activity, the reversibility of the relaxation is tested by increasing the noradrenaline concentration.

EVALUATION

The contractile force is determined before and after drug administration.

Percent inhibition of spasmogen-induced contraction by test drug is calculated as compared to the maximal contraction with a spasmogen alone (= 100%).

IC_{50} values are determined from the individual dose-response curves. IC_{50} is defined as the dose of drug leading to a 50% relaxation of noradrenaline-induced contraction.

MODIFICATIONS OF THE METHOD

The isolated vena cava of rabbits can be used for assaying α-adrenolytic activity. The rabbit is sacrificed by CO_2 anesthesia. The vena cava inferior is removed and cut into strips. The percent inhibition of epinephrine or norepinephrine induced contractions is determined.

The effects of bradykinin and bradykinin antagonists can be tested in isolated guinea pig artery and isolated rabbit aorta which contains predominantly the BK_1-receptor type (Regoli and Barabé 1980; Hock et al. 1991).

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated pulmonary artery. Endothelium-intact pulmonary artery rings were contracted with 3×10^{-8} M phenylephrine and a cumulative dose-response curve of relaxation was constructed.

REFERENCES AND FURTHER READING

Furchgott RF (1967) Techniques for studying antagonism and potentiation of sympathomimetic drugs an isolated tissues. In: Siegler PE, Moyer JH (eds) Animal and clinical pharmacologic techniques in drug evaluation. pp 256–266. Year Book Medical Publishers, Inc., Chicago

Green AF, Boura ALA (1964) Sympathetic nerve blockade. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press, London and New York, pp 370–430

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J, Schölkens BA (1991) Hoe 140 a new potent and long acting bradykinin antagonist: *in vitro* studies. Br J Pharmacol 102:769–773

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskolin derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydroxy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43(I):313–319

Regoli D, Barabé J (1980) Pharmacology of bradykinin and related peptides. Pharmacol Rev 32:1–46

Wisskirchen FM, Burt RP, Marshall I (1998) Pharmacological characterization of CGRP receptors of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens. Br J Pharmacol 123:1673–1683

A.1.2.2 α-Sympatholytic Activity in the Isolated Guinea Pig Seminal Vesicle

PURPOSE AND RATIONALE

The seminal vesicles of guinea pigs and rats are tubular organs whose longitudinal and annular muscles are innervated by the sympathetic system. The inhibition of contractions induced by norepinephrine or the α_1-selective agonist phenylephrine indicates α-sympatholytic activity. Sharif and Gokhale (1986) recommended the use the isolated rat seminal vesicle as a rather sensitive and specific model.

PROCEDURE

Male guinea pigs weighing 300 to 600 g are sacrificed by a blow to the neck. Both seminal vesicles are prepared and placed in Ringer's solution in an organ bath maintained at 32°C and being oxygenated with 95% O_2/5% CO_2. Isotonic or isometric registration is performed. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik Freiburg). Isometric force is measured with a force transducer (UC-2 Gould-Statham, Oxnard, USA).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 1 to 5 µg/ml or phenylephrine HCl in concentrations of 10 to 50 µg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine or phenylephrine is added. As standard phentolamine is used in concentrations of 3 to 30×10^{-7} M.

EVALUATION

Contractions of the seminal vesicle induced by the α-adrenergic agonists after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947)

MODIFICATION OF THE METHOD

Leitch (1954) recommended the use of isolated seminal vesicles of rats for the assay of sympatholytic drugs.

REFERENCES AND FURTHER READING

Brügger J (1945) Die isolierte Samenblase des Meerschweinchens als biologisches Testobjekt zur quantitativen Differenzierung der sympathikolytischen Wirkung der genuinen Mutterkornalkaloide und ihrer Dihydroderivate. Helv Physiol Acta 3:117–134

Green AF, Boura ALA (1964) Depressants of peripheral sympathetic nerve function. I Sympathetic nerve blockade. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press, London New York, pp 370–430

Leitch JL (1954) The use of the rat's isolated seminal vesicle for the assay of sympatholytic drugs. Br J Pharmacol 9:236–239

Schild HO (1947) pA, a new scale for the measurement of drug antagonism. Br J Pharmacol 2:189–206

Sharif SI, Gokhale SD (1986) Pharmacological evaluation of the isolated rat seminal vesicle preparation. J Pharmacol Meth 15:65–75

A.1.2.3 α-Sympatholytic Activity in the Isolated Vas Deferens of the Rat

PURPOSE AND RATIONALE

The vas deferens of the guinea pig or preferably the rat is used for quantitative evaluation of adrenergic antagonists. The response of this organ to α-adrenergic agonists consists of a strong rapid contraction followed

by quick relaxation on washing the agonists out of the tissue.

PROCEDURE

Male Wistar rats weighing about 300 g are used. The animals are sacrificed by a sharp blow to the neck, the vasa deferentia are dissected free from the extraneous tissues and suspended in a organ bath containing Tyrode solution being oxygenated with a 95% O_2 and 5% CO_2 mixture at 32°C. Isotonic registration is performed at a preload of 0.5 g. Changes in length are recorded isotonically using a lever transducer (e. g., 368 type B, Hugo Sachs Elektronik, Freiburg, FRG).

Following an equilibration period of 30 min, contractions are induced by repeated administration of (–)norepinephrine HCl in concentrations of 0.5, 1.0, 2.0, or 4.0 µg/ml. After obtaining a stable plateau of identical contractions, the test compound is added into the organ bath. Three min later, the previous concentration of norepinephrine is added. As standard phentolamine is used in concentrations of 3 to 30×10^{-7} M.

EVALUATION

Contractions of the vas deferens induced by the α-adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Electrical stimulation of the isolated ductus deferens results in the release of norepinephrine. Stimulation induced contractions of this organ are inhibited by clonidine which impairs adrenergic neurotransmission by activating inhibitory α-receptors. The ductus deferens is suspended in an organ bath bubbled with carbogen and maintained at 37°C. Tension is adjusted to 25 mN. Following a 45 min equilibration period, supramaximal amplitude stimulation by a HSE type 2 stimulator (Hugo Sachs Elektronik, Freiburg) is applied. After stabilization of the response, clonidine is added to the organ bath in accumulated doses. Test compounds are added 5 min prior to clonidine administration. The percent potentiation of clonidine induced inhibition of contractions is determined.

Taylor et al. (1983) used the rat deferens for pharmacological characterization of purinergic receptors.

Nerve-muscle preparations of the vas deferens have been reviewed by Holman (1975).

Hughes et al. (1974) used the electrically stimulated mouse vas deferens for assessment of the agonistic and antagonistic activities of narcotic analgesic drugs.

Ross et al. (2001) used the mouse vas deferens to study structure-activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors.

Oka et al. (1980) recommended the vas deferens from rabbits as a specific bioassay for opioid κ-receptor agonists.

Mutafova-Yambolieva and Radmirov (1993) studied the effects of endothelin-1 on electrically- or drug-induced contractile responses mediated by purinergic or adrenergic receptors in the isolated prostatic portion of rat vas deferens.

Ward et al. (1990) used isolated vasa deferentia preparations from rat and mouse to study the pharmacological profile of the analgesic pravadoline.

Hukovic (1961) described an isolated ductus deferens preparation together with the sympathetic hypogastric nerve of the guinea pig.

Cordellini and Sannomiya (1984) pretreated guinea pigs with reserpine. In the isolated vasa deferentia concentration-effects curves to phenylephrine were established in the presence of cocaine. The antagonistic effect of phenoxybenzamine was used for receptor occupancy studies.

Donoso et al. (1992) studied neurotransmission in epididymal and prostatic segments of isolated superfused rat vas deferens preparations.

Vaupel and Su (1987) used the vas deferens preparation of guinea pigs to study sigma and phencyclidine receptors.

Eltze (1988) used the field-stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) portion of rabbit vas deferens to study muscarinic M_1 and M_2-receptors.

Dumont et al. (1997) used the isolated guinea pig heart and the isolated rat vas deferens for *in vitro* bioassays of calcitonin gene-related peptide (CGRP) agonists and antagonists.

Poyner et al. (1999) found concentration-dependent inhibitions of the electrically stimulated twitch responses of guinea pig vas deferens by calcitonin gene-related peptide, amylin and adrenomedullin.

Couldwell et al. (1993) found that the rat prostate gland possesses a typical α_1-adrenoceptor similar to that found in the vas deferens.

Wisskirchen et al. (1998) tested agonists of calcitonin gene-related peptide, homologues and antagonists in rat isolated vas deferens. The prostatic half was

suspended under 0.5 g resting tension and equilibrated in Krebs solution at 37°C. Contractile responses of the prostatic vas were induced by electrical field stimulation at 0.2 Hz, 1.0 ms and 60 V through parallel platinum electrodes either side of the tissue.

REFERENCES AND FURTHER READING

Cordellini S, Sannomiya P (1984) The vas deferens as a suitable preparation for the study of α-adrenoreceptor molecular mechanisms. J Pharmacol Meth 11:97–107

Couldwell C, Jackson A, O'Brien H, Chess-Williams R (1993) Characterization of the α_1-adrenoceptors of rat prostate gland. J Pharm Pharmacol 45:922–924

Donoso MV, Montes CG, Lewin J, Fournier A, Calixto JB, Huidobro-Toro JP (1992) Endothelin-1 (ET)-induced mobilization of intracellular Ca^{2+} stores from the smooth muscle facilitates sympathetic cotransmission by potentiation of adenosine 5′-triphosphate (ATP) motor activity: studies in the rat vas deferens. Peptides 13:831–840

Dumont Y, Fournier A, St-Pierre S, Quirion R (1997) A potent and selective $CGRP_2$ agonist, [Cys(Et)^{2,7}]hCGRPα: comparison in prototypical $CGRP_1$ and $CGRP_2$ *in vitro* bioassays. Can J Physiol Pharmacol 75:671–676

Eltze M (1988) Muscarinic M_1 and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. Eur J Pharmacol 151:205–221

Holman ME (1975) Nerve-muscle preparations of the vas deferens. In: Daniel EE, Paton DM (eds) Methods in Pharmacology, Vol 3, Smooth Muscle. Plenum Press, New York and London, pp 403–417

Hughes J, Kosterlitz HW, Leslie FM (1974) Assessment of the agonistic and antagonistic activities of narcotic analgesic drugs by means of the mouse vas deferens. Br J Pharmacol 51:139P–140P

Hukovic S (1961) Responses of the isolated sympathetic nerve-ductus deferens preparation of the guinea pig. Br J Pharmacol 16:188–194

Lindner E (1963) Untersuchungen über das Verhalten des N-(3′-phenylpropyl-(2′))-1,1-diphenylpropyl-(3)-amins (Segontin) gegenüber den Wirkungen des Noradrenalins. Arch Int Pharmacodyn 146:475–484

Lotti VJ, Taylor DA (1982) α_2-adrenergic agonist and antagonist activity of the respective (–)- and (+)-enantiomers of 6-ethyl-9-oxaergoline (EOE). Eur J Pharmacol 85:211–215

Moore PK, Griffiths RT (1982) Pre-synaptic and post-synaptic effects of xylazine and naphazoline on the bisected rat vas deferens. Arch Int Pharmacodyn 260:70–77

Mutafova-Yambolieva V, Radomirov R (1993) Effects of endothelin-1 on postjunctionally-mediated purinergic and adrenergic components of rat vas deferens contractile responses. Neuropeptides 24:35–42

Ohlin P, Stromblad BCR (1963) Observations on isolated vas deferens. Br J Pharmacol 20:299–306

Oka T, Negishi K, Suda M, Matsumiya T, Inazu T, Ueki M (1980) Rabbits vas deferens: a specific bioassay for opioid κ-receptor agonists. Eur J Pharmacol 73:235–236

Poyner DR, Taylor GM, Tomlinson AE, Richardson AG, Smith DM (1999) Characterization of receptors for calcitonin gene-related peptide and adrenomedullin on the guinea pig vas deferens. Br J Pharmacol 126:1276–1282

Ross RA, Gibson TM, Brockie HC, Leslie M, Pashmi G, Craib SJ, DiMarzo V, Pertwee RC (2001) Structure-activity relationship for the endogenous cannabinoid, anadamide, and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. Br J Pharmacol 132:631–640

Schild HO (1947) pA, a new scale for the measurement of drug antagonism. Br J Pharmacol 2:189–206

Taylor DA, Wiese S, Faison EP, Yarbrough GGl (1983) Pharmacological characterization of purinergic receptors in the rat deferens. J Pharmacol Exp Ther 224:40–45

van Rossum JM (1965) Different types of sympathomimetic α-receptors. J Pharm Pharmacol 17:202–216

Vaupel DB, Su TP (1987) Guinea pig vas deferens preparation may contain both sigma receptors and phencyclidine receptors. Eur J Pharmacol 139:125–128

Wisskirchen FM, Burt RP, Marshall I (1998) Pharmacological characterization of CGRP receptors of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens. Br J Pharmacol 123:1673–1683

A.1.2.4 α-Sympatholytic Activity in the Isolated Rat Spleen

PURPOSE AND RATIONALE

α-Stimulant agents (such as epinephrine, norepinephrine) or electrical stimulation induce contractions in sympathetically innervated organs such as spleen smooth muscle. These effects can be antagonized by drugs with α-blocking activities such as phentolamine.

PROCEDURE

Male Sprague-Dawley rats weighing 180–220 g are used. The animal is sacrificed in CO_2 anesthesia. The spleen is removed and cut longitudinally into two halves. Each part is placed in an organ bath containing nutritive solution. The bath solution is bubbled with carbogen and maintained at 37°C. Following a 30 min incubation period under a tension of 0.5 g, contractions are elicited by administration of epinephrine (10^{-6} g/ml) or norepinephrine 10^{-6} g/ml). After obtaining 3 approximately identical spasms, the test compound is administered followed by the addition of the spasmogen 5 min later. The contractile response is allowed to plateau and recorded.

Standard compound:

- phentolamine

EVALUATION

The contractile force is recorded at its maximal level before and after drug administration. The percent inhibition of epinephrine or norepinephrine induced contraction is determined.

REFERENCES AND FURTHER READING

DiPalma (1964) Animal techniques for evaluating sympathomimetic and parasympathomimetic drugs. In: Nodine JH,

Siegler PE (eds) Animal and pharmacologic techniques in drug evaluation. Vol I, pp 105–110. Year Book Medical Publ., Inc. Chicago

Swamy VC (1971) α-adrenergic blocking agents. In: Turner RA, Hebborn P (eds) Screening Methods in Pharmacology, Vol II, pp 1–19, Academic Press, New York and London

Turner RA (1965) Sympatholytic agents. In: Screening methods in pharmacology, Vol I, pp 143–151, Academic Press, New York and London

A.1.2.5 α-Sympatholytic Activity in the Isolated Rat Anococcygeus Muscle

PURPOSE AND RATIONALE

The rat anococcygeus muscle as pharmacological tool was introduced by Gillespie (1972, 1980), Gibson and Gillespie (1973). This smooth muscle has a dense adrenergic innervation and contracts to noradrenaline, acetylcholine, 5-hydroxytryptamine, but not to histamine. Moreover, the muscle contracts to field stimulation or stimulation of extrinsic nerves. The preparation can be used to assess the pre- and post-synaptic α-adrenoceptor blocking activity of drugs (Doggrell 1980, 1983).

PROCEDURE

The two anococcygeus muscles arise from the upper coccygeal vertebra close to one another in the midline of the pelvic cavity. The muscles pass caudally, lying first behind and the to one side of the colon, finally joining together to form a ventral bar in front of the colon a few mm from the anus. The extrinsic nerves pass in a branch of the perineal nerve on either side to enter the deep surface of each muscle just before the formation of the ventral bar.

After sacrifice, the abdomen of rats is opened in the mid-line, the pelvis split and the bladder and urethra removed. Care is required in clearing the lower part of the urethra to avoid damage to the ventral bar of muscle, the only region lying ventral to the colon. The colon is then cut through at the pelvic brim, the pelvic portion pulled forward and the delicate connective tissue behind cleared until the anococcygeus muscles come into view. The muscles are isolated, in some instances with the extrinsic nerve intact. The extrinsic nerves on either side run in the posterior scrotal branch of the perineal nerve and leave it to enter the deep surface of the anococcygeal muscles as the lie on the lateral surface of the colon. The ventral bar is cut through and each muscle mounted in a 100 ml bath containing Krebs solution at 36°C. The solution is gassed with 95% O_2+5% CO_2. Tension is measured with isometric transducers and displayed on a polygraph. Field stimulation of the intramural nerves is applied after drawing the muscles through a pair of electrodes similar to those described by Burn and Rand (1960); when the muscles are stimulated through their extrinsic nerves the nerves are drawn through similar electrodes. Stimulation of either intramural or extrinsic nerves is with 1 ms pulses at 20 Hz, and at a supramaximal voltage.

Dose-response curves are established with doses of 2×10^{-7} to 4×10^{-6} M noradrenaline, 4×10^{-7} to 4×10^{-5} M acetylcholine or with graded frequencies of electrical stimulation.

The effect of noradrenaline is abolished by α-adrenergic antagonists, such as 10^{-6} M phentolamine. Dose-response curves show a parallel shift characteristic of competitive antagonism.

EVALUATION

Contractions of the anococcygeus muscle induced by an α-adrenergic agonist after addition of the test compound are compared with the initial values and expressed as percentage thereof. For in depth analysis, full dose-response curves of the agonist are recorded before and after addition of various doses of the antagonist. A parallel shift to the right indicates competitive antagonism which can be evaluated as pA_x – values according to Schild (1947).

MODIFICATIONS OF THE METHOD

Gibson et al. (1990) found L-N^G-nitroarginine to be a potent inhibitor of non-adrenergic, non-cholinergic relaxations in the rat anococcygeus muscle.

Oliveira and Bendhack (1992) found that dopamine has a dual effect in the rat anococcygeus muscle: a partial effect due to an indirect sympathomimetic action and a partial effect due the interaction with postjunctional receptors.

Brave et al. (1993) investigated the interaction between motor sympathetic and inhibitory non-adrenergic, non-cholinergic nerves in the rat anococcygeus muscle using L-N^G-nitroarginine, an inhibitor of L-arginine:NO synthase.

Cakici et al. (1993) described a coaxial bioassay system consisting of guinea pig trachea as donor organ for epithelial derived-relaxing factors and phenylepinephrine-precontracted rat anococcygeus muscle as assay tissue.

Iravani and Zar (1993) found differential effects of nifedipine on nerve-mediated and noradrenaline-evoked contractions of rat anococcygeus muscle.

Rand and Li (1993) studied the modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves.

Mudumbi and Leighton (1994) investigated the mechanisms of action of relaxation induced by bradykinin and by electrical field stimulation in isolated rat anococcygeus muscle, where contractile tone had been elevated with clonidine.

Gwee et al. (1995) investigated the prejunctional and postjunctional inhibition of adrenergic transmission in the rat isolated anococcygeus muscle by cimetidine.

Najbar et al. (1996) found that smooth muscle cells in the rat anococcygeus muscle are endowed with two distinct P-2-purinoreceptors which subserve contractions.

De Godoy et al. (2003) evaluated the inhibitory effects of atropine and hexamethonium on the angiotensin-II-induced contraction of rat anococcygeus muscles. Pettibone et al. (1993) examined the inhibitory potency and selectivity of an oxytocin antagonist against oxytocin-stimulated contractions if the **mouse** anococcygeus muscle.

Dehpour et al. (1993) and Radjaee et al. (1996) used isolated anococcygeus muscles from **rabbits** and found an extremely regular activity induced by methoxamine or clonidine.

REFERENCES AND FURTHER READING

Brave SR, Bhat S, Hobbs AJ, Tucker JF, Gibson A (1993) Influence of L-N^G-nitroarginine on sympathic nerve induced contraction and noradrenaline release in the rat anococcygeus muscle. J Auton Pharmacol 13:219–225

Burn HJ, Rand MJ (1960) The relation of circulating noradrenaline to the effect of sympathetic stimulation. J Physiol 150:295–305

Cakici I, Tunctan B, Abacioglu N, Kanzik I (1993) Epithelium-dependent responses of serotonin in a coaxial bioassay system. Eur J Pharmacol 236:97–105

De Godoy MAF, Accorsi-Mendonça D, de Oliviera AM (2003) Inhibitory effects of atropine and hexamethonium on the angiotensin II-induced contraction of the rat anococcygeus muscles. Naunyn-Schmiedebergs Arch Pharmacol 367:176–182

Dehpour AR, Tajkhorshid E, Radjaee-Behbahani N, Kheirollahi K (1993) Methoxamine-induced rhythmic activity in rabbit anococcygeus muscle. Gen Pharmacol 24:841–845

Doggrell SA (1980) The assessment of pre- and post-synaptic a-adrenoceptor blocking activity of drugs using the rat anococcygeus muscle. J Pharmacol Meth 3:323–331

Doggrell SA (1983) On the assessment of the potency of antagonists using the rat isolated anococcygeus muscle. J Pharmacol Meth 10:243–254

Gibson A, Hobbs AJ, Mirzazadeh D (1990) L-N^G-nitroarginine is a potent inhibitor of non-adrenergic, non-cholinergic relaxations in the rat anococcygeus muscle. Eur J Pharmacol 183:1793

Gillespie JS (1972) The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. Br J Pharmacol 45:404–416

Gillespie JS (1980) The physiology and pharmacology of the anococcygeus muscle. Trends Pharmacol Sci 1:453–457

Gibson A, Gillespie JS (1973) The effect of immunosympathectomy and of 6-hydroxydopamine on the response of the rat anococcygeus muscle to nerve stimulation and to some drugs. Br J Pharmacol 47:261–267

Gwee MCE, Cheahj LS, Shoon ML (1995) Prejunctional and postjunctional inhibition of adrenergic transmission in the rat isolated anococcygeus muscle by cimetidine. J Auton Pharmacol 15:177–185

Iravani MM, Zar MA (1993) Differential effects of nifedipine on nerve-mediated and noradrenaline-evoked contractions of rat anococcygeus muscle. Eur J Pharmacol 250:193–195

Kulkarni SK, Sharma A (1994) Rat anococcygeus: A dynamic smooth muscle preparation for experimental pharmacology. Methods Find Exp Clin Pharmacol 16:379–385

Mudumbi RV, Leighton HJ (1994) Analysis of bradykinin-induced relaxations in the rat isolated anococcygeus muscle. Life Sci 54:813–821

Najbar ATZ, Li CG, Rand MJ (1996) Evidence for two distinct P-2-purinoreceptors subserving contraction of the rat anococcygeus smooth muscle. Br J Pharmacol 118:537–542

Oliveira AM, Bendhack LM (1992) Dopamine-induced contractile responses of the rat anococcygeus muscle. Arch Int Pharmacodyn Ther 316:97–104

Pettibone DJ, Clineschmidt BV, Guidotti MT, Lis EV, Reiss DR, Woyden CJ, Bock MG, Evans BE, Freidinger RM, Hobbs DW, Veber DF, Williams PD, Chiu SHL, Thompson KL, Schorn TW, Siegl PKS, Kaufman MJ, Cukierski MA, Haluska GJ, Cook MJ, Novy MJ (1993a) L-368,899, a potent orally active oxytocin antagonist for potential use in preterm labor. Drug Dev Res 30:129–142

Radjaee-Behbahani N, Dehpour AR, Tajkhorshid E, Kheirollahi K (1993) Clonidine-induced rhythmic activity in rabbit anococcygeus muscle. Gen Pharmacol 27:525–528

Rand MJ, Li CG (1993) Modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves. Br J Pharmacol 110:1479–1482

Schild HO (1947) pA, a new scale for the measurement of drug antagonism. Br J Pharmacol 2:189–206

A.1.2.6 β_1-Sympatholytic Activity in Isolated Guinea Pig Atria

PURPOSE AND RATIONALE

The β-agonist isoprenaline (isoproterenol) induces an increase in the frequency and force of contraction of spontaneously beating isolated right atrias and potentiates contractions of electrically stimulated isolated left atria. Drugs with β-sympatholytic activity inhibit these isoprenaline-induced effects. β-receptor blocking activity of drugs can be evaluated in isolated right (a) and left (b) guinea pig atria. Since the heart contains predominantly β_1-adrenoreceptors, β_1-blocking activity is assessed by this test.

PROCEDURE

Pirbright White guinea pigs of either sex weighing 250–300 g are used. The animal is sacrificed by stunning and exsanguination. The heart is removed, the right or the left atrium is cut off and mounted in a 50 ml organ bath with a preload of 100 mg. The Krebs-

Henseleit solution is maintained at 32°C and aerated with 95% O_2/5% CO_2. Contractions are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronik, Freiburg).

Right Atrium

After an equilibration period of 30 min, isoprenaline is administered into the organ bath to potentiate inotropy and frequency of the isolated right atrium. Cumulative doses of isoprenaline are added starting from a concentration of 0.05 µg/ml; consecutive doses are administered at 3 min intervals.

When a stable maximum plateau of the effect is achieved, the organ bath is thoroughly flushed for 1 min; flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same isoprenaline concentrations (control baseline values = 100%).

The test compound is then added into the organ bath and 5 min later, again isoprenaline is given at cumulative doses.

If the test compound has β-receptor blocking activity (β-sympatholytic),

1. higher isoprenaline concentrations are necessary to induce the same potentiation of inotropy and frequency or
2. at the same isoprenaline concentrations added as before, the increase in inotropy and frequency is reduced.

At the end of the experiment, again a cycle without test drug is performed.

Left Atrium

The left atrium is stimulated by a square wave stimulator with 2 impulses/s at a voltage of 15 V and an impulse duration of 1 ms. After an equilibration period of 30 min, the β-agonist isoprenaline is added at concentrations of 0.05–0.1 mg/ml. The organ bath is then thoroughly flushed for 1 min. Flushing is repeated twice 5 and 20 min later. The whole procedure is repeated with the same cumulative isoprenaline concentrations (control baseline values = 100%) and flushing procedure.

When a stable plateau of contractions is achieved, the test compound is added into the organ bath and 3 min later, isoprenaline is added again at cumulative concentrations.

If the test compound has β-receptor blocking activity (β-sympatholytic), the isoprenaline-induced effects are inhibited.

In addition, refractory period is determined before and after drug administration.

EVALUATION

a) Percent inhibition of (a) isoprenaline-induced or (b) electrically-induced and isoprenaline-potentiated increased inotropy and frequency by test drug is calculated as compared to pre-drug activity (= 100%).
b) Percent change in refractory period is calculated.

IC_{50} values are determined from the individual dose-response curves.

Statistical evaluation is performed by means of the paired *t*-test.

Standard compounds:

- propranolol HCl,
- amrinon,
- nifedipine,
- and milrinone.

MODIFICATIONS OF THE METHOD

A detailed description of the use of isolated atrial preparations has been given by Levy (1971).

Instead of the right atrium, Doggrell and Hughes (1986) used the isolated right ventricle of the rat for the assessment of the β-adrenoreceptor blocking activity of propranolol and investigated the competitive nature of the isoproterenol antagonism at various doses with Schild-plot analysis. Doggrell (1988) used the isolated left atria of the rat for simultaneous assessment of membrane-stabilizing and β-adrenoreceptor blocking activity.

Berthold et al. (1990) described a method for testing cardiotonic sodium channel activators in isolated, electrically stimulated left guinea pig atria after potassium depolarization.

Olson et al. (1995) studied the function of isolated rat left atria and papillary muscles and quantified the voltage-response relationship between punctate and field electrical stimulation after pretreatment with reserpine or β-blockers.

REFERENCES AND FURTHER READING

Berthold H, Scholtysik G, Schaad A (1990) Identification of cardiotonic sodium channel activators by potassium depolarization in isolated guinea pig atria. J Pharmacol Meth 24:121–135

Doggrell DH (1988) Simultaneous assessment of membrane-stabilizing and β-adrenoreceptor blocking activity of drugs with the rat isolated left atria. J Pharmacol Meth 19:93–107

Doggrell S, Hughes EW (1986) On the assessment of the β-adrenoreceptor blocking activity of propranolol using the rat isolated right ventricle. J Pharmacol Meth 15:119–131

Furchgott RF (1967) Techniques for studying antagonism and potentiation of sympathomimetic drugs an isolated tissues. In: Siegler PE, Moyer JH (eds) Animal and clinical phar-

macologic techniques in drug evaluation. pp 256–266. Year Book Medical Publishers, Inc., Chicago
Grodzinska L, Gryglewski R (1971) Action of beta-adrenolytics on the isolated guinea pig atria. Arch Int Pharmacodyn 191:133–141
Grupp IL, Grupp G (1984) Isolated heart preparations perfused or superfused with balanced salt solutions. In: Schwartz A (ed) Methods in Pharmacology, Vol 5: Myocardial Biology. pp 111–128. Plenum Press, New York London
Levy JV (1971) Isolated atrial preparations. In: Schwartz A (ed) Methods in Pharmacology Vol 1, pp 77–104. Appleton-Century-Crofts, Meredith Corporation, New York
Olson RD, Vestal RE, Mednhall WA, Mudumbi RV (1995) Quantification of the voltage-response relationship between punctate and field electrical stimulation and the function of isolated rat left atria and papillary muscles. J Pharmacol Toxicol Meth 34:225–230

A.1.2.7 β_2-Sympatholytic Activity in the Isolated Tracheal Chain

PURPOSE AND RATIONALE

Contraction of bronchial smooth muscle is induced by the cholinergic agonist carbachol. The carbachol effect can be antagonized by the β-agonist isoprenaline (isoproterenol). A compound has β-sympatholytic activity if the spasmolytic action of isoprenaline is inhibited. The β-sympatholytic effect of drugs can be evaluated in an *in vitro* model. Since the trachea contains predominantly β_2-adrenoreceptors, β_2-blocking activity can be assessed by this test.

PROCEDURE

Male Pirbright White guinea pigs weighing 250–300 g are used. The animals are sacrificed by stunning and exsanguination. The trachea is removed and cut into individual rings. Six rings are connected in series by means of short loops of silk thread. The tracheal chain is mounted in an 50 ml organ bath with a preload of 1 g for isotonic registration. To the nutritive solution (Tyrode) containing ascorbic acid and 1.0 g/L glucose, the α-receptor blocking agent phentolamine (0.1 µg/ml) and the spasmogen carbachol (80 ng/ml) are added. The solution is maintained at 34°C and aerated with 95% O_2, 5% CO_2

Experimental Course

After an equilibration period of 30 min, cumulative doses of 10^{-10} to 10^{-7} M of the spasmolytic agent isoprenaline are added. When maximal relaxation is obtained, the organ bath is flushed and the procedure repeated. After the two control relaxations with isoprenaline, the tissue is rinsed thoroughly and the first dose of the test compound is administered. Three min later, cumulative doses of isoprenaline are administered as before. Following a 10 min washout and recovery period, the next dose of the test compound is given. Up to 10 drug concentrations can be tested with one organ.

Standard compounds:

- propranolol
- practolol

EVALUATION

Percent inhibition of isoprenaline-induced relaxation under drug treatment is calculated compared to maximal relaxation induced by isoprenaline alone (control = 100%).

A competitive antagonism of test compound is evaluated and can be quantitated from the dose-response curve.

MODIFICATIONS OF THE METHOD

O'Donnell and Wanstall (1980) used guinea pig tracheal preparations, where K^+-depolarization was achieved by replacing all the Na^+ in Krebs solution by an equivalent amount of K^+ causing a sustained contraction of the preparations. A dose-dependent relaxation effect of isoprenaline could be obtained provided that the preparations were repolarized by washing in normal Krebs solution between curves. pA_2 values were in good agreement with values obtained in other types of tracheal preparations.

Guinea pig superfused trachea and dispersed tracheal cells have been used by Buckner et al. (1995) to compare the effects of isoproterenol and forskolin on immunologic and nonimmunologic histamine release.

Lundblad and Persson (1988) found that epithelial removal is of little consequence for the pharmacology of the guinea pig tracheal open ring preparation *in vitro*.

The rat portal vein has been recommended as model for assessment of β_2 adrenoceptor blocking activity of drugs by Doggrell (1990).

REFERENCES AND FURTHER READING

Buckner CK, Fishleder RI, Conklin R, Graziano FM (1995) A comparison of the effects of isoproterenol and forskolin on immunologic and nonimmunologic release of histamine from guinea-pig superfused trachea and dispersed tracheal cells. J Pharmacol Toxicol Meth 33:47–52
Castillo JC, de Beer EJ (1947). The tracheal chain. I. A preparation for the study of antispasmodics with particular reference to bronchodilator drugs. J Pharmacol Exp Ther 90:104–109
Doggrell SA (1990) Assessment of the β_2 adrenoceptor and Ca^{2+} channel-blocking activity of drugs with the rat portal vein. J Pharmacol Meth 24:145–156

Foster RW (1965) The nature of the adrenergic receptors of the trachea of the guinea-pig. J Pharm Pharmac 18:1–12
Green AF, Boura ALA (1964) Sympathetic nerve blockade. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press, London and New York, pp 370–430
Longmore J, Miller M, Trezise DJ, Weston AL (1991) Further studies on the mechanism of action of isoprenaline in bovine tracheal smooth muscle. Br J Pharmacol 102:Proc Suppl 182P
Lundblad KA, Persson CG (1988) The epithelium and the pharmacology of guinea-pig tracheal tone *in vitro*. Br J Pharmacol 93:909–917
O'Donnell SR, Wanstall JC (1980) The use of guinea pig K^+-depolarized tracheal chain preparations in β-adrenoreceptor studies. J Pharmacol Meth 4:43–50
Trendelenburg P (1912) Physiologische und pharmakologische Untersuchungen an der isolierten Bronchialmuskulatur. Naunyn-Schmiedeberg's Arch Exp Pathol Pharmakol 69:79–107
Van Rossum JM (1963) Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch Int Pharmacodyn 143:299–300
Waldeck B, Widmark E (1985) Comparison of the effects of forskolin and isoprenaline on tracheal, cardiac and skeletal muscle from guinea-pig. Eur J Pharmacol 112:349–353

A.1.2.8 Angiotensin Converting Enzyme Inhibition in the Isolated Guinea Pig Ileum

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the formation of the active angiotensin II from the inactive angiotensin I. The same enzyme is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and inhibition of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The guinea pig ileum contracts in response to both peptides, angiotensin II and bradykinin, and can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine which is then severed above the cord. The intestine is gradually removed, and the mesentery is being cut away as necessary. When the colon is reached, the intestine is cut free. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 3 cm length are cut. Preferably, the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into an organ bath with Tyrode's solution at 37°C being oxygenated with O_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). Responses are recorded on a polygraph.

Angiotensin I Antagonism

After an equilibrium time of 30 min, angiotensin I is added in a concentration of 10 ng/ml bath solution. The force of contraction is recorded and the angiotensin I dosage is repeated once or twice until the responses are identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again angiotensin I is added. The contraction is diminished depending on the activity of the ACE inhibitor.

Bradykinin Potentiation

Pieces of guinea pig ileum are prepared as described before. After an equilibrium time of 30 min bradykinin is added in a concentration of 15 ng/ml bath solution. The force of contraction is recorded and bradykinin additions are repeated once or twice until the response is identical. Then the potential ACE inhibitor is added. After 5 min incubation time, again bradykinin is added. The contraction is potentiated depending on the activity of the ACE inhibitor.

EVALUATION

Angiotensin I Antagonism

The contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. Using various doses of the ACE inhibitor IC_{50} values (concentrations inducing 50% inhibition) are calculated. As standards ramipril, enalapril, and captopril are used.

Bradykinin Potentiation

The increase of the contraction after addition of the ACE inhibitor is expressed as percentage of contraction without the ACE inhibitor. As standard ramiprilate is used.

CRITICAL ASSESSMENT OF THE METHOD

The classical method of the isolated guinea pig ileum has been proven to be a reliable method for screening of potential ACE inhibitors.

REFERENCES AND FURTHER READING

Rubin B, Laffan RJ, Kotler DG, O'Keefe EH, Demaio DA, Goldberg ME (1978) SQ 14,225 (D-3-mercapto-2-methylpropanoyl-L-proline), a novel orally active inhibitor of angiotensin I-converting enzyme. J Pharmacol Exp Ther 204:271–280

A.1.2.9 Contractile and Relaxing Activity on Isolated Blood Vessels Including Effects of Potassium Channel Openers

PURPOSE AND RATIONALE

The contractile process within the vascular smooth muscle results from an increase in the concentration of intracellular Ca^{2+}. Inhibition of vasoconstriction occurs by addition of calcium antagonists or by removal of extracellular calcium. The vasorelaxing effects of compounds can be tested in isolated rodent arteries (pulmonary artery, thoracic aorta). Arterial rings or strips with or without endothelial lining are contracted with different agents, e. g., extracellular K^+ and Ca^{2+}, the α-adrenoceptor agonists phenylephrine and noradrenaline, the Ca^{2+} ionophore A23187 or the thromboxane receptor agonist U46619. Compounds with vasodilating activity antagonize the induced contractions.

Potassium channel openers such as cromakalin, nicorandil, pinacidil or HOE 234 induce relaxation of contracted smooth musculature (Bolton et al. 1998). These effects are explained by data from patch clamp technique and ion flux experiments as well as by antagonism against potassium channel blockers. They indicate the potential use as antihypertensive and anti-asthmatic drugs (Hamilton and Weston 1989; Edwards and Weston 1990, 1993; Weston and Edwards 1992). The studies are complicated by the high diversity of potassium channels including ATP-sensitive, voltage-sensitive and Ca^{2+}-activated channels (Mourre et al. 1986; Blatz and Magleby 1987; Ashcroft and Ashcroft 1990; Jan and Jab 1990; Pongs 1992; Wann 1993). Since each functional channel appears to consist of four different subunits, the possibility exists that there may be hundreds of different voltage-sensitive K channels, depending on their subunit composition. Ashcroft and Gribble (2000) discussed new windows on the mechanism of action of K_{ATP} channel openers.

Glibenclamide is an antagonist of the ATP-modulated K^+ channel allowing the localization of the binding sites (Eltze 1989; French et al. 1990; Mourre et al. 1990; Miller et al. 1991).

PROCEDURE

Male Pirbright White guinea pigs weighing about 400 g, or Chinchilla rabbits weighing about 3.5 kg, or Sprague-Dawley rats weighing 250–400 g are used as donor animals. The tested vessels are the thoracic aorta or the arteria pulmonalis.

Materials and solutions

Physiological salt solutions (PSS) [mM]			
	PSS I	PSS II	PSS III
NaCl	122	112	72 (92)
KCl	5.0	5	40 (20)
$CaCl_2$	1.2	–	–
$MgSO_4$	0.56	0.56	0.56
KH_2PO_4	1.2	1.2	1.2
$NaHCO_3$	25	25	25
EDTA	–	0.2	–
glucose	12	12	12

Contracting agents	
$K^+ + Ca^{2+}$	40 mM + 0.5 mM
$K^+ + Ca^{2+}$	20 mM + 0.5 mM
U 46619 (thromboxane A_2 analogue)	1 µM
A 23187 (calcium ionophore)	5 µM
noradrenaline	1 µM
phenylephrine	0.1 µM
acetylcholine	1 µM
oxyhemoglobin	10 µM
methylene blue	10 µM

Animals are sacrificed by stunning and exsanguination. At least 4 isolated organs are tested per drug. The heart and the pulmonary artery are quickly removed and immersed in PSS I at room temperature. The artery is dissected into rings and endothelial cells are removed by gently rubbing the intimal surface. Spirally cut strips of 15–20 mm length and 1–1.5 mm width are suspended at a resting force of 380 mg in an organ bath containing 20 ml oxygenated (95% O_2, 5% CO_2) PSS I at 37°C. Changes in length are recorded isotonically using a lever transducer (368 type B, Hugo Sachs Elektronic, Freiburg).

To test the effect of compounds on vessels with intact endothelial lining, the thoracic aorta of rats is isolated and dissected free from surrounding tissue. Rings of 3 mm width are cut and suspended in the organ bath containing PSS I. Isometric force is measured with a force transducer (UC-2, Gould-Statham, Oxnard, USA) under a resting tension of 500 mg.

The functional integrity of the endothelium is tested before drug administration. One µM acetylcholine in the organ bath should result in a transient relaxation.

After an equilibration period of 1 h, contraction of each vessel strip or ring is induced by addition of one of the contracting agents into the organ bath.

To induce contractions of potassium-depolarized vessels, three different PSS solutions are used (PSS I for 30 min, PSS II for 3×15 min and PSS III). Contraction is induced in the presence of PSS III by adding 0.5 mM Ca^{2+} into the organ bath.

When a stable plateau of contraction is achieved, cumulative concentrations of the test compound are added into the organ bath to obtain drug-response curves. Consecutive concentrations are added either at 1 h-intervals or when the response of the previous dose has reached a steady state level.

In order to study the time course of relaxation and the duration of action, only one concentration is tested.

To test whether the mechanism of action of a vasorelaxing agent is related to the liberation of nitric oxide, methylene blue or oxyhemoglobin (10 μM) are added to the organ bath 15–30 min prior to the cumulative administration of the test compound. Methylene blue or oxyhemoglobin block selectively NO induced relaxation.

EVALUATION

Mean values of relaxation ± SEM are calculated. The height of contraction before the first drug administration is taken as 100%.

IC^{50} values are determined from the individual dose-response curves. IC^{50} is defined as the dose of drug leading to a 50% relaxation of the contraction induced by KCl or other agonists.

Statistical evaluation is performed by means of the *t*-test.

MODIFICATIONS OF THE METHOD

Calderone et al. (1996) compared four **rat** aortic preparation (single ring, spiral strip, zig-zag strip, and multiple ring) on the basis of responses to noradrenaline and acetylcholine. They recommended the multiple ring preparation as the most suitable of all four for the study of vasoactive drugs because of the reproducibility of both contracturant and relaxing responses.

Kent et al. (1982) used rat aortic strips contracted to a stable tension by either phenylephrine or barium chloride for comparison of vasodilators.

Wilson et al. (1988) studied in isolated rings of rat aorta precontracted with noradrenaline the antagonism of glibenclamide against the vasorelaxation induced by cromakalin.

Löhn et al. (2002) cannulated cerebral arteries from mice with glass cannulas on both sides, allowing an application of hydrostatic pressure to the vessel. Diameter was measured by using a videomicroscopic system (Nikon Diaphot, Düsseldorf, Germany) connected to a personal computer with appropriate software for detection of changes of vessel diameter (TSE, Bad Homburg, Germany).

Nishimura and Suzuki (1995) tested the contractile responses to 5-HT in basilar arteries, superior mesenteric arteries and thoracic aortas from **stroke-prone spontaneously hypertensive rats** in comparison to normal Wistar–Kyoto rats and found that that the hyperresponsiveness to 5-HT is mediated by different 5-HT receptor subtypes.

Fouda et al. (1991) used **the isolated tail arteries from rats**. Differences of the vasoconstrictor response to potassium and norepinephrine between tail arteries from spontaneously hypertensive, renovascular hypertensive, and various strains of normotensive rats were found.

Hamilton et al. (1986), Dacquet et al. (1987) studied the effects of calcium entry blockers in **rat portal vein**.

Bråtveit and Helle (1984) studied the inhibition of vascular smooth muscle by vasoactive intestinal peptide (VIP) in the isolated rat portal vein.

Shetty and Weiss (1987) studied the inhibition of spontaneous rhythmic movements and norepinephrine-induced tension responses in the rat portal vein.

Edwards et al. (1991) compared the effects of several potassium-channel openers on rat bladder and rat portal vein *in vitro*.

Smith et al. (1993) tested the ability of C-terminally truncated fragments of human α-calcitonin gene-related peptide to relax **mesenteric arteries** precontracted with norepinephrine

Chen et al. (1996) studied the contractile effects of noradrenaline and neuropeptide Y given alone or in combination on isolated rat mesenteric resistance vessels.

Gurden et al. (1993) used **guinea pig** aorta relaxation for functional characterization of adenosine receptor types.

Eltze (1989) studied the antagonism of glibenclamide against potassium channel openers in the isolated **guinea pig pulmonary artery**.

Szentmiklósi et al. (1995) used circular segments from the proximal part of the main pulmonary artery of guinea pigs to study contractile and relaxant effects of adenosine receptors.

Pikkers and Hughes (1995) examined the effect of hydrochlorothiazide on intracellular calcium concentration $[Ca^{2+}]_i$ and tone in **guinea pig mesenteric arteries**. Vessels were mounted on a microvascular myograph and loaded with the Ca^{2+}-sensitive fluorescent dye, Fura-2.

Nishimura et al. (1998) used isolated aorta rings from **Syrian hamsters**. Contractile responses were recorded with an isometric transducer (TSE, Bad Homburg, Germany) and stored (TSE Data acquisition software).

Meisheri et al. (1990) recommended the use of the isolated **rabbit mesentery artery** as a sensitive *in vitro* functional assay to detect K^+-channel-dependent vasodilators.

Mironneau and Gargouil (1979) studied the influence on electrophysiological and mechanical parameters of longitudinal smooth muscle strips isolated from **rabbit portal vein** by means of a double sucrose gap method associated with a photoelectric device for recording contractions.

Lauth et al. (2001) performed superfusion assays with **rabbit jugular vein**. Four venous ring segments (3–4 mm long) were tested simultaneously by mounting them between force transducers and a rigid support for measurement of isometric force. Increasing doses of bradykinin were applied as bolus injections and the ensuing constrictor response was monitored with the aid of a PC-operated analysis System (Biosys, TSE, Bad Homburg, Germany).

McBean et al. (1986, 1988) used isolated segments of the arteria basilaris of **pigs** to detect compounds with antivasoconstrictive properties. Contraction is elicited by $PGF_{2\alpha}$, serotonin or norepinephrine. Specimens are obtained from adult pigs (strain: Deutsche Landrasse) within 30 min after slaughter from the local slaughter house and stored in nutritive solution. The vessels are trimmed to a length of 4 mm, and the segments are suspended between 2 L-shaped metal hooks in a bath containing 20 ml modified Krebs Henseleit solution (NaCl 148 mM, KCl 5.4 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 12 mM, glucose 12 mM). The bath solution is maintained at 37°C and continuously gassed with carbogen to produce a resulting pH of 7.35–7.45. The preparation is incubated under a tension of 37.28 mN (optimal passive load producing the largest contractile response to 3×10^{-6} M $PGF_{2\alpha}$). Following a 60 minute stabilization period, the vessels are sensitized with 30 mM KCl for 10 min. The vessels are washed for 1 minute, and allowed to recover for 30 min with additional 1 minute washes at 15 and 30 min. Thereafter, contractions of the vessels are induced by adding $PGF_{2\alpha}$ at 3×10^{-6} M. The contractile response is allowed to plateau, then the test compound is administered at cumulative doses.

For each test compound a dose-response curve is recorded. The EC_{50} is obtained graphically or by means of a Hill plot. The EC_{50} is defined as the dose of drug producing half maximal response.

Werner et al. (1991) studied the vascular selectivity of calcium antagonists using **porcine** isolated ventricular trabeculae and right **coronary arteries**.

Merkel et al. (1992) used isolated porcine coronary artery rings precontracted with prostaglandin $F_{2\alpha}$ to demonstrate the vasorelaxant activity of an A_1-selective adenosine agonist.

Miwa et al. (1993) compared the effect of a K^+-channel opener with cromakalim, nitroglycerin and nifedipine on endothelin-1-induced contraction of porcine coronary artery.

Satoh et al. (1993) investigated in isolated porcine large coronary arteries whether or not the vasorelaxant actions of nicorandil and cromakalim would be selective using seven different vasoconstrictor agonists.

Yokoyama et al. (1994) studied the vasodilating mechanisms of several pyridinecarboximidamide derivatives in isolated porcine coronary arteries.

Makujina et al. (1995) described a procedure that facilitates the eversion of vascular smooth muscle. Vascular segments of porcine coronary artery, approximately 2 cm in length, were sutured to portions of polyethylene tubing inserted into the lumen of the vessel. After being secured and stabilized by the tubing, the vessel was everted while immersed in physiological buffer. Intracellular calcium concentrations (measured by fura-2AM fluorometry) and tension were registered simultaneously in everted rings denuded of endothelium.

Izumi et al. (1996) tested a K^+-channel opener and related compounds in isolated porcine coronary arteries contracted with 25 mM KCl.

Frøbert et al. (1996) described impedance planimetry as a new catheter-based technique to measure porcine coronary artery pharmacodynamics and compared the results with the commonly used wire-mounted isometric tension technique after *in vitro* application of nifedipine in various concentrations. A four-electrode impedance measuring system was located inside a 12-mm long balloon which was introduced into 3–4 cm long segments of the left anterior descending coronary artery obtained from 70–90-kg Danish Landrace-Yorkshire pigs.

Hamel et al. (1993) dissected segments (3–4 mm long) of temporal ramifications of the middle cerebral

artery from **bovine** brains and mounted them between two L-shaped metal prongs in a tissue bath containing Krebs-Ringer solution at 37°C. Changes in muscle tension were measured by a force displacement transducer and recorded on a polygraph. Several 5-HT receptor agonists were tested for their ability to induce vasoconstriction and their potencies were compared to that of 5-HT. The authors concluded that bovine pial arteries appear to be the best available model for the human cerebrovascular 5-HT_{1D} receptor.

De la Lande et al. (1996) used isolated segments from proximal (4.5 mm i.d.) and distal (0.5 mm i.d.) bovine coronary arteries and found a heterogeneity of response to glyceryl trinitrate.

The isolated **human** coronary artery was used to study the vasoconstriction by acutely acting antimigraine drugs (Saxena et al. 1996a, b, 1997)

REFERENCES AND FURTHER READING

Ashcroft JH, Ashcroft FM (1990) Properties and functions of ATP-sensitive K-channels. Cell Signal 2:197–214

Ashcroft FM, Gribble FM (2000) New windows on the mechanism of action of K_{ATP} channel openers. Trends Pharmacol Sci 21:439–445

Barhanin J, Duprat F, Fink M, Guillemare E, Heurteaux C, Honoré E, Lesage F, Patel A, Reyes R, Romey G, Lazdunski M (1998) Novel structural and functional types of K^+ channels. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1, R 23

Blatz AL, Magleby KL (1987) Calcium-activated potassium channels. Trends Neurosci 10:463–467

Bolton TB, Prestwich SA, Zhang HL (1998) The target channel for potassium channel opener drugs in vasodilatation. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1, R 200

Bråtveit M, Helle KB (1984) VIP inhibition of vascular smooth muscle: complementary to β_2-adrenoceptor mediated relaxation in the isolated rat portal vein. Acta Physiol Scand 121:269–276

Calderone V, Martinotti E, Scatizzi R, Pelegrini A, Breschi MC (1996) A modified aortic multiple-ring preparation for functional studies. J Pharmacol Toxicol Meth 35:131–138

Cheng H, Fetscher C, Schäfers RF, Wambach G, Philipp Th, Michel MC (1996) Effects of noradrenaline and neuropeptide Y on rat mesenteric microvessel contraction. Naunyn-Schmiedeberg's Arch Pharmacol 353:314–323

Cook NS (1988) The pharmacology of potassium channels and their therapeutic potential. TIPS 9:21–28

Dacquet C, Mironneau C, Mironneau J (1987) Effects of calcium entry blockers on calcium-dependent contractions of rat portal vein. Br J Pharmac 92:203–211

De la Lande IS, Stafford I, Horowitz JD (1996) Heterogeneity of glyceryl trinitrate response in isolated bovine coronary arteries. Eur J Pharmacol 318:65–71

Edwards G, Weston AH (1990) Potassium channel openers and vascular smooth muscle relaxation. Pharmac Ther 48:237–258

Edwards G, Henshaw M, Miller M, Weston AH (1991) Comparison of the effects of several potassium-channel openers on rat bladder and rat portal vein in vitro. Br J Pharmacol 102:679–680

Edwards G, Weston AH (1993) The pharmacology of ATP-sensitive potassium channels. Annu Rev Pharmacol Toxicol 33:597–637

Eltze M (1989) Glibenclamide is a competitive antagonist of cromakalim, pinacidil and RP 49356 in guinea-pig pulmonary artery. Eur J Pharmac 165:231–239

Fouda AK, Capdeville C, Henrion D, Thorin-Trescases N, Thorin E, Atkinson J (1991) Differences between the *in vitro* vasoconstrictor responses of the tail artery to potassium and norepinephrine between spontaneously hypertensive, renovascular hypertensive, and various strains of normotensive rats. J Pharmacol Meth 25:61–68

French JF, Riera LC, Sarmiento JG (1990) Identification of high and low (GTP-sensitive) affinity [^{3}H]glibenclamide binding sites in cardiac ventricular membranes. Biochem Biophys Res Comm 167:1400–1405

Frøbert O, Mikkelsen EO, Gregersen H, Nyborg NBC, Bagger JP (1996) Porcine coronary artery pharmacodynamics *in vitro* evaluated by a new intravascular technique: relation to axial stretch. J Pharmacol Toxicol Meth 36:13–19

Gurden MF, Coates J, Ellis F et al (1993) Functional characteristics of three adenosine receptor types. Br J Pharmacol 109:693–698

Hamel E, Grégoire L, Lau B (1993) 5-HT_1 receptors mediating contractions in bovine cerebral arteries: a model for human cerebrovascular '5-$HT_{1D\beta}$' receptors. Eur J Pharmacol 242:75–82

Hamilton TC, Weston AH (1989) Cromakalim, nicorandil and pinacidil: novel drugs which open potassium channels in smooth muscle. Gen Pharmacol 20:1–9

Hamilton TC, Weir SW, Weston AH (1986) Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. Br J Pharmac 88:103–111

Izumi H, Tanaka Y, Okada N, Izawa T (1996) Structure-activity relationship of a novel K^+-channel opener and related compounds in porcine coronary artery. Gen Pharmacol 27:985–989

Jan LY, Jan YN (1990) How might the diversity of potassium channels be generated? Trends Neurosci 13:415–419

Kent RL, Harakal C, Santamore WP, Carey RA, Bove AA (1982) An index for comparing the inhibitory action of vasodilators. Eur J Pharmac 85:85–91

Kurachi Y (1998) Molecular pharmacology of potassium channels. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1, R 6

Langer SZ, Trendelenburg U (1969) The effect of a saturable uptake mechanism on the slopes of dose-response curves for sympathomimetic amines and on the shifts of dose-response curves produced by a competitive antagonist. J Pharmacol. Exp Ther 167:117–142

Lauth M, Cattaruzza M, Hecker M (2001) ACE inhibitor and AT_1 antagonist blockade of deformation-induced gene expression in the rabbit jugular vein through B_2 receptor activation. Arterioscler Thromb Vasc Biol 21:61–66

Löhn M, Kämpf D, Gui-Xuan C, Haller H, Luft FC, Goll Nishimura H, Buikema H, Baltatu O, Ganten D, Urata H (1998) Regulation of arterial tone by smooth muscle myosin type II. Am J Physiol 283:C1383–C1389

Makujina SR, Abebe W, Ali S, Mustafa SJ (1995) Simultaneous measurement of intracellular calcium and tension in vascular smooth muscle: validation of the everted ring preparation. J Pharmacol Toxicol Meth 34:157–163

Martin W, Villani GM, Jothianandan D, Furchgott RF (1985) Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by

methylene blue in the rabbit aorta. J Pharmacol Exp Ther 232:708–716
McBean DE, Harper AM, Rudolphi KA (1986) Effects of adenosine and its analogues on the cerebrovasculature and their antagonism by 8-phenyltheophylline: Identification of the receptor(s) involved. Pfluegers Arch 407 Suppl. 1:31
McBean DE, Harper AM, Rudolphi KA (1988) Effects of adenosine and its analogues on porcine basilar arteries: Are only A_2 receptors involved? J Cerebr Blood Flow Metab 8:40–45
Meisheri KD, Dubray LAC, Olynek JJ (1990) A sensitive *in vitro* functional assay to detect K^+-channel-dependent vasodilators. J Pharm Meth 24:251–261
Merkel LA, Lappe RW, Rivera LM, Cox BF, Perrone MH (1992) Demonstration of vasorelaxant activity with an A_1-selective adenosine agonist in porcine coronary artery: Involvement of potassium channels. J Pharmacol Exp Ther 260:437–443
Miller JA, Velayo NL, Dage RC, Rampe D (1991) High affinity [^{3}H]glibenclamide binding sites in rat neuronal and cardiac tissue: localization and development characteristics. J Pharm Exp Ther 256:358–364
Mironneau J, Gargouil YM (1979) Action of indapamide on excitation-contraction coupling in vascular smooth muscle. Eur J Pharmacol 57:57–67
Miwa A, Kasai H, Motoki K, Jinno Y, Yokoyama T, Fukushima H, Ogawa N (1993) Effect of KRN2391, a novel vasodilator, on endothelin-1-induced contraction of porcine coronary artery. Comparison with cromakalim, nitroglycerin and nifedipine. Arch Int Pharmacodyn Ther 326:52–61
Mourre C, Hugues M, Lazdunski M (1986) Quantitative autoradiographic mapping in rat brain of the receptor of apamin, a polypeptide toxin specific for one class of Ca^{2+}-dependent K^+ channels. Brain Res 382:239–249
Mourre C, Widman C, Lazdunski M (1990) Sulfonylurea binding sites associated with ATP-regulated K^+ channels in the central nervous system: autoradiographic analysis of their distribution and ontogenesis, and their localization in mutant mice cerebellum. Brain Res 519:29–43
Nishimura H, Buikema H, Baltatu O, Ganten D, Urata H (1998) Functíonal evidence for alternative ANG II-forming pathways in hamster cardiovascular system. Am J Physiol 275:H1307–H1312
Nishimura Y, Suzuki A (1995) Enhanced contractile responses mediated by different 5-HT receptor subtypes in basilar arteries, superior mesenteric arteries and thoracic aortas from stroke-prone spontaneously hypertensive rats. Clin Exper Pharmacol Physiol Suppl 1:S99–S101
O'Donnell SR, Wanstall JC (1987) Choice and concentration of contractile agent influence responses of rat aorta to vascular relaxant drugs. J Pharm Pharmacol 39:848–850
Pikkers P, Hughes AD (1995) Relaxation and decrease in $[Ca^{2+}]_i$ by hydrochlorothiazide in guinea pig isolated mesenteric arteries. Br J Pharmacol 114:703–707
Pongs O (1992) Structural basis of voltage-gated K^+ channel pharmacology. TIPS 13:359–365
Rehm H, Lazdunski M (1988) Purification and subunit structure of a putative K^+-channel protein identified by its binding properties for dendrotoxin I. Proc Natl Acad Sci USA 85:4919–4923
Satoh K, Mori T, Yamada H, Taira N (1993) Nicorandil as a nitrate, and cromakalim as a potassium channel opener, dilate isolated porcine large coronary arteries in an agonist-nonselective manner. Cardiovasc Drugs Ther 7:691–699
Saxena PR, Maassen van den Brink A, Heiligers JPC, Scalbert E, Guardiola-Lemaitire B (1996a) Effects of S20794, a close analogues of sumatripan, on porcine carotid haemodynamics and human isolated coronary artery. Pharmacol Toxicol 79:199–204
Saxena PR, De Vries P, Heiligers JPC, Maassen van den Brink A, Bax WA, Barf T, Wikström H (1996b) Investigations with GMC2021 in experimental models predictive of antimigraine activity and coronary side-effect potential. Eur J Pharmacol 312:53–62
Saxena PR, De Vries P, Wang W, Heiligers JPC, Maassen van den Brink A, Bax WA, Yocca FD (1997) Effects of avitriptan, a new 5-$HT_{1B/1D}$ receptor agonist, in experimental models predictive of antimigraine activity and coronary side-effect potential. Naunyn-Schmiedeberg's Arch Pharmacol 355:295–302
Scherf H, Pietsch R, Landsberg G, Kramer HJ, Düsing R (1986) Converting enzyme inhibitor ramipril stimulates prostacyclin synthesis by isolated rat aorta: evidence for a kinin-dependent mechanism. Klin Wschr 64:742–745
Shetty SS, Weiss GB (1987) Dissociation of actions of BRL 34915 in the rat portal vein. Eur J Pharmacol 141:485–488
Smith DD, Li J, Wang Q, Murphy RF, Adrian TE, Elias Y, Bockman CS, Abel PW (1993) Synthesis and biological activity of C-terminally truncated fragments of human α-calcitonin gene-related peptide. J Med Chem 36:2536–2541
Szentmiklósi AJ, Ujifalusi A, Cseppentö A, Nosztray K, Kovács P, Szabó JZ (1995) Adenosine receptors mediate both contractile and relaxant effects of adenosine in main pulmonary artery of guinea pigs. Naunyn-Schmiedeberg's Arch Pharmacol 351:417–425
Wann KT (1993) Neuronal sodium and potassium channels: structure and function. Br J Anaesth 71:2–14
Werner G, Klaus W, Kojda G, Fricke U (1991) Hydrophobic properties of novel dihydronaphthyridine calcium antagonists and biological activity in porcine isolated cardiac and vascular smooth muscle. Naunyn-Schmiedeberg's Arch Pharmacol 344:337–344
Weston AH, Edwards G (1992) Recent progress in potassium channel opener pharmacology. Biochem Pharmacol 43:47–54
Wilson C, Buckingham RE, Mootoo S, Parrott LS, Hamilton TC, Pratt SC, Cawthorne MA (1988) *In vivo* and *in vitro* studies of cromakalim (BRL 34915) and glibenclamide in the rat. Br J Pharmacol 93:126P
Yokoyama T, Okada Y, Jinno Y, Izumi H, Izawa T, Ogawa N (1994) Comparative analysis of vasodilating mechanisms of Ki1769, Ki3315 and KRN2391, pyridinecarboximidamide derivatives, in porcine isolated coronary artery. Gen Pharmacol 25:941–945

A.1.2.10 Isolated Guinea Pig Ureter

PURPOSE AND RATIONALE

The isolated guinea pig ureter shows phasic-rhythmic contractions after addition of KCl to the organ bath. Inhibition of this effect can be explained as a modulation of potassium channels.

PROCEDURE

Male unfasted guinea pigs weighing 400–500 g are sacrificed and both ureters removed immediately without the part directly connected to the pelvis in order to exclude the pacemaker region responsible for spontaneous activity. Each segment of 2 cm length is

placed in a Petri dish containing Tyrode solution at 37°C, freed of surrounding connective tissue and then suspended at a baseline tension of 0.5 p in a 25 ml organ bath containing Tyrode solution at 37°C being aerated with 5% CO_2/95% O_2, pH 7.4. Contractions are measured isometrically using Gould/Statham UC 2 transducers. After a 15 min equilibration period, KCl is added to the bath in a final concentration of 3×10^{-2} Mol/l and left in the bath for 2 min. KCl induces a constant series of phasic-rhythmic contractions without a rise in baseline tone. Subsequent washing causes the immediate disappearance of the rhythmic contractions. This addition of KCl is repeated and the values of these two experiments are used as initial values. The antagonistic activity is studied by addition of the test drug one min prior to the KCl challenge. Percentage of the following parameters are determined: mean height of contractions, frequency of contractions and the product of mean height and frequency of contractions. For interaction studies the potassium channel blocker glibenclamide 10^{-6} mol/l is added 1 min prior to the test drug.

EVALUATION

Arithmetic means and standard deviations of the data are calculated and compared with initial values using Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The isolated guinea pig ureter stimulated with KCl can be used for studies on the modulation of potassium channels.

MODIFICATIONS OF THE METHOD

Yoshida and Kuga (1980) recorded electrical activities in a preparation consisting of the pelvic region and the upper ureter of the guinea pig. Train field stimulation of the pelvic region evoked a train of nerve action potentials followed by a multiphasic smooth muscle action potential after a latency of about 2.5–8.0 s. This smooth muscle response was abolished by tetrodotoxin and dibucaine, and also by cholinergic blocking agents.

The effects of veratridine and of yohimbine on the efflux of norepinephrine from electrically stimulated guinea pig ureters were studied by Kalsner (1992).

Maggi and Giuliani (1994) studied the excitability and refractory period of the guinea pig ureter to electrical field stimulation.

Roza and Laird (1995), Laird and Cervero (1996) studied the pressor responses to distension of the ureter in anesthetized rats as a model of acute visceral pain.

A simple method for measurement of ureteric peristaltic function *in vivo* in anesthetized rats was published by Kontani et al. (1993).

REFERENCES AND FURTHER READING

Kalsner S (1992) Adrenergic presynaptic antagonists and their mechanism of action in smooth muscle. Am J Physiol 262 (Regul Intergr Comp Physiol 31):R400–R406

Kontani H, Ginkawa M, Sakai T (1993) A simple method for measurement of ureteric peristaltic function *in vivo* and the effects of drugs acting on ion channels applied from the ureter lumen in anesthetized rats. Jap J Pharmacol 62:331–338

Laird JMA, Cervero F (1996) Effects of metamizol on nociceptive responses to stimulation of the ureter and on ureter motility in anaesthetized rats. Inflamm Res 45:150–154

Linz W, Englert H, Kaiser J, Klaus E, Metzger H, Wirth K, Schölkens BA (1992) Evidence for an involvement of potassium channels in the action of forskolin and 1,9-dideoxyforskolin. Pharm Pharmacol Lett 1:99–102

Maggi CA, Giuliani S (1994) Calcitonin gene-related peptide (CGRP) regulates excitability and refractory period of the guinea pig ureter. J Urology 152:520–524

Roza C, Laird JMA (1995), Pressor responses to distension of the ureter in anaesthetized rats: characterization of a model of acute visceral pain. Neurosci Lett 198:9–12

Susano S, Moriyama K, Shimamura K (1992) Potentiation of twitch contraction in guinea pig ureter by sodium vanadate. Am J Physiol Cell Physiol 263:C953–C958

Yoshida S, Kuga T (1980) Effects of field stimulation on cholinergic fibers of the pelvic region in the isolated guinea pig ureter. Jap J Physiol 30:415–426

Young CJ, Attele A, Toledano A, Núñez R, Moss J (1994) Volatile anesthetics decrease peristalsis in the guinea pig ureter. Anesthesiology 81:452–458

A.1.2.11 Isolated Corpus Cavernosum

PURPOSE AND RATIONALE

The isolated corpus cavernosum of rabbits has gained interest as pharmacological model since selective inhibitors of cyclic guanosine monophosphate (cGMP) phosphodiesterase type 5 (PDE5) were found to be effective in the treatment of erectile dysfunction in man (Ballard et al. 1996; Jeremy et al. 1997; Chuang et al. 1998; Liu et al. 1998; Turko et al. 1999; Wallis 1999; Wallis et al. 1999; Stief 2000; Aydin et al. 2001; Thompson et al. 2001; Lin et al. 2002).

PROCEDURE

Male New Zealand White rabbits weighing 3–4 kg are sedated with an intramuscular injection of 25 mg/kg ketamine+6 mg/kg xylazine. Anesthesia is maintained by intravenous injection of 25 mg/kg nembutal. The penis is removed at the level of the attachment of the corporal bodies to the ischium. The corpus cavernosum (total length about 20 mm) is sharply dissected from tunica albuginea and two longitudinal strips with

unstretched length about 10 mm are made from the proximal, more muscular portion.

Corporal strips are placed in organ baths containing 10 ml Tyrode's buffer (NaCl 124.9 mmol/l, KCl 12.5 mmol/l, $MgCl_2 \cdot 6H_2O$ 0.5 mmol/l, NaH_2PO_4 H_2O 0.4 mmol/l, $CaCl_2$ 1.8 mmol/l and glucose 5.5.mmol/l) at 37°C. Each tissue is equilibrated with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. One end of each strip is connected to a force displacement transducer, and changes in muscle tension are measured and recorded with a polygraph. After zeroing and balancing transducers and strip chart, 2.0 g of tension is placed on each strip, and the strips are allowed to equilibrate for 30 min.

Each strip is prestimulated with 10 µM phenylephrine, then relaxed by electrical field stimulation with square wave pulses of 80 V, 1 ms duration at 2–16 Hz frequency. Then sodium nitroprusside (0.01–100 µM) is added ad NO donor. Finally, the standard (sildenafil 1 nM to 1 µM) or the test compound is added.

EVALUATION

The dose-dependent increase of relaxation after test compound and standard is measured. From dose-response curves activity ratios can be calculated.

MODIFICATIONS OF THE METHOD

Wallis et al. (1999) studied the inhibition of human phosphodiesterases PDE1 to PDE6 by sildenafil in various tissues, such as cardiac ventricle, corpus cavernosum, skeletal muscle and retina.

Park et al. (1997) reported functional characterization of angiotensin II receptors in rabbit corpus cavernosum.

Yildirim et al. (1997) investigated the effects of castration and testosterone on the constricting effect of phenylephrine and endothelium-dependent and -independent relaxing effects of different agonists in the corpus cavernosum of male rabbits.

Liu et al. (1998) analyzed the pharmacological effects of *in vitro* ischemia on rabbit corpus cavernosum.

Gupta et al. (1998) found that activation of G_i-coupled postsynaptic α_2-adrenoceptors causes contraction of smooth muscles in the corpus cavernosum of rabbits.

Teixeira et al. (1998) used a bioassay cascade to study the effect of *Tityus serrulatus scorpion* venom on the rabbit isolated corpus cavernosum.

Cellec and Moncada (1998) used the clitoral corpus cavernosum of female rabbits to study the role of nitrergic neurotransmission in non-adrenergic non-cholinergic relaxation responses.

The isolated corpus cavernosum of **rats** has been used by Tong and Cheng (1997), Gemalmaz et al. (2001), and Wingard et al. (2003), of **mice** by Gocmen et al. (1997) and Mizusawa et al. (2001), of **dogs** by Hayashida et al. (1996), Comiter et al. (1997), of **monkeys** by Okamura et al. (1998), of **horses** by Recio et al. (1997).

Studies in isolated **human** corpus cavernosum were performed by Holmquist et al. (1992), Bush et al. (1992), Rajfer et al. (1992), Cellek and Moncada (1997), Ballard et al. (1998), Omote (1999), Wallis et al. (1999), Lin et al. (2000), and Stief et al. (1998, 2000).

***In vivo*studies** measuring intracavernous pressure in **rats** were performed by Ari et al. (1996), Chan et al. (1996), Moody et al. (1997), Reilly et al. (1997), Chang et al. (1998), Mills et al. (1998), Gemalmaz et al. (2001), Takagi et al. (2001), Rajasekaran et al. (2005), and Wingard et al. (2006).

Cashen et al. (2002) measured intracavernous pressure in anesthetized mice.

In vivo studies on penile erection were performed in **cats** by Champion et al. (1997).

Intracavernous pressure was measured *in vivo* in anesthetized **dogs** by Ayajiki et al. (1997), Sarikaya et al. (1997), Carter et al. (1998), and Noto et al. (2000).

Bischoff and Schneider (2000) decribed a simple and quantitative model to study agents that influence penile erection in **conscious rabbits**. Erection was assessed by measuring the length of uncovered mucosa before and after the intravenous administration of agents. Anmals did not require anesthesia during the course of the study.

REFERENCES AND FURTHER READING

Ari G, Vardi Y, Hoffman A, Finberg JPM (1996) Possible role of endothelins in penile erection. Eur J Pharmacol 307:69–74

Ayajiki K, Hayashida H, Okamura T, Toda N (1997) Pelvic nerve stimulation-induced pressure responses in corpus cavernosum of anesthetized dogs. Am J Physiol 273, Heart Circ Physiol 42:H2141–H2145

Aydin S, Ozbeck H, Yilmaz Y, Atilla MK, Bayrakli H, Catin H (2001) Effects of sildenafil citrate, acetylcholine, and sodium nitroprusside on the relaxation of rabbit cavernosal tissue in vitro. Urology 58:119–124

Ballard SA, Turner LA, Naylor AM (1996) Sildenafil, a potent selective inhibitor of type 5 phosphodiesterase, enhances nitric oxide-dependent relaxation of rabbit corpus cavernosum. Br J Pharmacol 118, Proc Suppl: 153P

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and the activities of cyclic nucleotide phosphodiesterase isozymes. J Urol 159:2164–2171

Bischoff E, Schneider K (2000) A conscious-rabbit model to study verdenafil hydrochloride and other agents that influence penile erection. Int J Impot Res 13:230–235

Bush PA, Aronson WJ, Buga GM, Ignarro LJ (1992) Nitric oxide is a potent relaxant of human and rabbit corpus cavernosum. J Urol 147:1650–1655

Carter AJ, Ballard SA, Naylor AM (1998) Effect of the selective phosphodiesterase type5 inhibitor sildenafil on erectile function in the anesthetized dog. J Urol 160:242–246

Cashen DE, McIntyre DE, Martin WJ (2002) Effects of sildenafil on erectile activity in mice lacking endothelial nitric oxide synthase. Br J Pharmacol 136:693–700

Cellec S, Moncada S (1997) Nitrergic control of peripheral sympathetic responses in the human corpus cavernosum. Proc Natl Acad Sci USA 94:8226–8231

Cellec S, Moncada S (1998) Nitrergic transmission mediates the non-adrenergic non-cholinergic responses in the clitoral corpus cavernosum of the rabbit. Br J Pharmacol 125:1627–1629

Champion HC, Wang R, Hellstrom WJG, Kadowitz PJ (1997) Nociceptin, a novel endogenous ligand for the ORL_1 receptor, has potent erectile activity in the cat. Am J Physiol 273, Endocrinol Metab 36:E214–E219

Chan YH, Huang C-L, Chan SHH (1996) Nitric oxide as a mediator of cocaine-induced penile erection in the rat. Br J Pharmacol 118:155–161

Chang AYW, Chan JYH, Chan SHH (1998) Participation of hippocampal formation in negative feedback inhibition of penile erection in rats. Brain Res 788:160–168

Chuang AT, Strauss JD, Murphy RA, Steers WD (1998) Sildenafil, a type-5 cGMP phosphodiesterase inhibitor, specifically amplifies cGMP-dependent relaxation in rabbit corpus cavernosum muscle *in vitro*. J Urol 160:257–261

Comiter CV, Sullivan MP, Yalla SV, Kifor I (1997) Effect of angiotensin II on corpus cavernosum smooth muscle in relation to nitric oxide environment: *In vitro* studies in canines. Int J Impotence Res 9:135–140

Gemalmaz H, Waldeck K, Chapman TN, Tuttle JB, Steers WD, Andersson KE (2001) In vivo and in vitro investigation of the effects of sildenafil on rat cavernous smooth muscle. J Urol 165:1010–1014

Gocmen C, Ucar P, Singirik E, Dikmen A, Baysal F (1997) An *in vitro* study of nonadrenergic-noncholinergic activity on the cavernous tissue of mouse. Urol Res 25:269–275

Gupta S, Moreland RB, Yang S, Gallant CM, Goldstein I, Traish A (1998) The expression of functional postsynaptic α_2-adrenoceptors in the corpus cavernosum smooth muscle. Br J Pharmacol 123:1237–1245

Hayashida H, Okamura T, Tomoyoshi T, Toda N (1996) Neurogenic nitric oxide mediates relaxation of canine corpus cavernosum. J Urol 155:1122–1127

Holmquist F, Hedlund H, Andersson KE (1991) L-*NG*-nitro arginine inhibits non-adrenergic, non-cholinergic relaxation of human isolated corpus cavernosum. Acta Physiol Scand 141:441–442

Jeremy JY, Ballard SA, Naylor AM, Miller MAW, Angelini GD (1997) Effects of sildenafil, a type-5 cGMP phsophodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum *in vitro*. Br J Urol 79:958–963

Lin C-S, Lau A, Tu R, Lue TF (2000) Expression of three isoforms of cGMP-binding cGMP-specific phsophodiesterase (PDE5) in human penile cavernosum. Biochem Biophys Res Commun 268:628–635

Lin RJ, Wu BN, Lo YC, Shen KP, Lin YT, Huang CH, Chen IJ (2002) KMUP-1 relaxes rabbit corpus cavernosum smooth muscle in vitro and in vivo: involvement of cyclic GMP and K^+ channels. Br J Pharmacol 135:1159–1166

Liu S-P, Horan P, Levin RM (1998) Digital analysis of the pharmacological effects of *in vitro* ischemia of rabbit corpus cavernosum. Pharmacology 56:216–222

Mills TM, Lewis RW, Stopper VS, Reilly CM (1998) Loss of alpha-adrenergic effect during the erectile response in the long-term diabetic rat. J Androl 19:473–478

Mizusama H, Hedlund P, Håkansson A, Alm P, Andersson KE (2001) Morphological and functional in vitro and in vivo characterization of the mouse corpus cavernosum. Br J Pharmacol 132:1333–1341

Moody JA, Vernet D, Laidlaw S, Rajfer J, Gonzalez-Cadavid HF (1997) Effects of long-term oral administration of L-arginine on the rat erectile response. J Urol 158:942–947

Noto T, Inoue H, Ikeo T, Kikkawa K (2000) Potentiation of penile tumescence by T-1032, a new potent and specific phosphodiesterase type V inhibitor, in dogs. J Pharmacol Exp Ther 294:870–675

Okamura T, Ayajiki K, Toda N (1998) Monkey corpus cavernosum relaxation mediated by NO and other relaxing factor derived from nerves. Am J Physiol 274, Heart Circ Physiol 43:H1075–H1081

Omote M (1999) Pharmacological profiles of sildenafil (VIAGRA^{TM}) in the treatment of erectile dysfunction: Efficacy and drug interaction with nitrate. Folia Pharmacol Jpn 114:213–218

Park J-K, Kim S-Z, Kim S-H, Park Y-K, Cho K-W (1997) Renin angiotensin system in rabbit corpus cavernosum: Functional characterization of angiotensin II receptors. J Urol 158:653–658

Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ (1992) Nitric oxide as a mediator of relaxation of the corpus cavernosum response to nonadrenergic, noncholinergic neurotransmission. N Engl J Med 326:90–94

Rajasekaran M, White S, Baquir A, Wilkes N (2005) Rho-kinase inhibition improves erectile function in aging male Brown-Norway rats. J Androl 26:182–188

Recio P, Lopez JLG, Garcia-Sacristan A (1997) Pharmacological characterization of adrenoceptors in horse corpus cavernosum penis. J Auton Pharmacol 17:191–198

Reilly CM, Zamorano P, Stopper VS, Mills TM (1997) Androgenic regulation of NO availability in rat penile erection. J Androl 18:110–115

Sarikaya S, Asci R, Aybek Z, Yilmaz AF, Buyukalpelli R, Yildiz S (1997) Effect of intracavernous calcium blockers in dogs. Int Urol Nephrol 29:673–680

Stief CG, Uckert S, Becker AJ, Truss MC, Jonas U (1998) The effect of the specific phosphodiesterase (PDE) inhibitors on human and rabbit cavernous tissue in vitro and in vivo. J Urol 159:1390–1393

Stief CG (2000) Phosphodiesterase inhibitors in the treatment of erectile dysfunction. Drugs Today 36:93–99

Stief CG, Uckert S, Becker AJ, Harringer W, Truss MC, Forssmann WG, Jonas U (2000) Effects of sildenafil on cAMP and cGMP levels in isolated human cavernous and cardiac tissue. Urology 55:146–150

Takagi M, Mochida H, Noto T, Yano K, Inoue H, Ikeo T, Kikkawa K (2001) Pharmacological profile of T-1032, a novel specific phosphodiesterase type 5 inhibitor, in isolated rat aorta and rat corpus cavernosum. Eur J Pharmacol 411:161–168

Teixeira CE, Bento AC, Lopes-Martins RAB, Teixeira SA, von Eickestedt V, Muscará MN, Arantes EC, Giglio JR, Antunes E, de Nucci G (1998) Effect of *Tityus serrulatus scorpion* venom on the rabbit isolated corpus cavernosum and

the involvement of NANC nitrergic nerve fibres. Br J Pharmacol 123:435–442
Thompson CS, Mumtaz FH, Khan MA, Wallis RM, Mikhailidis DP, Morgan RJ, Angelini GD, Jereremy JY (2001) The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. Eur J Pharmacol 425:57–64
Tong Y-C, Cheng J-T (1997) Subtyping of α_1-adrenoceptors responsible for the contractile response in the rat corpus cavernosum. Neurosci Lett 228:159–162
Turko IV, Ballard SA, Francis SH, Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (type 5) by sildenafil and related compounds. Mol Pharmacol 56:124–130
Wallis RM (1999) The pharmacology of sildenafil, a novel and selective inhibitor of phosphodiesterase (PDE) type 5: Folia Pharmacol Jpn 114/Suppl 1:22P–26P
Wallis RM, Corbin JD, Francis SH (1999) Tissue distribution of phosphodiesterase families and the effect of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings *in vitro*. Am J Cardiol 83:3C–12C
Wingard CJ, Johnson JA, Holmes A, Prikosch A (2003) Improved erectile function after Rho-kinase inhibition in a rat castrate model of erectile dysfunction. Am J Physiol 284:R1572–R1578
Yildirim MK, Yildirim S, Utkan T, Sarioglu Y, Yalman Y (1997) Effects of castration on adrenergic, cholinergic and nonadrenergic, noncholinergic responses of isolated corpus cavernosum from rabbit. Br J Urol 79:964–970

A.1.3
Cardiovascular Analysis in Vivo

A.1.3.1
Hemodynamic Screening in Anesthetized Rats

PURPOSE AND RATIONALE

The test is used to detect the effect of compounds on blood pressure and heart rate of anesthetized rats and to check for possible interference with adrenergic receptors. Antihypertensive agents with different mechanisms of action can be detected with this test.

PROCEDURE

Male Sprague-Dawley rats weighing 250–400 g are used. At least 2 animals are necessary for screening of one compound. The rats are anesthetized by intraperitoneal injection of 8 ml/kg of a solution of 8% urethane and 0.6% chloralose. The trachea is cannulated to facilitate spontaneous respiration. Body temperature is maintained at 38°C by placing the animal on a heating pad.

The left femoral vein is cannulated for drug administration, which is standardized to injections of 0.2 ml/100 g body weight over a period of 1 min. For measurement of hemodynamic parameters and for intra-arterial administration of test compound, a cannula is inserted retrogradely into the right carotid artery. The tip of the catheter is positioned close to the origin of the subclavian artery. This allows most of the injected substances to reach the CNS via the vertebral artery before going into the general circulation.

For continuous monitoring of blood pressure (systolic and diastolic pressure) and heart rate, the catheter is connected to a pressure transducer (Statham DB 23).

When stable hemodynamic conditions are achieved for at least 20 min (control values), test boli of adrenaline (1 mg/kg) and isoprenaline (0.25 mg/kg) are administered. When baseline values are again established, increasing doses of the test substance (0.01, 0.1, 3.0 mg/kg) are given intra-arterially. In case of no effect, the interval between successive doses is 15 min, otherwise 60 min. To check for α- or β-blocking activity, adrenaline and isoprenaline administration is repeated after injection of the highest dose of test compound. If the test compound shows no effect, a standard antihypertensive compound is administered for control purpose.

Hemodynamic parameters are recorded continuously during the whole experiment.

EVALUATION

Changes in blood pressure and heart rate after drug administration are compared to control values obtained during the 20 min pre-drug period.

Maximal changes in BP and HR and duration of the effect are reported.

The results are scored relative to the efficacy of standard compounds for the degree of the effect and the duration of the effect.

Statistical significance is not tested because of the small number of animals used ($n = 2$, sometimes 3 or 4) but larger numbers of animals have to be used for quantitative evaluation.

CRITICAL ASSESSMENT OF THE METHOD

Due to the administration of the test compounds via the right common carotid artery not only peripherally acting vasodilators and neuron blockers but also compounds affecting the blood pressure regulating mechanisms in the CNS are detected. Bolus injections of adrenaline and isoprenaline reveal possible α- or β-antagonistic effects.

Standard data:

The following compounds at the doses indicated lead to a strong decrease in blood pressure:

- Clonidine 0.008 mg/kg
- Dihydralazine 1.0 mg/kg
- Phentolamine 3.0 mg/kg

- Prazosin 0.1 mg/kg
- Propranolol 1.0 mg/kg
- Urapidil 1.0 mg/kg
- Verapamil 0.1 mg/kg

MODIFICATIONS OF THE METHOD

Several authors (Mervaala et al. 1999; Wallerath et al. 1999; Rothermund et al. 2000; Baltatu et al. 2001) monitored arterial pressure and heart rate using a pressure transducer system and continuously recorded on a computer-based registration system (TSE, Bad Homburg, Germany).

A procedure for differential intra-arterial pressure recordings from different arteries in the rat was described by Pang and Chan (1985).

DeWildt and Sangster (1983) described the evaluation of derived aortic flow parameters measured by means of electromagnetic flowmetry as indices of myocardial contractility in anesthetized rats.

Using a special Millar ultraminiature catheter pressure transducer and a thermodilution microprobe, Zimmer et al. (1987, 1988) measured right ventricular functional parameters in anesthetized, closed-chest rats.

Veelken et al. (1990) published improved methods for baroreceptor investigations in chronically instrumented rats.

Salgado and Krieger (1988), de Abreu and Salgado (1990), Da Silva et al. (1994) studied the function of the**baroreceptor reflex** in thiopental anesthetized rats. The left aortic nerve was isolated and supported by a bipolar stainless steel electrode and carefully insulated with silicone rubber. Carotid pressure was recorded simultaneously with aortic nerve discharges on an oscilloscope and monitored with a loudspeaker.

King et al. (1987) developed a cross circulation technique in rats to distinguish central from peripheral cardiovascular actions of drugs. The right common carotid arteries were ligated, and the left common carotid arteries and left and right external jugular veins of two phenobarbital-anesthetized rats were connected with polyethylene tubing so that peripheral blood from one rat, A, supplied the head of another rat, B, and then returned to the body of A, and vice versa, for peripheral blood from rat B. Each rat was artificially ventilated with O_2, the chest was opened, and both subclavian arteries were ligated. Prior to the ligation of the subclavian arteries, blood flow from rat A supplied its own brain and both brain hemispheres but not the brain stem of rat B. Following subclavian artery ligation, blood flow from rat A did not supply A's brain, but supplied both hemispheres and brain stem of rat B. The head of each rat was, therefore, rendered dependent on the carotid arterial blood supply from another rat. This rat cross-circulation preparation can be used to separate the central and peripheral cardiovascular actions of drugs.

Zavisca et al. (1994) studied the hypertensive responses to defined electrical and mechanical stimuli in anesthetized rats. Rats were given etomidate, 3.8 mg/kg/h intravenously following carotid artery and jugular vein cannulation. At 15 min after beginning the infusion, 4 types of noxious stimuli were administered sequentially at 1-min intervals: Type 1: Square electrical waves 125 cps, 1.6 ms, 2-s duration, varying current from 0.4 to 12 mA; Type 2: A single 10-mA electrical stimulus, 5-s train duration; Type 3: Tail clamping; Type 4: Skin incision. After each stimulus, maximum change in systolic blood pressure was measured. Graded electrical stimulation allowed the best quantitative evaluation of the hypertensive response to noxious stimuli.

Hyman et al. (1998) described a novel catheterization technique for the *in vivo* measurements of **pulmonary vascular responses** in rats. Male Charles River rats weighing 26–340 g were anesthetized and strapped in supine position to a fluoroscopic table. They breathed air enriched with oxygen through an endotracheal tube inserted by tracheostomy. Catheters were inserted into the femoral blood vessels. The venous catheters were passed to the right atrium under fluoroscopy. A F-1 thermistor catheter was passed from the left carotid artery into the ascending aorta under fluoroscopy, and a PE-50, 150-mm plastic catheter with a specially constructed curved tip was passed fluoroscopically from the left jugular vein into the main pulmonary artery. A plastic radiopaque 22-gauge catheter 100 mm in length with a curved tip was passed with a 0.025 mm soft-tip coronary guiding catheter from the right jugular vein through the right atrium to the inferior vena cava. The coronary soft-tip guide was then withdrawn. A specially curved 102.5-mm transseptal needle, 0.4 mm in diameter, was then passed though the catheter. Both the needle and catheter were withdrawn into the superior portion of the right atrium under fluoroscopic guidance so that the needle and catheter both rotated freely. With the rat in a slight left anterior oblique position, the catheter and needle were carefully rotated anteriorly to the intra-atrial septum. With gentle pressure, the catheter and needle can be felt and seen fluoroscopically to pass through the atrial septum. As the needle was withdrawn, the curve of the catheter permitted passage of the tip into the vein draining either the left or right

lower lobe. The catheter was carefully positioned near the pulmonary venoatrial junction and fixed in place. Mean pressures in the femoral artery, pulmonary artery and pulmonary vein at the venoatrial junction were measured with pressure transducers and recorded on a polygraph. Cardiac output was obtained in triplicate by delivering 0.1 ml normal saline at room temperature into the femoral venous catheter at the right venoatrial junction and determining thermodilution cardiac output with the thermistor catheter in the ascending aorta.

Hayes (1982) described a technique for determining contractility, intraventricular pressure, and heart rate in the **anesthetized guinea pig** by inserting a needle, attached to a pressure transducer, through the chest wall into the left ventricle.

Williams et al. (1995) used **castrated male ferrets** anesthetized by intramuscular injection of a mixture of 55 mg/kg ketamine and 4 mg/kg xylazine to measure the effects of a nonpeptidyl endothelin antagonist on endothelin-induced pressor responses.

REFERENCES AND FURTHER READING

Baltatu O, Fontes MAP, Campagnole-Santos MJ, Caligiorni S, Ganten D, Santos RAS, Bader M (2001) Alterations in the renin-angiotensin system, at the RVLM of transgenic rats with low brain angiotensinogen. Am J Physiol 280:R428–R433

Chu D, Hofmann A, Stürmer E (1978) Anesthetized normotensive rats for the detection of hypotensive activity of a β-adrenoceptor antagonist and other anti-hypertensive agents. Arzneim Forsch/Drug Res 28:2093–2097

Da Silva VJD, da Silva SV, Salgado MCO, Salgado HC (1994) Chronic converting enzyme inhibition facilitates baroreceptor resetting to hypertensive levels. Hypertension 23 (Suppl I):I-68–I-72

de Abreu GR, Salgado HC (1990) Antihypertensive drugs distinctly modulate the rapid resetting of the baroreceptors. Hypertension 15 (Suppl 1):I-63–I-67

DeWildt DJ, Sangster B (1983) An evaluation of derived aortic flow parameters as indices of myocardial contractility in rats. J Pharmacol Meth 10:55–64

Hayes JS (1982) A simple technique for determining contractility, intraventricular pressure, and heart rate in the anesthetized guinea pig. J Pharmacol Meth 8:231–239

Hyman AL, Hao Q, Tower A, Kadowitz PJ, Champion HC, Gumusel B, Lippton H (1998) Novel catheterization technique for the *in vitro* measurements of pulmonary vascular responses in rats. Am J Physiol 274 (Heart Circ Physiol 43):H1218–H1229

King KA, Tabrizchi R, Pang CCY (1987) Investigation of the central and peripheral actions of clonidine and methoxamine using a new *in vivo* rat preparation. J Pharmacol Meth 17:283–295

Mervaala E, Dehmel B, Gross V, Lippoldt A, Bohlender J, Milia AF, Ganten D, Luft FC (1999) Angiotensin-converting enzyme inhibition and AT_1 receptor blockade modify the pressure-natriuresis relationship by additive mechanisms in rats with human renin and angiotensinogen genes. J Am Soc Nephrol 10:1669–1680

Pang CCY, Chan TCK (1985) Differential intraarterial pressure recordings from different arteries in the rat. J Pharmacol Meth 13:325–330

Rothermund L, Friebe A, Paul M, Koesling D, Kreutz R (2000) Acute blood pressure effects of YC-1-induced activation of soluble guanylyl cyclase in normotensive and hypertensive rats. Br J Pharmacol 130:205–208

Salgado HC, Krieger EM (1988) Extent of baroreceptor resetting in response to sodium nitroprusside and verapamil. Hypertension 11 (Suppl 1):I-121–I-125

Veelken R, Unger Th, Medvedev OS (1990) Improved methods for baroreceptor investigations in chronically instrumented rats. J Pharmacol Meth 23:247–254

Wallerath T, Witte K, Schäfer SC, Schwarz PM, Prellwitz W, Wohlfart P, Kleinert H, Lehr HA, Lemmer B, Förstermann U (1999) Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. Proc Natl Acad Sci USA 96:13357–13362

Williams DL Jr, Murphy KL, Nolan NA, O'Brien JA, Pettibone DJ, Kivlighn SD, Krause SM, Lis EV Jr, Zingaro GJ, Gabel RA, Clayton FC, Siegl PKS, Zhang K, Naue J, Vyas K, Walsh TF, Fitch KJ, Chakravarty PK, Greenlee WJ, Clineschmidt BV (1965) Pharmacology of L-754,142, a highly potent, orally active, nonpeptidyl endothelin antagonist. J Pharmac Exp Ther 275:1518–1526

Zavisca FG, David Y, Kao J, Cronau LH, Stanley TH, David T (1994) A new method to evaluate cardiovascular response in anesthetized rats. Hypertension after variable intensity, brief electrical stimuli. J Pharmacol Toxicol Meth 31:99–105

Zimmer HG, Zierhut W, Marschner G (1987) Combination of ribose with calcium antagonist and β-blocker treatment in closed-chest rats. J Mol Cell Cardiol 19:635–639

Zimmer HG, Zierhut W, Seesko RC, Varekamp AE (1988) Right heart catheterization in rats with pulmonary hypertension and right ventricular hypertrophy. Basic Res Cardiol 83:48–57

A.1.3.2 Blood Pressure in Pithed Rats

PURPOSE AND RATIONALE

The pithed rat has been proposed for assessing pressor substances by Shipley and Tilden (1947). The preparation is frequently used to evaluate drug action on the cardiovascular system since this preparation is devoid of neurogenic reflex control that may otherwise modulate the primary drug effect.

PROCEDURE

Male rats weighing 250–350 g are prepared for pithing under halothane anesthesia. The left carotid artery is cannulated for blood pressure monitoring and blood sampling. Furthermore, the trachea and the right jugular vein are cannulated. The rats are pithed inserting a steel rod, 2.2 mm in diameter and about 11 cm in length, through the orbit and foramen magnum down the whole length of the spinal canal. Via the tra-

cheotomy tube, the animals are ventilated with a small animal ventilation pump. Inspirated air is oxygen-enriched by providing a flow of oxygen across a T-piece attached to the air intake of the ventilation pump (Harvard Apparatus model 680). The rats are ventilated at a frequency of 60 cycles/min with a tidal volume of 2 ml/100 body weight. Thirty min after pithing, a 0.3 ml blood sample is withdrawn from the carotid cannula and immediately analyzed for pO_2, pCO_2, pH, and derived bicarbonate concentration using an automatic blood gas analyzer. By alterations of the respiratory stroke volume of the pump, the values are adjusted to: pCO_2 30–43 mm Hg, pH 7.36–7.50, pO_2 87–105 mm Hg.

Continuous registration of blood pressure and cardiac frequency (Hellige He 19 device and Statham P 23 Db transducer) is performed via the left carotid artery.

In order to measure α_1 and α_2 antagonism, first dose-response curves are registered using doses of 0.1–30 µg/kg i.v. phenylephrine (a selective α_1 agonist), and 1–1000 µg/kg i.v. BHT 920, (a selective α_2 agonist). The test drug is administered intravenously and the agonist dose-response curves are repeated again 15 min later.

EVALUATION

If the curve of blood pressure response to the agonists is shifted, dose-response curves are plotted on a logarithmic probit scale and potency ratios are calculated.

MODIFICATIONS OF THE METHOD

Gillespie and Muir (1967) described a method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat by coating those parts of the pithing rod which lay in the sacral and cervical region of the spinal cord with high-resistance varnish to restrict stimulation to the thoracolumbar region. The steel rod is insulated with an adhesive throughout its length except for a 5 cm section which provides sufficient a stimulation area of the lower thoracolumbar nerves. For stimulating nerves fibers supplying exclusively the heart, a pithing rod is used which is insulated throughout its length except for a 0.5 cm section 7 cm proximal to the tip. The spinal cord is stimulated electrically using the pithing rod as the cathode and a hypodermic needle which is inserted under the skin near the right hind-limb, as the anode. Varying the intensity and/or the duration of the stimulation, dose-response curves can be registered which are altered after treatment with drugs.

Curtis et al. (1986) described an improved pithed rat method by mounting the preparation vertically with the head pointing downward resulting in considerably higher blood pressure and heart rate.

MacLean and Hiley (1988) studied the effect of artificial respiratory volume on the cardiovascular responses to an α_1- and α_2-adrenoceptor agonist in the air-ventilated pithed rat using microsphere technique and analysis of arterial blood gases and pH.

Trolin (1975) used decerebrated rats to study the clonidine-induced circulatory changes.

Balt et al. (2001) compared the angiotensin II type 1 (AT1) receptor blockers losartan, irbesartan, telmirsatan and the ACE inhibitor captopril on inhibition of angiotensin-II-induced facilitation of sympathetic neurotransmission in the pithed rat.

REFERENCES AND FURTHER READING

Balt JC, Mathy MJ, Pfaffendorf M, van Zwieten PA (2001) Inhibition of angiotensin II-induced facilitation of sympathetic neurotransmission in the pithed rat: a comparison between losartan, irbesartan, telmirsatan and captopril. J Hypertens 19:465–273

Curtis MJ, McLeod BA, Walker MJA (1986) An improved pithed rat preparation: the actions of the optical enantiomers of verapamil. Asia Pacific J Pharmacol 1:73–78

Fluharty SJ, Vollmer RR, Meyers SA, McCann MJ, Zigmond MJ, Stricker EM (1987) Recovery of chronotropic responsiveness after systemic 6-hydroxydopamine treatment: Studies in the pithed rat. J Pharm Exp Ther 243:415–423

Gillespie JS, Muir TC (1967) A method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat. Br J Pharmacol Chemother 30:78–87

Gillespie S, MacLaren A, Pollock D (1970) A method of stimulating different segments of the autonomic flow from the spinal column to various organs in the pithed cat and rat. Br J Pharmacol 40:257–267

MacLean MR, Hiley CR (1988) Effect of artificial respiratory volume on the cardiovascular responses to an α_1- and α_2-adrenoceptor agonist in the air-ventilated pithed rat. Br J Pharmacol 93:781–790

Majewski H, Murphy TV (1989) Beta-adrenoreceptor blockade and sympathetic neurotransmission in the pithed rat. J Hypertension 7:991–996

Milmer KE, Clough DP (1983) Optimum ventilation levels for maintenance of normal arterial blood pO_2, pCO_2, and pH in the pithed rat preparation. J Pharmacol Meth 10:185–192

Nichols AJ, Hamada A, Adejare A, Miller DD, Patil PN, Ruffolo RR (1989) Effect of aromatic fluorine substitution on the *alpha* and *beta* adrenoreceptor mediated effects of 3,4-dihydroxy-tolazoline in the pithed rat. J Pharmacol Exp Ther 248:617–676

Schneider J, Fruh C, Wilffert B, Peters T (1990) Effects of the selective β_1-adrenoreceptor antagonist, Nebivolol, on cardiovascular parameters in the pithed normotensive rat. Pharmacology 40:33–41

Shipley RE, Tilden JH (1947) A pithed rat preparation suitable for assaying pressor substances. Proc Soc Exp Med 64:453–455

Trolin G (1975) Effects of pentobarbitone and decerebration on the clonidine-induced circulatory changes. Eur J Pharmacol 34:1–7

Tung LH, Jackman G, Campell B, Louis S, Iakovidis D, Louis WJ (1993) Partial agonist activity of celiprolol. J Cardiovasc Pharmacol 21:484–488

Van Meel JCA, Wilfert B, De Zoeten K, Timmermans PBMWM, Van Zwieten PA (1982) The inhibitory effect of newer calcium antagonists (Nimodipine and PY-108–068) on vasoconstriction *in vivo* mediated by postsynaptic α_2-adrenoreceptors. Arch Int Pharmacodyn 260:206–217

Vargas HM, Zhou L, Gorman AJ (1994) Role of vascular alpha-1 adrenoceptor subtypes in the pressor response to sympathetic nerve stimulation in the pithed rat. J Pharm Exp Ther 271:748–754

A.1.3.3 Antihypertensive Vasodilator Activity in Ganglion-Blocked, Angiotensin II Supported Rats

PURPOSE AND RATIONALE

The method is used to demonstrate direct vasodilator activity of potential antihypertensive agents. The experimental model is an anesthetized, ganglion-blocked rat whose blood pressure is maintained by an intravenous infusion of angiotensin II. The test allows to differentiate between centrally acting antihypertensives and peripheral vasodilators.

PROCEDURE

Male Wistar rats weighing 275–450 g are anesthetized with a combination of urethane (800 mg/kg) and chloralose (60 mg/kg) administered intraperitoneally in a volume of 10 ml/kg. Following induction of anesthesia, chlorisondamine (2.5 mg/kg) is injected into the peritoneal cavity to abolish sympathetic and parasympathetic nerve activity. The right femoral artery is cannulated to monitor blood pressure (Statham pressure transducer P23Db) and heart rate. Both femoral veins are cannulated to administer drugs or infuse angiotensin II. The trachea is intubated and animals are allowed to breathe spontaneously. Following a stabilization interval of 10–15 min, angiotensin II is infused at a rate of 0.25 or 3.5 µg/min in a volume equivalent to 0.05 ml/min (Harvard infusion pump).

After an increase of blood pressure, a new elevated steady-state pressure is established within 15–20 min. Drugs are subsequently injected intravenously over an interval of 3 min in a volume of 2 ml/kg. Mean arterial pressure is recorded on a polygraph at 5, 10, 15, 20 and 30 min after initiation of drug administration. Seven to 9 animals are used for each drug and dose level studied.

α-adrenoreceptor blockade can be determined in ganglion-blocked rats. Pressor responses to graded doses of phenylephrine injected intravenously are obtained before and 15 min after administration of test compounds. Sufficient concentrations of phenylephrine have to be given to ensure a rise in mean arterial blood pressure of 50 mm Hg or more. Data obtained from 5 or 6 animals are averaged and resultant dose-response curves plotted. The dose of phenylephrine required to elicit a 50 mm Hg increase in mean arterial blood pressure is interpolated from dose response curves.

Standard data:

The following compounds are used as standards and, at the doses indicated, lower mean arterial blood pressure by about 50 mm Hg:

- Cinnarizine 3.0 mg/kg, i.v.
- Hydralazine 1.0 mg/kg, i.v.
- Minoxidil 10.0 mg/kg, i.v.
- Saralasine 0.03 mg/kg, i.v.
- Molsidomine 0.1 mg/kg, i.v.

EVALUATION

Mean values ± SEM are given for mean arterial blood pressure and heart rate. Changes of these parameters after drug administration are compared to control values obtained immediately before the application of the test compound. Statistical significance is assessed by means of the paired *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

A hypotensive response in this model appears to correlate more closely with antihypertensive activity in DOCA-salt hypertensive rats than does a vasodilator response in the perfused hind limb of anesthetized dogs and allows a distinction between central anti-hypertensive and vasodilators.

MODIFICATIONS OF THE METHOD

Santajuliana et al. (1996) developed a standard ganglionic blockade protocol to assess neurogenic pressor activity in conscious rats. Rats were instrumented with arterial and venous catheters for measurement of arterial pressure and heart rate and for administration of three different ganglionic blockers (trimethaphan, hexamethonium, and chlorisondamine).

REFERENCES AND FURTHER READING

Deitchman D, Braselton JP, Hayes DC, Stratman RL (1980) The ganglion-blocked, angiotensin II-supported rat: A model for demonstrating antihypertensive vasodilator activity. J Pharmacol Meth 3:311–321

Santajuliana D, Hornfeldt BJ, Osborn JW (1996) Use of ganglionic blockers to assess neurogenic pressor activity in conscious rats. J Pharmacol Toxicol Meth 35:45–54

A.1.3.4 Blood Pressure in Conscious Hypertensive Rats (Tail Cuff Method)

PURPOSE AND RATIONALE

Rats with spontaneous or experimentally induced hypertension are widely used for screening of potentially antihypertensive compounds. The indirect tail cuff method allows the determination of systolic blood pressure according to the following principle: The cuff is quickly inflated to well above suspected systolic blood pressure; the pulse will then be obliterated. Thereafter, pressure in the cuff is slowly released and, as the pressure falls below systolic blood pressure, the pulse will reappear. The method is analogous to sphygmomanometry in human and can be applied not only at the tail of awake rats but also in dogs and small primates. The indirect tail cuff method is widely used to evaluate the influence of antihypertensive drugs in spontaneously and experimentally hypertensive rats.

PROCEDURE

Male spontaneous hypertensive rats (Charles River) weighing 300–350 g or rats with experimentally induced hypertension are used.

Surgical Procedure to Induce Renal Hypertension

Male Sprague-Dawley rats weighing 80–100 g are anesthetized by intraperitoneal injection of 0.8 ml 4% chloralhydrate solution. Both kidneys are exposed retroperitoneally. To induce renal hypertension, a silver clip (0.2 mm diameter, 4 mm length) is placed onto both renal arteries, the kidneys are reposed and the wound is closed by suture.

Within 5–6 weeks, operated animals attain a renal hypertension with a systolic blood pressure (BPs) of 170–200 mm Hg (mean normal physiological BPs for rats is 100 mm Hg). Only animals with a BPs = 180 mm Hg are used for the tests.

Test Procedure

The procedure is the same for spontaneously and experimentally hypertensive rats. Groups of 6 animals are used per dose. The control group receives saline only. To reduce spontaneous variations in blood pressure, animals are adjusted to the experimental cage by bringing them into the restraining cage which is enclosed in a 31–32°C measuring chamber 3–4 times before the start of the experiment for a period of 30–60 min.

To measure blood pressure, a tubular inflatable cuff is placed around the base of the tail and a piezoelectric pulse detector is positioned distal to the cuff. The cuff is inflated to approximately 300 mm Hg. As the pressure in the cuff is slowly released, the systolic pressure is detected and subsequently recorded on a polygraph.

The test substance is administered intraperitoneally or by gavage once per day over a period of 5 days. The usual screening dose of a new compound is 25 mg/kg. Blood pressure and heart rate measurements are taken at the following times:

day 1: predose and 2 h postdrug
day 3: predose and 2 h postdrug
day 5: predose, 2 h postdrug and 4 h postdrug.

Between measurements, animals are returned to their home cages.

Standard compounds:

- endralazine (3 mg/kg p.o.)
- nifedipine (3 mg/kg p.o.)
- urapidil (5 mg/kg p.o.)

EVALUATION

Mean values in systolic blood pressure before and after drug administration and the duration of the effect are determined. Percent decrease in systolic blood pressure under drug treatment is calculated. Statistical significance is assessed by the Student's *t*-test.

Scores for % decrease in systolic blood pressure and for the duration of the effect are allotted.

CRITICAL ASSESSMENT OF THE METHOD

The indirect tail-cuff method is being used in many laboratories with many modifications of the devices. Pfeffer et al. (1971) found a good correlation between values obtained with the indirect tail-cuff method and values measured directly with indwelling carotid arterial cannulae, whereas Buñag et al. (1971) reported a lack of correlation between direct and indirect measurement of arterial pressure in unanesthetized rats and Patten and Engen (1971) found difficulties to measure accurate systolic values at higher blood pressure. A good correlation between direct blood pressure data from the carotid artery in rats and readings with the tail cuff method was found by Matsuda et al. (1987) who developed a six-channel automatic blood pressure measuring apparatus with a highly sensitive photoelectric sensor for the detection of tail arterial blood flow and a microcomputer system for automatic measurement of systolic blood pressure and heart rate and for data acquisition and processing.

MODIFICATIONS OF THE METHOD

Details of the tail-cuff method in rats have been discussed by Stanton (1971).

Special equipment for measuring blood pressure in rats is commercially available (e. g., TSE GmbH, Bad Homburg, Germany).

Widdop and Li (1997) described a simple versatile method for measuring tail cuff systolic blood pressure in conscious rats. A tail cuff consisting of a metal T-piece tube with latex rubber inside the tube is placed around the tail at the proximal end. A piezoelectric transducer (model MLT1010) is strapped to the ventral surface of the tail to record the pulse signal from the caudal artery and connected directly to a MacLab data-acquisition system ADInstruments Pty Ltd.).

The tail-cuff method for measurement blood pressure has been adapted for dogs, monkeys (Wiester and Iltis 1976), and cats (Mahoney and Brody 1978).

Blood pressure can be measured from the hind leg of the rat using a leg cuff and a photoelectric cell situated at the dorsal surface of the foot (Kersten et al. 1947). When the leg is occluded, the foot swells and the amount of light striking the photocell is reduced. When the pressure in the cuff is released, the arterial blood flow is restored, the increase of foot volume is decreased and the amount of light transversing the paw increases.

REFERENCES AND FURTHER READING

Buñag RD (1984) Measurement of blood pressure in rats. In: de Jong W (ed) Handbook of Hypertension. Vol 4. Experimental and Genetic Models of Hypertension. Elsevier Science Publ., New York, pp 1–12

Buñag RD, McCubbin JW, Page IH (1971) Lack of correlation between direct and indirect measurement of arterial pressure in unanesthetized rats. Cardiovasc Res 5:24–31

Kersten H, Brosene WG Jr, Ablondi F, Subba Row Y (1947) A new method for the indirect measurement of blood pressure in the rat. J Lab Clin Med 32:1090–1098

Mahoney LT, Brody MJ (1978) A method for indirect recording of arterial pressure in the conscious cat. J Pharmacol Meth 1:61–66

Matsuda S, Kurokawa K, Higuchi K, Imamura N, Hakata H, Ueda M (1987) A new blood pressure measuring apparatus equipped with a microcomputer system for conscious rats. J Pharmacol Meth 17:361–376

Patten JR, Engen RL (1971) The comparison of an indirect method with a direct method for determining blood pressure on rats. Cardiovasc Res Center Bull 9:155–159

Pernot F (1991) Blood pressure on conscious rats, non-invasive method: tail-cuff. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany, pp 30–32

Pfeffer JM, Pfeffer MA, Frohlich ED (1971) Validity of an indirect tail-cuff method for determining systolic arterial pressure in unanesthetized normotensive and spontaneously hypertensive rats. J Lab Clin Med 78:957–962

Stanton HC (1971) Experimental hypertension. In: Schwartz A (ed) Methods in Pharmacology, Vol 1, pp 125–150. Appleton-Century-Crofts, Meredith Corporation. New York

Widdop RE, Li XC (1997) A simple versatile method for measuring tail cuff systolic blood pressure in conscious rats. Clin Sci 93:191–194

Wiester MJ, Iltis R (1976) Diastolic and systolic blood pressure measurements in monkeys determined by a non invasive tail-cuff technique. J Lab Clin Med 87:354–361

A.1.3.5 Direct Measurement of Blood Pressure in Conscious Rats with Indwelling Catheter

PURPOSE AND RATIONALE

The method first described by Weeks (1960) allows the direct measurement of arterial pressure in conscious rats eliminating the influence of anesthesia on cardiovascular regulation.

PROCEDURE

Preparation of Cannulae

In order to prepare the cannulae 7 cm and 12 cm long pieces are cut from PE 10 and PE 20 tubings respectively. A stylet wire is inserted into the PE 10 tubing and the PE 20 tubing is also slipped over the stylet wire. The ends of the tubings are heated in a current of hot air and fused together. Ridges are made to anchor the cannula in the animal's tissue. In order to make a ridge, the stylet wire is left inside the cannula and the cannula is heated in a fine jet of hot air. When the polyethylene at the point of heating becomes soft, the cannula is pressed slightly and thus a ridge is formed. One ridge is formed at the PE 20 tubing, about 0.5 cm away from the junction with the PE 10 tubing, and 3 more ridges are formed on the PE 20 tubing at a distance of about 1 cm from each other, first one being situated about 3 cm away from the free end of the PE 20 tubing. The stylet wire is then removed from the cannula and the PE 10 portion of the cannula near the junction with the PE 20 tubing is wound around a glass rod with a diameter of 4 mm. Two rounds are made. Then it is dipped in a boiling water bath for about 5 s. When taken out of the bath, the cannula retains its circles, forming a spring-like structure.

Implantation of Cannulae

Male Sprague-Dawley rats weighing about 300 g are used. The rat is anesthetized with 45 mg/kg pentobarbital i.p. The area of the neck and the abdomen are shaved and cleaned with 70% alcohol. The viscera are exposed through a midline abdominal incision. A segment of the abdominal aorta is exposed just above the bifurcation. A trocar is passed through the psoas muscles adjacent to this segment of the aorta, through the muscles of the back and under the skin until it emerges

from the skin of the neck. Then the cannula is inserted into the trocar and the trocar is withdrawn from the body. The end of the cannula thus comes out from the neck, being anchored by silk sutures to the neck skin and to the psoas muscle. The cannula is filled with heparin solution and the end which is projecting out from the neck skin is blocked with a tight fitting stainless steel needle. Then the other end of the cannula is implanted into the aorta. The aorta is wiped with a cotton-tipped applicator stick above the bifurcation, occluded above this segment and punctured with a bent 27 gauge hypodermic needle. The tip of the PE 10 catheter is inserted through the needle and advanced up the aorta. The intestines are replaced and the wound sutured. The rats are allowed to recover for one week.

Measurement of Blood Pressure

The occluding stainless steel needle is removed and the cannula flushed with diluted heparin solution. The rat is placed in a small cage to restrict its movements, even so it is free to move. The cannula is connected to a Statham P 23 Db pressure transducer and blood pressure is recorded on a polygraph. Test drugs or standards are administered either subcutaneously or orally. Recordings are taken before and after administration of drug over a period of 1 h.

EVALUATION

Changes of blood pressure are measured for degree and duration. Five rats are used for each dose and compound. The maximal changes of each group are averaged and compared with the standard.

CRITICAL ASSESSMENT OF THE METHOD

Direct measurement of arterial blood pressure in unanesthetized rats originally introduced by Weeks (1960) has become a valuable and widely used tool in cardiovascular research.

MODIFICATIONS OF THE METHOD

A detailed description of a slightly modified Week's method has been given by Stanton (1971).

Improvements of the method for continuous direct recording of arterial blood pressure and heart rate in rats have been described by Buñag et al. (1971), Laffan et al. (1972), Buckingham (1976), Garthoff and Towart (1981), Garthoff (1983). A detailed description of permanent cannulation of the iliolumbar artery was given by Remie et al. (1990).

Wixson et al. (1987) described a technique for chronic catheterization of the carotid artery in the rat.

Prepared cannulas are commercially available (IRC Life Science, Woodland Hills CA).

A newer modification uses the access to the aorta via the common carotid artery (Linz et al. 1992). Rats are prepared under thiopental anesthesia with arterial PE-50 lines (Intramedic from Clay Adams, USA). The lines are introduced into the ascending aorta via the right carotid artery for direct measurement of arterial blood pressure and into the jugular vein for i.v. application of test compounds. Both lines, filled with saline containing heparin, are surfaced on the neck. The animals are allowed to recover for at least 2 days. Blood pressure is monitored through Statham R P23 Db transducers connected to a recording device. During measurements the lines are kept open with counter current saline infusion at a rate of 1 ml/h.

Bao et al. (1991) placed one catheter via the right femoral artery in the abdominal aorta in rats for recording mean arterial pressure and two additional catheters via the left carotid artery into the descending aorta for application of bradykinin and bradykinin antagonists.

Arterial pressure was recorded in unanesthetized rats after induction of severe hypertension by complete ligation of the aorta between the origin of the renal arteries by Sweet and Columbo (1979).

Hilditch et al. (1978) described a device for the direct recording of blood pressure in conscious dogs.

Akrawi and Wiedlund (1987) described a method for chronic portal vein infusion in unrestrained rats. Hepatic drug metabolism can be studied by infusion into the portal vein and blood collection from the femoral vein.

Robineau (1988) described a method for recording electrocardiograms in conscious, unrestrained rats. Electrodes were implanted subcutaneously and a socket connector was sutured on the head of the animal. A flexible cord leading to a swivel collector was linked to an ECG amplifier.

Kurowski et al. (1991) reported on an improved method to implant, maintain, and protect arterial and venous catheters in conscious rats for extended periods of time.

Schenk et al. (1992) measured cardiac left ventricular pressure in conscious rats using a fluid-filled catheter.

Tsui et al. (1991) recommended a reliable technique for chronic carotid arterial catheterization in the rat.

Hagmüller et al. (1992) described a tail-artery cannulation method for the study of blood parameters in freely moving rats.

Liebmann et al. (1995) described an *in vivo* long-term perfusion system which is based on au-

tomated, computer-controlled high-frequency heparin (10 U/ml) flushing of a cannula inserted into the tail artery of freely moving rats.

Santajuliana et al. (1996) used conscious rats instrumented with arterial and venous catheters to assess neurogenic pressor activity after administration of ganglionic blockers.

Rezek and Havlicek (1975) described simple cannula systems for the infusion of experimental substances in chronic, unrestrained animals. A cannula with a removable cap is used for infusions into various parts of the digestive tract. Intravenous infusions can be performed through a closed system cannula which avoids a possible introduction of air into the circulation.

Kimura et al. (1988) described a method for chronic portal venous, aortic, and gastric cannulation to determine portal venous and aortic glucose and lactate levels in conscious rats.

REFERENCES AND FURTHER READING

Akrawi SH, Wiedlund PJ (1987) A method for chronic portal vein infusion in unrestrained rats. J Pharmacol Meth 17:67–74

Bao G, Qadri F, Stauss B, Stauss H, Gohlke P, Unger T (1991) HOE 140, a new highly potent and long-acting bradykinin antagonist in conscious rats. Eur J Pharmacol 200:179–182

Buckingham RE (1976) Indwelling catheters for direct recording of arterial blood pressure and intravenous injection of drugs in the conscious rat. J Pharm Pharmac 28:459–461

Buñag RD, McCubbin JW, Page IH (1971) Lack of correlation between direct and indirect measurement of arterial pressure in unanesthetized rats. Cardiovasc Res 5:24–31

Garthoff B (1983) Twenty-four hour blood pressure recording in aortic coarctation hypertensive rats. Naunyn Schmiedeberg's Arch Pharmacol 322:R22

Garthoff B, Towart R (1981) A new system for the continuous direct recording of blood pressure and heart rate in the conscious rat. J Pharmacol Meth 5:275–278

Hagmüller K, Liebmann P, Porta S, Rinner I (1992) A tail-artery cannulation method for the study of blood parameters in freely moving rats. J Pharm Toxicol Meth 28:79–83

Hilditch A, Newberry A, Whithing S (1978) An improved device for the direct recording of blood pressure in conscious dogs. J Pharmacol Meth 1:89–90

Kimura RE, Lapine TR, Gooch III WM (1988) Portal venous and aortic glucose and lactate changes in a chronically catheterized rat. Pediat Res 23:235–240

Kurowski SZ, Slavik KJ, Szilagyi JE (1991) A method for maintaining and protecting chronic arterial and venous catheters in conscious rats. J Pharmacol Meth 26:249–256

Laffan RJ, Peterson A, Hitch SW, Jeunelot C (1972) A technique for prolonged, continuous recording of blood pressure of unrestrained rats. Cardiovasc Res 6:319–324

Liebmann PM, Hofer D, Absenger A, Zimmermann P, Weiss U, Porta S, Schauenstein K (1995) Computer-controlled flushing for long-term cannulation in freely moving rats. J Pharmacol Toxicol Meth 34:211–214

Linz W, Klaus E, Albus U, Becker R, Mania D, Englert HC, Schölkens BA (1992) Cardiovascular effects of the novel potassium channel opener (3S,4R)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulfonylchromane hemihydrate. Arzneim Forsch/Drug Res 42:1180–1185

Remie R, van Dongen JJ, Rensema JW (1999) Permanent cannulation of the iliolumbar artery. In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ, pp 231–241

Rezek M, Havlicek V (1975) Chronic multipurpose cannulas and a technique for the cannulation of small veins and arteries. Physiol Behav 15:623–626

Robineau F (1988) A simple method for recording electrocardiograms in conscious, unrestrained rats. J Pharmacol Meth 19:127–133

Santajuliana D, Hornfeldt BJ, Osborn JW (1966) Use of ganglionic blockers to assess neurogenic pressor activity in conscious rats. J Pharmacol Toxicol Meth 35:45–54

Schenk J, Hebden A, McNeill JH (1992) Measurement of cardiac left ventricular pressure in conscious rats using a fluid-filled catheter. J Pharm Toxicol Meth 27:171–175

Stanton HC (1971) Experimental hypertension. In: Schwartz A (ed) Methods in Pharmacology, Vol 1, pp 125–150. Appleton-Century-Crofts, Meredith Corporation. New York

Sweet CS, Columbo JM (1979) Cardiovascular properties of antihypertensive drugs in a model of severe renal hypertension. J Pharmacol Meth 2:223–239

Tsui BCH, Mosher SJ, Yeung PKF (1991) A reliable technique for chronic carotid arterial catheterization in the rat. J Pharm Meth 25:343–352

Weeks JR, Jones JA (1960) Routine direct measurement of arterial pressure in unanesthetized rats. Proc Soc Exp Biol Med 104:646–648

Wixson SK, Murray KA, Hughes HC Jr (1987) A technique for chronic arterial catheterization in the rat. Lab Anim Sci 37:108–110

A.1.3.6 Cannulation Techniques in Rodents

PURPOSE AND RATIONALE

Cardiovascular pharmacology requires special techniques for catheterization and permanent cannulation of vessels. A few methods are described below.

A comprehensive literature survey on methods for vascular access and collection of body fluids from the laboratory rat was written by Cocchetto and Bjornsson (1983).

REFERENCES AND FURTHER READING

Cocchetto DM, Bjornsson TD (1983) Methods for vascular access and collection of body fluids from the laboratory rat. J Pharmaceut Sci 72:465–492

A.1.3.6.1 Permanent Cannulation of the Jugular Vein in Rats

PURPOSE AND RATIONALE

Permanent cannulation of the jugular vein in rats in combination with a head attachment apparatus allowing easy connection of cannulae was first introduced by Steffens (1969). Modifications were described by Brown and Hege (1972), Nicolaidis et al. (1974) and

by Dons and Havlik (1986). A detailed description was given by Remie et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. The shaven neck of the animal on the right side is disinfected with chlorhexidine solution. The incision is made just above the right clavicle. Connective and adipose tissue are pushed aside with blunt forceps and the jugular vein is exposed. The external jugular vein is followed and the division into the maxillary vein, the facial and the linguofacial vein identified. The largest vein is chosen and mobilized for a distance of about 5 mm. Small artery forceps are used to clamp the vessel. The vein is then ligated rostral to the clamp with 6–0 silk, and a second ligature is put loosely around the vessel, but not tightened. Using iridectomy scissors, a V-shaped hole is cut in the vein 2 mm rostral from the bifurcation. Prior to its insertion into the vessel, a sterile cannula is connected to a 1 ml syringe filled with a heparinized saline solution. The vessel is dilated by means of a sharp pointed jeweler's forceps, the cannula slit between the legs of the forceps and gently pushed into the vessel until the tip is at the level of the right atrium. Then the forceps is removed, the caudal ligature gently tied, and the rostral ligature used to anchor the cannula to the vessel. The cannula is tunneled to emerge at the top of the head. While the skin in the neck is held firmly, the artery forceps is inserted subcutaneously in caudal direction over a distance of about 3 cm, then turned anti-clockwise in the direction of the incision in the neck. The cannula is grasped with the forceps. Then the forceps is pulled back until the cannula emerges at the crown of the head and closed by a small microvascular clamp. The cannula is slid over the short end of a 20G stainless steel needle bent to a 90° angle. The catheter is flushed with saline and filled with polyethylene/heparin solution. The long end of the L-shaped stainless steel adapter is closed with a piece of heat-sealed PE-tubing and the wounds are closed with sutures.

MODIFICATIONS OF THE METHOD

Hutchaleelaha et al. (1997) described a simple apparatus for serial blood sampling from the external jugular vein which permits simultaneous measurement of locomotor activity in freely moving rats.

REFERENCES AND FURTHER READING

Brown MR, Hedge GA (1972) Thyroid secretion in the unanesthetized, stress-free rat and its suppression by pentobarbital. Neuroendocrinology 9:158–174

Dons RF, Havlik R (1986) A multilayered cannula for long-term blood sampling in unrestrained rats. Lab Anim Sci 36:544–547

Hutchaleelaha A, Sukbuntherng J, Mayersohn (1997) Simple apparatus for serial blood sampling in rodents permitting simultaneous measurement of locomotor activity as illustrated with cocaine. J Pharmacol Toxicol Meth 37:9–14

Nicolaidis S, Rowland N, Meile MJ, Marfaing-Jallat P, Pesez A (1974) A flexible technique for long term infusion in unrestrained rats. Pharmacol Biochem Behav 2:131–136

Remie R, van Dongen JJ, Rensema JW (1990) Permanent cannulation of the jugular vein (acc. to Steffens). In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ., pp 159–169

Steffens AB (1969) A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. Physiol Behav 4:833–936

A.1.3.6.2 Permanent Cannulation of the Renal Vein in Rats

PURPOSE AND RATIONALE

A detailed description for permanent cannulation of the renal vein in rats was given by Remie et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. This provides an excellent view to the vena cava. At its confluence with the vena cava, the right renal vein is stripped of its adipose tissue and the peritoneum is opened. Using small anatomical forceps the peritoneum is detached from the vena cava by making small spreading movements with the forceps just beneath the peritoneum. Subsequently, the vena cava and the renal vein are mobilized for approximately 1.5 cm, to allow for clamping of the vessel. A four or five fine-stitch purse-string is placed in the vessel at the confluence of the vena cava and the right renal vein. Using a Barraquer needle holder and a cotton-wool stick, the 7–0 silk suture, armed with a BV-1 needle, is guided through the vessel. After each stitch, any bleeding has to be immediately arrested by applying light pressure using a cotton-wool stick. Having completed the suture a single knot is made with the drawstrings. Three microvascular clips are then placed on the vena cava and the renal vein; first the proximal clip on the vena cava, followed by the clip on the renal vein and finally the distal vena cava clip. A small aperture is cut immediately inside the purse-string suture using iridectomy scissors and jeweler's forceps. The cannula, which is filled with a heparinized saline solution, is pushed into the opening as far as possible. Subse-

quently, the purse-string suture is pulled taut and the clip of the renal vein removed, while pushing the cannula further. The proximal clip on the vena cava is now removed as quickly as possible. The patency of the cannula is checked and the drawstrings of the purse-string suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

REFERENCES AND FURTHER READING

Remie R, Rensema JW, van Dongen JJ (1990) Permanent cannulation of the renal vein. In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ., pp 223–230

A.1.3.6.3 Permanent Cannulation of the Portal Vein in Rats

PURPOSE AND RATIONALE

Several techniques have been described for cannulation of the portal vein in rats (Hyun et al. 1967; Pelzmann and Havemeyer 1971; Suzuki et al. 1973; Sable-Amplis and Abadie 1973; Helman et al. 1984). A detailed description for permanent cannulation of the portal vein in rats was given by Remie et al. (1990). After additional application of platinum electrodes around the portal vein in close proximity to the catheter tip, this model can also be used to study the presynaptic regulation of neurotransmitter release from nonadrenergic nerve terminals (Remie and Zaagsma 1986; Remie et al. 1988, 1989).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal wall, the intestines are lifted out and laid next to the animal on the right side (as viewed by the surgeon) on gauze moistened with warm saline solution. Using a micro needle holder and a cotton-wool stick, a four or five, fine stitch purse-string suture (7–0 silk suture armed with a BV-1 needle) is placed in the wall of the portal vein at the side opposite the gastroduodenal vein. The diameter of the purse-string should be about 1 mm. After the suture has been completed a single knot is made with the drawstrings. The portal vein is clamped with a small curved hemostatic bulldog clamp. Using iridectomy scissors and a pair of jeweler's forceps the center of the purse-string is cut, a cannula filled with heparinized saline is inserted into the vessel and pushed upwards. The purse-string is gently tightened taking care not to obstruct the cannula. The drawstrings of the suture are used to anchor the cannula. The cannula is laid kink-free in the abdominal cavity and sutured to the internal abdominal cavity near the xiphoid cartilage. The abdomen is closed in two layers and the cannula tunneled to the top of the head. The cannula together with a L-shaped adapter is fixed to the skull.

REFERENCES AND FURTHER READING

Helman A, Castaing D, Morin J, Pfister-Lemaire N, Assan R (1984) A new technique for hepatic portal vein catheterization in freely moving rats. Am J Physiol 246 (Endocrinol Metab 9):E544–E547

Hyun SA, Vanhouny GV, Treadwell CR (1967) Portal absorption of fatty acids in lymph and portal vein cannulated rats. Biochem Biophys Acta 137:296–305

Pelzmann KS, Havemeyer RN (1971) Portal blood vein sampling in intestinal drug absorption studies. J Pharm Sci 60:331–332

Remie R, Zaagsma J (1986) A new technique for the study off vascular presynaptic receptors in freely moving rats. Am J Physiol 251:H463–H467

Remie R, Knot HJ, Kolker HJ, Zaagsma J (1988) Pronounced facilitation of endogenous noradrenaline release by presynaptic β_2-adrenoceptors in the vasculature of freely moving rats. Naunyn-Schmiedeberg's Arch Pharmacol 338:215–220

Remie R, Coppes RP, Zaagsma J (1989) Presynaptic muscarinic receptors inhibiting endogenous noradrenaline release in the portal vein of the freely moving rat. Br J Pharmacol 97:586–590

Remie R, van Dongen JJ, Rensema JW, van Wunnik GHJ (1990) Permanent cannulation of the portal vein. In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ., pp 213–221

Sable-Amplis R, Abadie D (1975) Permanent cannulation of the hepatic portal vein in rats. J Appl Physiol 38:358–359

Suzuki T, Sattoh Y, Isozaki S, Ishida R (1973) Simple method for portal vein infusion in the rat. J Pharm Sci 62:345–347

A.1.3.6.4 Permanent Cannulation of the Thoracic Duct in Rats

PURPOSE AND RATIONALE

Collection of lymph is rather difficult and has performed mainly in dogs (Biedl and Offer 1907; Gryaznova 1962, 1963; Vogel 1963). Some techniques have been described for the rat (Bollman et al. 1948; Girardet 1975). Remie et al. (1990) did not obstruct the duct by placing a purse-string suture in the wall of the duct, by which the cannula is secured. The animal's lymph can be collected during the experiment, and after refilling the cannula the lymph flow remains undisturbed.

PROCEDURE

Rats are anesthetized with N_2O_2/O_2/halothane. After opening the abdominal cavity, the intestines are placed

in gauze moistened with warm saline and laid to the left of the animal. The suprarenal abdominal artery is located and mobilized by gently tearing the connective tissue. Using blunt dissection technique, the thoracic duct is mobilized along the dorsolateral surface of the aorta. A small three to four fine stitch purse-string suture is placed in the wall of the duct, using a 9–0 Ethilon suture. A hole is cut inside the purse-string with a very fine pair of scissors, while holding the wall with angled jeweler's forceps. The cannula which is filled with heparinized saline solution and is inserted into the duct using anatomical forceps. After the tip of the cannula has been inserted into the thoracic duct, the curved forceps are removed and the total tip is pushed into the duct. The ligature is then closed and some lymph will flow into the cannula. The cannula is secured within the abdominal cavity by attaching it to the abdominal muscle near the xiphoid cartilage with a 7–0 silk suture. Following the closure of the abdominal wall and the tunneling of the cannula to the crown of the head, a L-shaped adapter is placed on the cannula, filled with PVP-solution and closed with a heat-sealed polyethylene cap.

REFERENCES AND FURTHER READING

Biedl A, Offer TR (1907) Über Beziehungen der Ductuslymphe zum Zuckerhaushalt. Hemmung von Adrenalinwirkung durch die Lymphe. Wien Kin Wschr

Bollman JL, Cain JC, Grindlay JH (1948) Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J Lab Clin Med 33:1349–1352

Girardet RE (1975) Surgical technique for long-term studies of thoracic duct circulation in the rat. J Appl Physiol 39:682–688

Gryaznova AV (1962) Ligation of the thoracic duct in dogs. Arkhiv Anatomii, Gistologii i Embriologii 42:90–95

Gryaznova AV (1963) Ligation of the thoracic duct in dogs. Fed Proc 22/II,T886

Remie R, van Dongen JJ, Rensema JW (1990) Permanent cannulation of the thoracic duct. In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ., pp 243–253

Vogel HG (1963) Unpublished data

A.1.3.6.5 Portacaval Anastomosis in Rats

PURPOSE AND RATIONALE

In 1877 the Russian surgeon Eck reported the achievement of successful portacaval shunts in dogs. Lee and Fischer (1961), Funovics et al. (1975), de Boer et al. (1986), described portacaval shunt in the rat. A detailed description of surgery for portacaval anastomosis in rats was given by van Dongen et al. (1990).

PROCEDURE

Rats are anesthetized with N_2O_2/O_2 in combination with either enflurane, methoxyflurane or isoflurane. After opening the abdominal wall, the intestines are placed left to the animal on gauze moistened with warm saline solution. Proximally and distally to the animal's right renal vein, the vena cava is then stripped of its adipose and connective tissue, and the retroperitoneal cavity is opened. Using anatomical forceps the peritoneum is dissected from the vena cava by making small spreading movements with the forceps just above the vena cava. The portal vein is pulled slightly to the left using straight anatomical forceps and freed from the hepatic artery and the gastroduodenal artery with curved anatomical forceps. Rostral to the celiac artery, the abdominal artery which is covered with peritoneum is freed from its lateral muscle bed over a length of approximately 5 mm providing enough space for placing a small bulldog clamp at a later stage of the operation. Without occlusion a six fine-stitch purse-string is placed in the wall of the vena cava close to its confluence with the right renal vein. Using a Barraquer needle holder and a cotton wool stick, the 7–0 silk suture armed with a BV-1 needle is guided through the vessel. After each stitch, bleeding has to be arrested immediately, by applying light pressure on the area, again using the cotton wool stick. After the suture has been completed, a single knot is made with the drawstrings. The drawstrings should come together at the rostral part of the purse-string. A bulldog clamp, modified to resemble a Satinsky vascular clamp, is then placed on the vena cava.

Before clamping the abdominal aorta rostral to the celiac artery with a small bulldog clamp, a ligature (7–0 silk) is placed around the portal vein as close as possible to the hilus of the liver. Subsequently, the clamp is placed on the aorta and the ligature tightened. A Heifetz clip is then placed transversely onto the portal vein at its confluence with the gastroduodenal vein. The portal vein is cut just distally from the ligature. A prepared button is slipped over the left-hand straight small anatomical forceps, while the right-hand forceps are used to pass the portal vein to the left-hand anatomical forceps. The vein is then grasped and pulled through the button. Subsequently, the button is pushed as close as possible to the Heifetz clip, and clamped to the clip using a Pilling bulldog clamp.

Using small straight, and curved anatomical forceps the portal vein is reversed around the button and fixed with a previously prepared 7–0 silk suture. The Pilling bulldog clamp is then removed and replaced at the end of the Satinsky clamp for reasons of stability. The vena

cava is then somewhat elevated, bringing it into closer contact with the portal vein button.

A longitudinal cut is made in the purse-string suture using iridectomy scissors and jeweler's forceps. One drawstring of the suture is clamped with a small hemostat and put under slight tension in a rostral direction. The button manipulated by its grip, is pushed into the vena cava. The purse-string is tightened with the left hand whilst the right hand still holds the button in position. The button is released and two additional knots tied. The Satinsky clamp is removed first followed by the Heifetz clamp and the bulldog clamp on the aorta. After replacing the intestines the abdominal wall is closed in two layers.

REFERENCES AND FURTHER READING

De Boer JEG, Janssen MA, van Dongen JJ, Blitz W, Oostenbroek RJ, Wesdorp RIC, Soeters PB (1986) Sequential metabolic characteristics following portacaval shunt in rats. Eur Soc Surg Res 18:96–106

Eck NV (1877) On the question of ligature of the portal vein. Voen Med J; St. Petersburg 130:1–2

Funovics JM, Cummings MG, Shuman L, James JH, Fischer JE (1975) An improved non suture method for portacaval anastomosis in the rat. Surgery 77:668–672

Lee SH, Fischer B (1961) Portacaval shunt in the rat. Surgery 50:668–672

van Dongen JJ, Remie R, Rensema JW, van Wunnik GHJ (1990) Portacaval anastomosis. In: Van Dongen, Remie, Rensema, van Wunnik (eds) Manual of Microsurgery on the Laboratory Rat. Elsevier Science Publ., pp 171–199

A.1.3.7 Cardiovascular Analysis in Anesthetized Mice

PURPOSE AND RATIONALE

To fully utilize the potential of mouse models with specific gene mutations, it is necessary to study the functional consequences of genetic manipulations in fully intact mice. Lorenz and Robbins (1997) developed and validated a methodology to study cardiovascular parameters in closed-chest mice.

PROCEDURE

Adult mice of either sex weighing 25–35 g are anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 100 mg/kg thiobutabarbital. After the mice are placed on a thermally controlled surgical table with body temperature continually monitored via a rectal probe, a tracheotomy is performed with a short length (< 1 cm) of PE-90 tubing. The right femoral artery is then cannulated with polyethylene tubing which is pulled over a flame to a small diameter (∼0.4 mm OD). The catheter is advanced ∼1 cm, near the level of the aorta, and connected directly to a low-compliance COBE CDXIII fixed-dome pressure transducer for the measurement of arterial blood pressure. The right femoral vein is then cannulated with the same type of small-diameter tubing and connected to a microinjection pump for the infusion of experimental drugs. To assess myocardial performance, the right carotid artery is cannulated with a 2F Millar MIKRO-TIP transducer (Model SPR-407, Millar Instruments, Houston TX). This high-fidelity transducer, which has a tip diameter of ∼0.67 mm, has a reported frequency response that is flat up to 10,000 Hz and therefore can be used to accurately monitor the high frequency of the mouse ventricular pulse pressure. During continual monitoring of the blood pressure wave to ascertain the anatomic position of the catheter, the tip of the transducer is carefully advanced through the ascending aorta and into the left ventricle. When the stable waveform of the ventricular pressure profile is achieved, the transducer is anchored in place with 7–0 silk sutures. After completion of the surgery, all wounds are closed with cyanoacrylate to minimize evaporative loss of fluid, and the animals are allowed to stabilize for 30–45 min.

EVALUATION

Blood pressure signals from the COBE transducer and from the Millar transducer are amplified and the output is recorded and analyzed with a MacLab 4/s data acquisition system connected to a Macintosh 7100/80 computer which allows the calculation of the following parameters:

dP/dt	first derivative of the ventricular pressure wave,
MAP	mean arterial pressure,
HR	heart rate,
LVP	systolic and diastolic left ventricular pressure,
LVEDP	left ventricular enddiastolic pressure.

Further indices of ventricular performance can be calculated from dP/dt.

MODIFICATIONS OF THE METHOD

Champion et al. (2000) described a **right-heart catheterization technique** for *in vivo* measurement of vascular responses in lungs of intact mice. CD1 mice weighing 25–38 g were anesthetized with thiopentobarbital (85–95 mg/kg i.p.) and ketamine 3 mg/kg i.p.) and were strapped in supine position to a thermoregulated fluoroscopic table. The trachea was cannulated and the animals breathed with room air enriched with 95% O_2/5% CO_2. A femoral artery was cannulated

for the measurement of systemic arterial pressure. Heart rate was electronically monitored from the systolic pressure pulses with a tachometer (Grass model 7P44A). The left jugular vein was cannulated for the administration of agonists and antagonists.

For measuring pulmonary arterial pressure, a special single lumen catheter was constructed. The catheter was 145 mm in length and 0.25 mm in outer diameter, with a specially curved tip to facilitate passage through the right heart, main pulmonary artery, and the left or right pulmonary artery. Before the catheter was introduced, the catheter curve was initially straightened with a 0.010-in. straight angioplastic guide wire to facilitate passage from the right jugular vein into the right atrium at the tricuspid valve under fluoroscopic guidance. As the straight wire was removed, the natural curve facilitated entry of the catheter into the right ventricle. A 0.010-in. soft-tip coronary artery guide wire was then inserted, and the catheter was passed over the guide wire into the main pulmonary artery under fluoroscopic guidance. Pressure in the main pulmonary artery was measured with a pressure transducer, and mean pulmonary artery pressure was derived electronically and recorded continuously.

Cardiac output was measured by the thermodilution technique. A known volume (20 µl plus catheter dead space) of 0.9% NaCl solution at 23°C was injected into the right atrium, and changes in blood temperature were measured at the root of the aorta. A cardiac output computer equipped with a small-animal interface was used. The thermistor microprobe was inserted into the right carotid artery and advanced to the aortic arch, where changes in aortic blood temperature were measured. A catheter placed in the right jugular vein was advanced to the right atrium or main pulmonary artery for rapid bolus injection of saline. The saline solution was injected with a constant-rate syringe to ensure rapid and repeatable injection of the saline indicator solution. Thermodilution curves were recorded on a chart recorder and pulmonary and systemic blood pressure monitored continuously. Catheter placement was verified by postmortem examination.

REFERENCES AND FURTHER READING

Champion HC, Villnave DJ, Tower A, Kadowitz PJ, Hyman AL (2000) A novel right-heart catheterization technique for *in vivo* measurement of vascular responses in lungs of intact mice. Am J Physiol, Heart Circ Physiol 278:H8–H15

Lorenz JN, Robbins J (1997) Measurement of intraventricular pressure and cardiac performance in the intact, closed-chest mouse. Am J Physiol 272 (Heart Circ Physiol 41):H1137–H1146

A.1.3.8 Blood Pressure in Anesthetized Cats

PURPOSE AND RATIONALE

Cats are the most sensitive species of cardiovascular regulation. They were used extensively for cardiovascular screening. Recently, experiments in dogs are preferred since this species can be bred more easily in homogeneous strains.

PROCEDURE

Adult cats of either sex weighing 2.5 to 4 kg are anesthetized by intraperitoneal injection of 35 mg/kg pentobarbital sodium. Tracheotomy is performed and a tracheal cannula is inserted so that the cat can be mechanically ventilated with room air. A femoral artery and two femoral veins are cannulated for measurement of arterial blood pressure and systemic administration of drugs. The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler. Rectal temperature is monitored and maintained between 37°C and 38°C with a heating pad.

The following drugs are injected i.v. as challenges:

- epinephrine 0.1, 0.3, 0.5 µg/kg,
- norepinephrine 0.1, 0.3, 0.5 µg/kg,
- isoproterenol 0.1, 0.2, 0.4 µg/kg,
- carbachol 0.1, 0.2, 0.5 µg/kg.

At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline.

Test drugs are injected at various doses followed by injections of the challenging drugs.

EVALUATION

Dose-response curves of challenging drugs are established before and after injections of the test drugs.

CRITICAL ASSESSMENT OF THE METHOD

Blood pressure experiments in anesthetized cats are very valuable as screening techniques for cardiovascular agents. Moreover, potentiation of norepinephrine response has been used as screening procedure for antidepressants with norepinephrine uptake inhibiting activity.

MODIFICATIONS OF THE METHOD

Sander (1965) investigated the vasoconstrictor and vasodilator effects of procaine in spinal cats. The animals

were anesthetized with ether and ventilated with a positive pressure pump via a tracheal cannula. The spinal cord was then cut between the second and third vertebrae, and ether administration stopped. The remaining portion of the spinal cord above the transection was destroyed by passing a curette through the spinal canal.

Yardley et al. (1989) studied cardiovascular parameters in spinal cats. The animals were anesthetized with 80 mg/kg intravenously administered α-chloralose. The spinal cord was transected or crushed at the first cervical segment after tetracaine hydrochloride (0.125 mg in 0.1 ml) had been injected into this region of the cord. Systemic blood pressure was supported at a level sufficient to maintain constricted pupils (mean value 45 ± 5 mm Hg) by volume expansion with blood from a donor (10–20 ml) or an infusion of dextran.

REFERENCES AND FURTHER READING

Chen K, Hernandez Y, Dertchen KI, Gillis RA (1994) Intravenous NBQX inhibits spontaneously occurring sympathetic nerve activity and reduces blood pressure in cats. Eur J Pharmacol 252:155–160

Corman LE, diPalma JR (1967) Animal techniques for measuring drug effects on various peripheral vascular beds. In: Siegler PE, Moyer JH (eds) Animal and clinical pharmacologic techniques in drug evaluation, Vol II, pp 434–443. Year Book Medical Publ., Inc., Chicago

Crumb WJ Jr, Kadowitz PJ, Xu YQ, Clarkson CW (1990) Electrocardiographic evidence for cocaine cardiotoxicity in cat. Canad J Physiol Pharmacol 68:622–625

Henderson TR, DeLorme EM, Takahashi K, Gray AP, Dretchen KI (1988) Cardiovascular effects of 1-methyl-4-(1-naphthyl-vinyl)piperidine hydrochloride. Eur J Pharmacol 158:149–152

Mian MA, Malta E, Raper C (1986) Cardiovascular actions of Xamoterol (ICI 118,587) in anaesthetized cats, rats, and guinea pigs. J Cardiovasc Pharmacol 8:314–323

Pichler L, Kobinger W (1985) Possible function of α_1-adrenoceptors in the CNS in anesthetized and conscious animals. Eur J Pharmacol 107:305–311

Sander HD (1965) The vasoconstrictor and vasodilator effects of procaine. Can J Physiol Pharmacol 43:39–46

Yardley CP, Fitzsimonis CL, Weaver LC (1989) Cardiac and peripheral vascular contributions to hypotension in spinal cats. Am J Physiol 257 (Heart Circ Physiol 26): H1347–H1353

A.1.3.9 Cardiovascular Drug Challenging Experiments in Anesthetized Dogs

PURPOSE AND RATIONALE

Sympathomimetic and cholinomimetic compounds as well as angiotensin II and carotid occlusion exert characteristic responses in blood pressure of anesthetized dogs. Antagonism or potentiation of these responses allow to characterize the cardiovascular activity of a new compound.

PROCEDURE

Adult Beagle dogs of either sex weighing between 8 and 15 kg are anesthetized with 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 75 mg/kg sodium pentobarbital. Additional doses of sodium pentobarbital are given as needed. The dogs are intubated with a cuffed endotracheal tube and placed on a Harvard respirator (20 ml/kg, 10–15 cycles/min). A femoral vein and artery are cannulated using polyethylene tubing for drug administration and determination of arterial blood pressure, respectively. The animals are bilaterally vagotomized.

The arterial cannula is connected to a Statham model P23Gb transducer. All recordings are made on a polygraph. Heart rate is determined with a Beckman Cardiotach connected to a voltage/pressure-pulse coupler.

Drug challenges: One of the following combinations of drugs is administered i.v. to the dogs. The challenges are given in a fixed order: at least twice prior to test drug administration to insure consistent responses and again starting 15 min post test drug. Epinephrine and norepinephrine (1 µg/kg), isoproterenol (0.25 µg/kg), carbachol (0.25 µg/kg), tyramine (100 µg/kg) are used; bilateral carotid occlusion (45 s), phenylephrine (10 µg/kg), isoproterenol (0.25 µg/kg) angiotensin II (0.2 µg/kg) and carbachol (0.25 µg/kg) for cardiovascular drugs. At least 5 min are allowed between challenge doses to permit the measured parameters to return to baseline. Challenge drug doses are sometimes varied to keep the mean arterial pressure within the following limits: epinephrine (+30 to +60 mm Hg), norepinephrine (–30 to +70 mm Hg), tyramine (+30 to +70 mm Hg), isoproterenol (–30 to –50 mm Hg), carbachol (–30 to –50 mm Hg), phenylephrine (–30 to +70 mm Hg), angiotensin II (+30 to +50 mm Hg), and bilateral carotid occlusion (+30 to +70 mm Hg).

EVALUATION

The recordings are studied to detect any changes in the arterial pressure response to the challenge drug before and after test-drug administration and to observe any changes in blood pressure and heart rate. Results are expressed as the percentage change from the predrug response.

REFERENCES AND FURTHER READING

Corman LE, diPalma JR (1967) Animal techniques for measuring drug effects on various peripheral vascular beds.

In: Siegler PE, Moyer JH (eds) Animal and clinical pharmacologic techniques in drug evaluation, Vol II, pp 434–443. Year Book Medical Publ., Inc., Chicago

Hoppe JO, Brown Jr TG (1964) Animal techniques for evaluating autonomic blocking agents, antihypertensive and vasodilator drugs. In: Nodine JE, Siegler PE (eds) Animal and clinical pharmacologic techniques in drug evaluation. Vol I, pp 116–121. Year Book Medical Publ., Inc., Chicago

A.1.3.10 Hemodynamic Analysis in Anaesthetized Dogs

PURPOSE AND RATIONALE

The hemodynamic effects of compounds supposed to affect the cardiovascular system are evaluated by measuring preload and afterload of the heart, contractility, heart rate, cardiac output and peripheral or coronary flow. To measure these cardiovascular parameters accurately, the use of larger animals such as dogs or pigs is necessary.

This experimental model allows the classification of test drugs according to their action as having:

- positive inotropic effects
- negative inotropic effects (Ca^{2+}-antagonist, anti-arrhythmic?)
- hypertensive effects
- hypotensive effects
- coronary-dilating effects
- β-blocking effects
- α-blocking effects
- anti-anginal effects
- peripheral-vasodilating effects

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, and continued with an infusion of 4–6 mg/kg/h. A catheter is placed into the cephalic vein for intravenous injections. Another catheter is placed into the duodenum for enteral administration. Respiration is maintained with room air through a tracheal tube using a positive pressure respirator, e. g., Bird-Mark-7-respirator. Blood gas analyses are performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannula inserted into the left femoral artery and connected to a Statham pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left common carotid artery into the left ventricle. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/dt_{max} is differentiated and heart rate is counted. The LVP-signal also triggers a cardiotachometer.

Cardiac output, pulmonary artery pressure (PAP) and stroke volume are measured by a thermodilution technique using a Cardiac Output Computer (Gould/Statham SP 1245) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

Myocardial oxygen consumption (MVO_2) is calculated as pressure-work-index according to Rooke and Feigl (1982).

Femoral blood flow and coronary flow are measured with electromagnetic flow probes attached to the femoral artery and the circumflex branch of the left coronary artery (LCX), respectively.

Experimental Course

When stable hemodynamic conditions and blood gas values of pO_2>100 mm Hg and pCO_2 < 35 mm Hg are achieved for at least 20 min (control values), the test substance is administered through a catheter inserted into a cephalic vein in doses of 0.1, 0.3, 1.0, and 3.0 mg/kg or into the duodenum in doses of 0.3, 1.0, 3.0, and 10.0 mg/kg.

All parameters are recorded continuously during the whole experiment.

Characteristics

- blood pressure
  - systolic, BPs
  - diastolic, BPd
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- maximal rate of pressure rise, dp/dt max
- heart rate, HR
- peripheral blood flow in A. femoralis, PF
- blood pressure A. pulmonalis, PAP
- coronary flow, CF
- cardiac output, CO
- stroke volume, SV
- total peripheral resistance, TPR
- left ventricular stroke work, LVSW
- left ventricular minute work, LVMW
- left ventricular myocardial oxygen consumption, MVO_2

CALCULATION OF RESULTS AND EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formulae

- stroke volume [ml/beat],

$$SV = \frac{CO}{HR}$$

- total peripheral resistance [dyns/cm^5],

$$TPR = \frac{BPm}{CO} \times 79.9$$

- left ventricle stroke work [J/beat],

$$LVSW = (BPm - LVEDP) \times SV \times 0.333 \times 10^{-3}$$

- left ventricular minute work [J/min],

$$LVMW = LVSW \times HR$$

- left ventricular myocardial oxygen consumption [ml O_2/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \times \frac{(0.8BPs + 0.2BPd) \times HR + SV}{BW} + 1.43$$

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25 \times 10^{-4}$
BPs = systolic blood pressure [mm Hg]
BPd = diastolic blood pressure [mm Hg]
BPm = mean blood pressure [mm Hg]
HR = heart rate [beats/min]
CO = cardiac output [ml/min]
SV = stroke volume [ml/beat]
$LVEDP$ = left ventricular enddiastolic pressure [mm Hg]
BW = body weight [kg]

Changes in parameters measured after drug administration are compared to control values obtained during the 20 min pre-drug period.

Results are presented as mean ± SEM with $n > 3$.

Statistical significance is assessed by means of the paired *t*-test.

MODIFICATIONS OF THE METHOD

The effect of drugs on the carotid artery occlusion effect can be studied in anesthetized dogs. The occlusion of right and left common carotid arteries is performed by squeezing them between a polyethylene tubing and a twine which is passed inside the tubing and around the carotid artery. An occlusion of the carotid arteries for 30 s causes an increase of systolic blood pressure by 40–50 mm Hg. Inhibition of this effect by drugs is tested.

Studies in anesthetized dogs can be used to determine the influence of cardiotonic drugs on propranolol induced cardiac insufficiency (Rajagopalan et al. 1993)

Instead of dogs, **pigs (German landrace)** weighing between 20–35 kg can be used. They are pretreated with ketamine 500 mg/5 ml i.m., methomidate hydrochloride 200 mg/4 ml i.p., xylazine 60 mg/3 ml i.m., and anaesthetized with 15–20 mg/kg pentobarbital sodium, followed by continuous infusion of 12 mg/kg/h. The parameters are evaluated similarly to the experiments in dogs.

Measurement of cardiac output by the thermodilution method in **rats** was described by Richardson et al. (1962) and Müller and Mannesmann (1981).

Thermodilution methods were used by Rosas et al. (1964) in anesthetized rats, by Carbonell et al. (1985) and by Salyers et al. (1988) in conscious rats to determine hemodynamic parameters.

Oxygen pressure, carbon dioxide pressure and pH in coronary venous and common carotid arterial blood of anesthetized dogs has been measured using a blood gas analyzer (Aisaka et al. 1988).

Acute ischemic left ventricular failure can be induced in anesthetized dogs by repeated injections of plastic microspheres into the left coronary artery (Smiseth and Mjøs 1982; Sweet et al. 1984; Schölkens et al. 1986). A coronary catheter was introduced through the right femoral artery and advanced under fluoroscopy to the left coronary ostium, guided by injection of small amounts of contrast medium. After reaching baseline values, acute left ventricular failure was induced by subsequent intra-coronary injections of plastic microspheres (52.9 ±2.48 µm non-radioactive tracer microspheres). The microspheres were suspended in saline with a drop of Tween 80 and sonified before use, 1 mg microspheres/1 ml saline corresponding to approximately 12,000 microspheres. (13–16 injections of microspheres or 3.4–5.0 mg/kg). Microspheres were injected every 5 min for 70–90 min. Each microsphere injection effected an immediate and stepwise increase in LVEDP. With this procedure, LVEDP can be increased to a desired level in a very controlled manner. In the 30 min following embolization, LVEDP continued to increase by approximately 5 mm Hg. Animal with arrhythmias had to be excluded from the study. Thirty min after the end of embolization, when hemodynamic parameters had stabilized, drug administrations were started.

Valdes-Cruz et al. (1984) developed an **open-chest preparation in dogs** to validate the accuracy of a two-dimensional Doppler echocardiographic method for

estimating pressure drops across discrete stenotic obstructions.

In order to assess the potential of a single breath technique (using freon-22) as an effective way to estimate cardiac output non-invasively, Franks et al. (1990) measured simultaneously with the single breath technique the aortic flow using an electromagnetic flowmeter in anesthetized dogs.

REFERENCES AND FURTHER READING

Aisaka K, Hidaka T, Hattori Y, Inomata N, Ishihara T, Satoh F (1988) General pharmacological studies on N-(2,6-dimethyl-phenyl)-8-pyrrolizidineacetamide hydrochloride hemihydrate. 3rd Communication: Effect on cardiovascular system. Arzneim Forsch/Drug Res 38:1417–1425

Bohn H, Martorana PA, Schönafinger K (1992) Cardiovascular effects of the new nitric oxide donor, pirsidomine. Hemodynamic profile and tolerance studies in anesthetized and conscious dogs. Eur J Pharmacol 220:71–78

Carbonell LF, Salom MG, Salazar FJ, Garcia-Estañ J, Ubeda M, Queseda T (1985) Normal hemodynamic parameters in conscious Wistar rats. Revista Española Fisiologia 41:437–442

Franks PJ, Hooper RH, Humphries RG, Jones PR, O'Connor SE (1990) Effective pulmonary flow, aortic flow and cardiac output: *in vitro* and *in vivo* comparisons in the dog. Exp Physiol 75:95–106

Martorana PA, Kettenbach B, Bohn H, Schönafinger K, Henning R (1994) Antiischemic effects of pirsidomine, a new nitric oxide donor. Eur J Pharmacol 257:267–273

Millard RW (1984) Cardiac and vascular measurements in conscious and anesthetized animals. In: Schwartz A (ed) Methods in Pharmacology, Vol 5, Myocardial Biology., Plenum Press, New York and London, pp 167–174

Müller B, Mannesmann G (1981) Measurement of cardiac output by the thermodilution method in rats. II. Simultaneous measurement of cardiac output and blood pressure in conscious rats. J Pharmacol Meth 5:29–34

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-tri-hydroxy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43(I):313–319

Richardson AW, Cooper T, Pinakatt T (1962) Thermodilution method for measuring cardiac output of rats by using a transistor bridge. Science 135:317–318

Rooke GA, Feigl EO (1982) Work as a correlate of canine left ventricular oxygen consumption, and the problem of catecholamine oxygen wasting. Circ Res 50:273–286

Rosas R, Montague D, Gross M, Bohr DF (1964) Cardiac action of vasoactive polypeptides in the rat. I. Bradykinin. II Angiotensin. Circ Res 16:150–161

Salyers AK, Rozek LF, Bittner SE, Walsh GM (1988) Simultaneous determination of ventricular function and systemic hemodynamics in the conscious rat. J Pharmacol Meth 19:267–274

Schölkens BA, Becker RHA, Kaiser J (1984) Cardiovascular and antihypertensive activities of the novel non-sulfhydryl converting enzyme inhibitor 2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (Hoe 498). Arzneim Forsch/Drug Res 34:1417–1425

Schölkens BA, Martorana PA, Göbel H, Gehring D (1986) Cardiovascular effects of the converting enzyme inhibitor ramipril (Hoe 498) in anesthetized dogs with acute ischemic left ventricular failure. Clin Exper Theory and Practice A8 (6) 1033–1048

Smiseth OA, Mjøs OD (1982) A reproducible and stable model of acute ischemic left ventricular failure in dogs. Clin Physiol 2:225–239

Sweet CS, Ludden CT, Frederick CM, Ribeiro LGT (1984) Hemodynamic effects of angiotensin and renin inhibition in dogs with acute left ventricular failure. Amer J Med 77:7–12

Valdes-Cruz LM, Horowitz S, Sahn DJ, Larson D, Lima CO, Mesel E (1984) Validation of a Doppler echocardiographic method for calculating severity of discrete stenotic obstructions in a canine preparation with a pulmonary arterial band. Circulation 69:1177–1181

A.1.3.11 Hemodynamic Measurements in Conscious Dogs

PURPOSE AND RATIONALE

The potency of a cardiovascular drug depends on the direct effects at the cellular level and on the response of the cardiovascular control mechanisms. The latter are often markedly influenced by anesthesia. The chronically instrumented conscious dog with renal hypertension is therefore a more realistic test model to evaluate the effects of antihypertensive, anti-anginal and cardiotonic compounds. The test is used to evaluate hemodynamic drug effects in conscious dogs, an experimental model with chronic arterial and ventricular catheterization and renal artery constriction.

PROCEDURE

Male or female Labrador-Harrier dogs weighing 15–25 kg are used. They are anaesthetized with 1 mg/kg xylazine i.m., followed by 1 mg/kg xylazine i.v. and 18 mg/kg pentobarbital sodium i.v. For chronic instrumentation and induction of renal hypertension, fluid-filled catheters are implanted into the abdominal aorta and into the left ventricle. The catheters are tunneled subcutaneously and exteriorized on the nape of the neck dorsally. Renal hypertension is induced by placing silastic constrictors around both renal arteries. Hemodynamic measurements are performed after a two-week recovery period or later.

To familiarize the dogs to the test surroundings, they are brought into the laboratory 2–3 times before the start of the study. Thus, drug testing is possible without sedation. During the experiment the animal rests quietly on a laboratory table.

Experimental Protocol

Hemodynamic measurements are performed by connecting the two implanted catheters to Statham pressure transducers. Pressure signals, electronically dif-

ferentiated LVP d*p*/d*t* max and heart rate are recorded with a polygraph.

After reaching stable hemodynamic conditions for at least 20 min (control baseline values), the test compound is administered either orally in a gelatin capsule or by intravenous injection into the cephalic vein.

Hemodynamic parameters are recorded continuously starting 30 min before to 120 min after drug administration, and thereafter at 1 h intervals until 6 h after dosage.

EVALUATION

The following parameters are monitored:

- systolic blood pressure [mm Hg]
- diastolic blood pressure [mm Hg]
- left ventricular enddiastolic blood pressure, LVEDP [mm Hg]
- left ventricular pressure at d*p*/d*t* max [mm Hg/s]
- heart rate [beats/min]

Mean values ± SEM are calculated with $n > 3$ as differences to pre-drug control values.

MODIFICATIONS OF THE METHOD

Mann et al. (1987) described a simple procedure for direct blood pressure measurement in conscious dogs using the Vascular-Access-Port™, consisting of a 33 × 13 mm reservoir body affixed to a silicon rubber catheter.

Müller-Schweinitzer (1984) described a method for the assessment of vasoconstrictor agents by recording venous compliance in the conscious dog. Changes in the diameter of the canine saphenous vein, produced by inflation to 45 mm Hg of a sphygmomanometer cuff placed on the upper hind leg, were recorded.

Hintze and Vatner (1983) compared the effects of nifedipine and nitroglycerin in conscious dogs, instrumented for instantaneous and continuous measurements of coronary arterial and left ventricular diameters with an ultrasonic dimension gauge, arterial and left ventricular pressure with implanted miniature gauges, and coronary blood flow with an electromagnetic flowmeter or a Doppler ultrasonic flowmeter.

Shimshak et al. (1986) studied the recovery of regional myocardial contractile function after a 10 min coronary artery occlusion in chronically instrumented conscious dogs.

Wright et al. (1987) described a minimally invasive technique which allows assessment of histamine H_1-receptor antagonist activity in conscious dogs based on the inhibition of tachycardia caused by intravenous administration of the H_1-receptor agonist, 2-pyridylethylamine.

Hashimoto et al. (1991) studied the coronary effects of nicorandil in comparison with nitroglycerin in chronic conscious dogs instrumented with ultrasonic crystals and electromagnetic flowmeters in the circumflex coronary artery.

Hartman and Warltier (1990) described a model of multivessel coronary artery disease using conscious, chronically instrumented dogs. A hydraulic occluder and Ameroid constrictor were implanted around the left anterior descending and the left circumflex coronary arteries. Pairs of piezoelectric crystals were implanted within the subendocardium of the left anterior descending and the left circumflex coronary artery perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion.

Hof et al. (1990) used the Doppler method for measuring cardiac output in **conscious rabbits**.

Grohs et al. (1993) simultaneously assessed cardiac output with pulsed Doppler and electromagnetic flowmeters during cardiac stimulation.

REFERENCES AND FURTHER READING

Bohn H, Rosenstein B (1986) Technical notes on chronic fluid-filled catheters and renal artery constrictors for testing hemodynamic drug effects in conscious hypertensive dogs. J Pharmacol Meth 16:227–238

Grohs JG, Huber S, Raberger G (1993) Simultaneous assessment of cardiac output with pulsed Doppler and electromagnetic flowmeters during cardiac stimulation. J Pharmacol Toxicol Meth 30:33–38

Hartman JC, Warltier DC (1990) A model of multivessel coronary artery disease using conscious, chronically instrumented dogs. J Pharmacol Meth 24:297–310

Hashimoto K, Kinoshita M, Ohbayashi Y (1991) Coronary effects of nicorandil in comparison with nitroglycerin in chronic conscious dogs. Cardiovasc Drugs Ther 5:131–138

Hintze TH, Vatner SF (1983) Comparison of effects of nifedipine and nitroglycerin on large and small coronary arteries and cardiac function in dogs. Circ Res 52 (Suppl I):139–146

Hof RP, Hof A, Stürm RP (1990) The Doppler method for measuring cardiac output in conscious rabbits: Validation studies, uses, and limitations. J Pharmacol Meth 24:263–276

Mann WA, Landi MS, Horner E, Woodward P, Campbell S, Kinter LB (1987) A simple procedure for direct blood pressure measurement in conscious dogs. Lab Anim Sci 37:105–108

Müller-Schweinitzer E (1984) The recording of venous compliance in the conscious dog: A method for the assessment of vasoconstrictor agents. J Pharmacol Meth 12:53–58

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydroxy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43(I) 313–319

Sarazan RD (1991) The chronically instrumented conscious dog model. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany. pp 37–44

Shimshak TM, Preuss KC, Gross GJ, Brooks HL, Warltier DC (1986) Recovery of contractile function in postischemic reperfused myocardium of conscious dogs: influence of nicorandil, a new antianginal agent. Cardiovasc Res 20:621–626

Vatner SF, Higgins CB, Franklin D, Braunwald E (1971) Effect of a digitalis glycoside on coronary and systemic dynamics in conscious dogs. Circ Res 28:470–479

Wright A, Raval P, Eden RJ, Owen DAA (1987) Histamine H_1-receptor antagonist activity assessed in conscious dogs. J Pharm Meth 18:123–129

A.1.3.12 Hemodynamic Studies in Monkeys

PURPOSE AND RATIONALE

Prior to studies in human beings, studies of cardiovascular effects of new drugs in monkeys are necessary in some instances.

PROCEDURE

Rhesus monkeys of either sex, weighing between 5 and 8 kg are anesthetized with 20 mg/kg ketamine hydrochloride followed by 50 mg/kg pentobarbital-Na given slowly i.v. A small side-branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure recordings using a blood pressure transducer (P23 ID). Heart rate is determined from a conventional ECG lead by a biotachometer. Compounds are administered either intravenously or via a gastric fibroscope, e. g., Olympus XP 10, into the duodenum under visual control. The cardiovascular parameters are registered for a pretest period of 30 min and then during 60 min after intravenous administration or 2 h after intragastric administration of the test drug. Three to 6 animals are used for evaluation.

EVALUATION

Mean values ± SD are calculated for the pretest period and for the cardiovascular effects every min for 5 min after i.v. administration and then every 5 min. After intragastric administration the values are registered every 5 min up to 30 min and then every 10 min. The values after administration of the test compound are compared statistically with the pretest values using the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Lacour et al. (1993) studied cardiovascular parameters in conscious **cynomolgus monkeys** (*Macaca fascicularis*). A silicone catheter (internal and external diameter 0.64 and 1.19 mm, respectively) was implanted under aseptic conditions into the thoracic aorta via a carotid artery after the monkeys had been anesthetized with 40 mg/kg ketamine and 0.5 mg/kg acepromazine intramuscularly. The vascular catheter (filled with an aqueous solution of 40% polyvinylpyrolidone and 20% heparin) was inserted into a carotid artery. A patch of silicone was sewn around the artery to maintain the catheter in position, the latter being routed subcutaneously and exteriorized at the top of the head into a stainless steel connector. This connector was fixed to the skull with screws and dental cement, and sealed with a plug to protect the catheter from damage. The monkeys were permitted a 3-week minimum recovery period. Before the experiment was performed the monkeys were placed in a primate-restraining chair on several occasions, of gradually increasing duration, for experiment acclimatization.

Pulsatile arterial pressure was recorded by connecting the arterial catheter to a polygraph via a Statham P23Id pressure transducer. Mean arterial pressure and heart rate were derived from the pulse pressure signal and recorded. A catheter was inserted acutely into a saphenous vein for administration of compounds.

REFERENCES AND FURTHER READING

Lacour C, Roccon A, Cazaubon C, Segondy D, Nisato D (1993) Pharmacological study of SR 47436, a non-peptide angiotensin II AT^1-receptor antagonist, in conscious monkeys. J Hypertens 11:1187–1194

Linz W, Klaus E, Albus U, Becker R, Mania D, Englert HC, Schölkens BA (1992) Cardiovascular effects of the novel potassium channel opener (3S,4R)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulfonyl-chromane hemihydrate. Arzneim-Forsch/Drug Res 42:1180–1185

A.1.3.13 Measurement of Cardiac Output and Regional Blood Flowwith Microspheres

PURPOSE AND RATIONALE

The microsphere technique allows the measurement of cardiac output and regional blood flow. Using different radionuclides, repeated determinations are possible. The method is applicable not only for dogs, cats, and minipigs (Hof et al. 1980) but also for rats (McDevitt and Nies 1976; Bonnacrossi et al. 1978; Ishise et al. 1980; Stanek et al. 1985) using microspheres of appropriate size.

PROCEDURE

Male Sprague-Dawley rats weighing 265–375 g are anesthetized with 35 mg/kg i.p. pentobarbital. The

right carotid and right femoral arteries are cannulated. Using pressure monitoring, a carotid cannula is manipulated into the left ventricle. Carbonized microspheres ($15 \pm 5\,\mu$ diameter) labelled with ^{85}Sr are drawn into a glass injection chamber and suspended in 0.3 ml 6% dextran so that each chamber contains 60000 to 80,000 microspheres. The radioactivity in each chamber is determined by gamma scintillation counting before and after microsphere injection, the difference being the amount of radioactivity injected. The microspheres are injected into the left ventricle in a total volume of 0.8 ml 6% dextran over 20 s. Simultaneously, arterial blood from the femoral artery is withdrawn at 0.8 ml/min for 90 s with a syringe withdrawal pump.

EVALUATION

This reference blood sample is used to calculate the cardiac output by the formula:

$$\text{cardiac output} = \text{counts injected} \times \frac{\text{reference sample withdrawal rate}}{\text{reference sample counts}}$$

After obtaining the reference sample, the animals are sacrificed with pentobarbital and the organs dissected, placed in counting vials, and counted for 5 min. Regional distribution of the cardiac output is calculated by comparing the radioactivity in each organ with the total injected radioactivity. Organ flow is determined by multiplying the cardiac output by the fractional distribution of the cardiac output to the organ.

CRITICAL ASSESSMENT OF THE METHOD

Problems associated with the microsphere technique in rats are the hemodynamic effects of the solutions used to inject the microspheres and the effects of blood withdrawal after repeated determinations (Stanek et al. 1985).

MODIFICATIONS OF THE METHOD

For repeated determinations, other nuclides have been used, such as ^{46}Sc, ^{51}Cr, ^{141}Ce, ^{125}I (Hof et al. 1980).

Kovách et al. (1992) used up to 5 radiolabelled microspheres (^{57}Co, ^{113}Sn, ^{85}Sr, ^{95}Nb and ^{46}Sc) for measurement of regional cerebral blood flow in cats.

Faraci and Heistad (1992) measured blood flow with radioactive microspheres (15 μ diameter) labeled with ^{46}Sc, ^{95}Nb, ^{153}Gd, ^{85}Sr, and ^{141}Ce in anesthetized rabbits.

Grover et al. (1990), Gross et al. (1992) measured myocardial blood flow in dogs with the radioactive microsphere technique.

Kowallik et al. (1991) measured regional myocardial blood flow with multiple colored microspheres. The method yielded values very similar to those obtained with radioactive microspheres.

REFERENCES AND FURTHER READING

Bonnacrossi A, Dejana E, Qunintana A (1978) Organ blood flow measured with microspheres in the unanesthetized rat: effects of three room temperatures. J Pharmacol Meth 1:321–328

Faraci FM, Heistad DD (1992) Does basal production of nitric oxide contribute to regulation of brain-fluid balance? Am J Physiol 262:H340–H344

Flaim SF, Nellis SH. Toggart EJ, Drexler H, Kanda K, Newman ED (1984) Multiple simultaneous determinations of hemodynamics and flow distribution in conscious rats. J Pharmacol Meth 11:1–39

Gross GJ, Auchampach JA, Maruyama M, Warltier DC, Pieper GM (1992) Cardioprotective effects of nicorandil. J Cardiovasc Pharmacol 20 (Suppl 3):S22–S28

Grover GJ, Sleph PG, Dzwonczyk S (1990) Pharmacological profile of chromakalim in the treatment of myocardial ischemia in isolated rat hearts and anesthetized dogs. J Cardiovasc Pharmacol 16:853–864

Heymann MA, Payne BD, Hoffmann JIE, Rudolph AM (1977) Blood flow measurements with radionuclide-labeled particles. Prog Cardiovasc Dis 20:55–79

Hof RP, Wyler F, Stalder G (1980) Validation studies for the use of the microsphere method in cats and young minipigs. Basic Res Cardiol 75:747–756

Ishise S, Pegram BL, Yamamoto J, Kitamura Y, Frohlich ED (1980) Reference sample microsphere method: cardiac output and blood flows in conscious rat. Am J Physiol 239:H443–449

Kovách AGB, Szabó C, Benyó Z, Csaki C, Greenberg JH, Reivich M (1992) Effects of N^G-nitro-L-arginine and L-arginine on regional cerebral blood flow in the cat. J Physiol 449:183–196

Kowallik P, Schulz R, Guth BD, Schade A, Pfaffhausen W, Gross R, Heusch G (1991) Measurement of regional myocardial blood flow with multiple colored microspheres. Circulation 83:974–982

McDevitt DG, Nies AS (1976) Simultaneous measurement of cardiac output and its distribution with microspheres in the rat. Cardiovasc Res 10:494–498

Stanek KA, Coleman TG, Smith TL, Murphy WR (1985) Two hemodynamic problems commonly associated with the microsphere technique for measuring regional blood flow in rats. J Pharmacol Meth 13:117–124

A.1.3.14 Carotid Artery Loop Technique

PURPOSE AND RATIONALE

The carotid loop method, originally described by van Leersum (1911) for rabbits has been used by several authors (e. g., Child and Glenn 1938; Valli et al. 1967; O'Brien et al. 1971; Meyer et al. 1989) for measurement of blood pressure or blood sampling in conscious dogs and sheep (Lagutchik et al. 1992).

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, continued with an infusion of 4–6 mg/kg/h. The animal is placed on a heated operating table. The skin on the ventral side of the neck is carefully shaved and disinfected. The course of the carotid artery is outlined by palpation along the tracheal border. About 2 cm of skin is taken on each side marking the width of the flap. The medial incision is made slightly above the thyroid cartilage and is extended caudal to a point about 1 cm lateral and 1 cm above the manubrium sterni. The lateral incision again lies about 2 cm from the line of the carotid artery and parallel to it. The lateral incision is only half as long as the medial one. The incisions are made down to the subcutaneous tissue over the platysma muscle. Between the skin and the muscle, the flap is undermined. All bleeding points are carefully clamped and tied.

The subcutaneous tissue, the platysma myoides muscle and the anterior fascia of the neck are incised in the course of the midline incision down to the plane of cleavage between the sternohyoid and sternomastoid muscle. By blunt dissection, these muscles are separated, disclosing at their depth the neurovascular bundle over which lies the internal jugular vein. The floor of the space so isolated is formed by the longus capitis muscle. By careful dissection, these muscles are separated at least 1 cm above and below the limits of the incision in the skin. The superior thyroid artery marks the uppermost portion of the carotid artery suitable for exteriorization. The plane of cleavage is followed caudal to the origin of the sternocleidomastoid muscle at the manubrium sterni. Throughout the limits of the incision the artery is dissected free from the internal jugular vein and then from the vagus nerve.

The first step in the exteriorization of the artery is the reapproximation of the muscle borders beneath the vessel by mattress sutures. In order to prevent tension on the completed loop due to contraction of the sternomastoid and sternohyoid muscle, it is important to reapproximate these muscles throughout their course. Sutures are placed at the edges of skin. The tubular flap of skin is then approximated loosely around the carotid artery. It is essential that the skin flap fits loosely around the artery. A continuous suture of fine silk is started at the place where the vessel emerges from the muscle borders. The suture is so placed as to include the artery in a sling of skin which isolates the vessel from the line of suture of the underside of the completed loop. Finally, the proximal and distal quarters of the flap are closed with sutures, while the skin tube is closed with a continuous suture. Antibiotics are given locally and systemically.

One thickness of gauze is placed beneath the loop and along each border a strip of gauze in order to relieve the loop from the pressure caused by the reminder of dressings. Around the neck is wrapped a gauze bandage several turns of which have passed behind the forelimbs in order to prevent the dressing from riding upwards on the animal's neck. Over this is placed a plaster roll protecting the loop from the animal's efforts of scratching. The dressings are changed on the fifth and seventh day when the sutures can be removed.

Blood pressure measurements can be made according to Riva-Rocci's principle by placing a inflatable cuff around the loop.

CRITICAL ASSESSMENT OF THE METHOD

The carotid artery loop method needs some surgical experience and very meticulous caretaking of the animals.

MODIFICATIONS OF THE METHOD

Lewis et al. (1980) placed a CO_2 sensor using mass spectrometry and its through flow cuvette in a common carotid artery-to-jugular vein loop in anesthetized cats.

Meyer et al. (1989a, b) studied pulmonary gas exchange in panting dogs with an exteriorized carotid artery loop.

Kaczmarczyk et al. (1979) used conscious, chronically instrumented dogs with electric flow probes around the left renal artery and a carotid loop to study postprandial volume regulation.

REFERENCES AND FURTHER READING

Child CG, Glenn F (1938) Modification of van Leersum carotid loop for determination of systolic blood pressure in dogs. Arch Surg 36:381–385

Kaczmarczyk G, Schimmrich B, Mohnhaupt R, Reinhardt HM (1979) Atrial pressure and postprandial volume regulation in conscious dogs. Pflüger's Arch Eur J Physiol 38:143–150

Lagutchik MS, Sturgis JW, Martin DG, Bley JA (1992) Review of the carotid loop procedure in sheep. J Invest Surg 5:79–89

Lewis G, Ponte J, Purves MJ (1980) Fluctuations of P(a), (CO_2) with the same period as respiration in the cat. J Physiol (London) 298:1–11l

Meyer M, Hahn G, Buess Ch, Mesch U, Piiper J (1989) Pulmonary gas exchange in panting dogs. J Appl Physiol 66:1258–1263

Meyer M, Hahn G, Piiper J (1989) Pulmonary gas exchange in panting dogs: a model for high frequency ventilation. Acta Anaesthesiol Scand 33, Suppl 90:22–27

O'Brien DJ, Chapman WH, Rudd FV, McRoberts JW (1971) Carotid artery loop method of blood pressure measurement in the dog. J Appl Physiol 30:161–163
Valli VEO, McSherry BJ, Archibald J (1967) The preparation and use of carotid loops. Can Vet Jour 8:209–211
van Leersum EC (1911) Eine Methode zur Erleichterung der Blutdruckmessung bei Tieren. Arch ges Physiol 142:377–395

A.1.3.15 Measurement of Heart Dimensions in Anesthetized Dogs

PURPOSE AND RATIONALE

The measurement of the heart dimensions allows to localize the effect of a drug on the activity of the heart. An ultrasonic technique is used for continuous measurement of left ventricular dimensions. Compounds are tested with potential anti-anginal activity due to the reduction of left ventricular diameter. The test is used to evaluate the influence of drugs on left ventricular external and internal diameter in anesthetized dogs.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used for the test. The dog is anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium followed by subcutaneous injection of 2 mg/kg morphine. Respiration is maintained through a tracheal tube with N_2O/O_2 (3:1) using a positive pressure respirator.

Implantation of Ultrasonic Transducers

Ultrasonic transducers are constructed and implanted as described by Stinson et al. (1974).

To measure left ventricular external diameter (LVED), two ultrasonic transducers are fixed to the left ventricular wall. One crystal is sutured to the posterior wall within the rectangular area formed by the left circumflex coronary artery and the left posterior descending artery. The other one is placed near the first diagonal branch of the left anterior descending coronary artery. Exact positioning is assured with an oscilloscope.

To measure left ventricular internal diameter (LVID), the transducers are placed in the same anatomical area as for the epicardial crystals. However, they are pushed through the wall of the left ventricle through stab wound incisions. The crystals are positioned across the greatest transverse diameter of the left ventricle, one on the anterior and the other on the posterior endocardial wall.

Bleeding during the implantation procedure is controlled by umbilical tapes around the cranial and caudal veins and by purse string sutures at the implantation sites. The pericardial incision and the chest is closed by sutures and the transducer wires are connected to the recording equipment.

In each dog, either LVED or LVID is measured together with the other hemodynamic parameters.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannulated femoral artery by a pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left A. carotis communis. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dp/d$t$max is differentiated and heart rate is calculated.

Hemodynamic parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 30 min (control values), the test substance is administered by intravenous or intraduodenal injection.

Readings are taken at times 0, 15, 30, 45, 60, 75, 90 and 120 after drug administration. Left ventricular dimensions are measured at the end of the diastole and systole.

Characteristics:

- blood pressure
  - systolic blood pressure
  - diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure
- left ventricular contractility, dp/dt
- heart rate, HR
- left ventricular external diameter, LVED
- left ventricular internal diameter, LVID

EVALUATION

Hemodynamic parameters, LVED and LVID [mm] are determined.

Changes in parameters after drug administration are compared to control values obtained during the 30 min pre-drug period.

Statistical significance is assessed by means of the paired t-test.

Since a change in the diameter of the left ventricle is a reasonable accurate index of left ventricular volume, a reduction of LVED or LVID with no change in dp/dt and HR can be considered as a strong indicator

for "venous pooling" and thus an anti-anginal activity of a compound.

Scores are allotted relative to the efficacy of standard compounds assessing the intensity as well as the duration of the effect.

Standard data:

		LVED [mm]		LVID [mm]	
Nitroglycerin	0.005 mg/kg, i.v.	–0.9	20 min	–1.2	30 min
Isosorbide-dinitrate	0.1 mg/kg, i.v.			–0.6	120 min
Molsidomine	0.2 mg/kg, i.v.	–2.1	>60 min	–1.4	>120 min
Nifedipine	0.1 mg/kg, i.v.			+1.2	120 min

MODIFICATIONS OF THE METHOD

Novosel et al. (1992) measured the dimensions of the right ventricle with microsonometry in anesthetized rabbits.

REFERENCES AND FURTHER READING

Barnes GE, Horwith LD, Bishop VS (1979) Reliability of the maximum derivatives of left ventricular pressure and internal diameter as indices of the inotropic state of the depressed myocardium. Cardiovasc Res 13:652–662

Bishop VS, Horwitz LD (1971) Effects of altered autonomic control on left ventricular function in conscious dogs. Am J Physiol 221:1278–1282

Fiedler VB, Oswald S, Göbel H, Faber W, Scholtholt J (1980) Determination of left ventricular dimensions with ultrasound. J Pharmacol Meth 3:201–219

Horwitz LD, Bishop VS (1972) Left ventricular pressure-dimension relationships in the conscious dog. Cardiovasc Res 6:163–171

Novosel D, Hof A, Evenou JP, Hof PP (1992) Assessment of right ventricle dimensions with microsonometry in anesthetized rabbits. J Pharmacol Toxicol Meth 28:73–77

Stinson EB, Rahmoeller G, Tecklenberg PL (1974) Measurement of internal left ventricular diameter by tracking sonomicrometer. Cardiovasc Res 8:283–289

Suga H, Sagawa K (1974) Assessment of absolute volume from diameter of the intact canine left ventricular cavity. J Appl Physiol 36:496–499

A.1.3.16 Telemetric Monitoring of Cardiovascular Parameters in Rats

PURPOSE AND RATIONALE

Radiotelemetry allows the recording of cardiovascular parameters in conscious, free-moving animals. Several authors (Brockway et al. 1991; Mattes and Lemmer 1991; Guiol et al. 1992; Morimoto et al. 1992; Basil et al. 1993; Brockway and Hassler 1993; Lemmer et al. 1993, 1994, 1995; Calhoun et al. 1994; Diamant et al. 1993; Kramer et al. 1993a, 1995; Griffin et al. 1994; Kuwahara et al. 1994; Sato et al. 1994, 1995; van den Buuse et al. 1994; Kinter 1996; Becker et al. 1997; Witte et al. 1998) used commercially available systems with some modifications to study the circadian rhythm of blood pressure and the influence of drugs on heart rate, blood pressure and motility in rats.

PROCEDURE

The telemetry and data acquisition system (e. g., Data Sciences International, Inc., St Paul MN) consists of four parts:

1. the implantable transmitter, which measures the pressure. This device contains a highly stable, ion-implant, semiconductor, strain-gauge sensor and battery-powered electronics to process the information from the pressure sensor and to telemeter it from within the animal. Arterial pressure is transmitted to the sensor via a 0.7-mm diameter, fluid-filled catheter;
2. the receiver which detects the signal from the implanted transmitter and converts it to a form readable by computer;
3. the pressure reference module, which measures atmospheric pressure to allow for the telemetered absolute pressure to be converted to a gauge pressure;
4. the data acquisition software, which accepts data from the reference module and the receivers, filters corrupt samples from the incoming data stream, converts the telemetered pressure to millimeters of mercury, subtracts atmospheric pressure from the telemetered pressure, and stores the data for retrieval, plotting, and analysis.

Under pentobarbital anesthesia, the telemetry transmitter is implanted into rats. The descending aorta is exposed between the renal arteries. A vascular clamp is made by putting two surgical threads on the proximal and distal part of the artery. The catheter tip is inserted through an incision in the vessel. A drop of cyanoacrylate glue is applied to the dried entry point. The transmitter is sutured to the abdominal musculature.

EVALUATION

Data from individual animals are recorded over long periods of time which allow the investigator to follow the circadian rhythm under several experimental conditions.

MODIFICATIONS OF THE METHOD

Hess et al. (1996) monitored pulmonary arterial pressure in freely moving **rats** by inserting the sensing

catheter of a telemetric system through a small hole and pushing it into the pulmonary artery.

Further cardiovascular studies in rats using the telemetric system were reported by Sgoifo et al. (1998), Webb et al. (1998).

Kramer et al. (1993b) used telemetry to record electrocardiogram and heart rate in freely moving **mice**.

Carlson and Wyss (2000) used small telemetry probes for long term recording of arterial pressure and heart rate in mice after implantation to the carotid artery or the abdominal aorta.

DePasquale et al. (1994) used radiotelemetry to monitor cardiovascular function in conscious **guinea pigs**.

Telemetric ECG recordings in **cardiomyopathic hamsters** were reported by Desjardins et al. (1996).

Van den Buuse and Malpas (1997) studied cardiovascular parameters in **rabbits** by radiotelemetry.

Astley et al. (1991), Smith et al. (1993) used telemetric systems to monitor cardiovascular responses in **baboons**.

Schnell and Wood (1993) measured blood pressure and heart rate by telemetry in conscious, unrestrained **marmosets**.

An ultrasonic blood flowmeter telemetry system for **cats** and rabbits has been described by Yonezawa et al. (1989, 1992).

Telemetry was used by Symons et al. (1992) to monitor the severity of events representing myocardial dysfunction in **miniswine**.

Savory and Kostal (1997) applied the telemetric system for chronic measurement of cardiovascular and other parameters in **chicken**.

Radiotelemetry has also been used for other pharmacological experiments, such as field potential analysis by radioelectroencephalography (see Sect. E.1.2.6), step through passive avoidance (see Sect. F.3.1.2), shock-prod burying test in rats (see Sect. E.2.5.3), measurement of body temperature (see Sect. H.4.0.2) and motility in rats and mice (Clement et al. 1989; Guillet et al. 1990; Diamant et al. 1993; van den Buuse 1994).

REFERENCES AND FURTHER READING

Astley CA, Smith OA, Ray RD, Golanov EV, Chesney MA, Chalyan VG, Taylor DJ, Bowden DM (1991) Integrating behavior and cardiovascular response: the code. Am J Physiol, Regul Integr Comp Physiol 261:R172–R181

Basil MK, Krulan C, Webb RL (1993) Telemetric monitoring of cardiovascular parameters in conscious spontaneously hypertensive rats. J Cardiovasc Pharmacol 22:897–905

Becker RHA, Baldes L, Furst U, Schulze KJ (1997) Sustained diurnal blood pressure reduction in SHR with ramipril assessed by telemetric monitoring. Clinical and Experimental Hypertension. Vol 19:1233–1246

Brockway BP, Medvedev OS (1991) Circulatory studies on rats using telemetry instrumentation and methodology. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany. pp 142–147

Brockway BP, Hassler CR (1993) Application of radiotelemetry to cardiovascular measurements in pharmacology and toxicology. In: Salem H, Baskin SI (eds) New Technologies and Concepts for Reducing Drug Toxicities. CRC Press, Boca Raton, pp 109–132

Brockway BP, Mills PA, Azar SH (1991) A new method for continuous chronic measurement and recording of blood pressure, heart rate and activity via radiotelemetry. Clin Exp Hyper Theory Pract A13:885–895

Calhoun DA, Zhu S, Wyss JM, Oparil S (1994) Diurnal blood pressure variations and dietary salt in spontaneously hypertensive rats. Hypertension 24:1–7

Carlson SH, Wyss JM (2000) Long-term telemetric recording of arterial pressure and heart rate in mice fed basal and high NaCl diets. Hypertension 35:e1–e5

Clement JG, Mills P, Brockway B (1989) Use of telemetry to record body temperature and activity in mice. J Pharmacol Meth 21:129–140

DePasquale MJ, Ringer LW, Winslow RL, Buchholz R, Fossa AA (1994) Chronic monitoring of cardiovascular function in the conscious guinea pig using radiotelemetry. Hypertension 16:245–260

Desjardins S, Cauchy MJ, Kozliner A (1996) The running cardiomyopathic hamster with continuous telemetric ECG: A new heart failure model to evaluate 'symptoms', cause of death and heart rate. Exper Clin Cardiol 1:29–36

Diamant M, von Wolfswinkel L, Altorffer B, de Wied D (1993) Biotelemetry: Adjustment of a telemetry system for simultaneous measurement of acute heart rate changes and behavioral events in unrestrained rats. Physiol Behav 53:1121–1126

Griffin KA, Picken M, Bidani AK (1994) Radiotelemetric BP monitoring, antihypertensives and glomeruloprotection in remnant kidney model. Kidney Internat 46:1010–1018

Guillet MC, Molinié B, Laduron PM, Terlain B (1990) Effects of ketoprofen in adjuvant-induced arthritis measured in a new telemetric model test. Eur J Pharmacol 183:2266–2267

Guiol C, Ledoussal C, Surgé JM (1992) A radiotelemetry system for chronic measurement of blood pressure and heart rate in the unrestrained rat. Validation of the method. J Pharmacol Toxicol Meth 28:99–105

Hess P, Clozel M, Clozel JP (1996) Telemetric monitoring of pulmonary pressure in freely moving rats. J Appl Physiol 81:1027–1032

Kinter L (1996) Cardiovascular Telemetry and Laboratory Animal Welfare: New Reduction and Refinements Alternatives. SMD, Data Sciences International, Inc., pp 1–10

Kramer K, Dijkstra H, Bast A (1993a) Control of physical exercise in rats in a swimming basin. Physiol Behav 53:271–276

Kramer K, van Acker SABE, Voss HP, Grimbergen JA, van der Vijgh WJF, Bast A (1993b) Use of telemetry to record electrocardiogram and heart rate in freely moving mice. J Pharm Toxicol Meth 30:209–215

Kramer K, Grimbergen JA, van der Gracht L, van Jeperen DJ, Jonker RJ, Bast A (1995) The use of telemetry to record electrocardiogram and heart rate in freely swimming rats. Meth Find Exp Clin Pharmacol 17:107–112

Kuwahara M, Yayou KI, Ishii K, Hashimoto SI, Tsubone H, Sugano S (1994) Power spectral analysis of heart rate vari-

ability as a new method for assessing autonomic activity in the rat. J Electrocardiol 27:333–337
Lee JY, Brune ME, Warner RB, Buckner SB, Winn M, De B, Zydowsky TM, Opgenorth TJ, Kerkman DJ, DeBerhardis JF (1993) Antihypertensive activity of ABBOTT-81282, a nonpeptide angiotensin II antagonist, in the renal hypertensive rat. Pharmacology 47:176–187
Lemmer B, Mattes A, Böhm M, Ganten D (1993) Circadian blood pressure variation in transgenic hypertensive rats. Hypertension 22:97–101
Lemmer B, Witte K, Makabe T, Ganten D, Mattes A (1994) Effects of enalaprilat on circadian profiles in blood pressure and heart rate of spontaneously and transgenic hypertensive rats. J Cardiovasc Pharmacol 23:311–314
Lemmer B, Witt K, Minors D, Waterhouse J (1995) Circadian rhythms of heart rate and blood pressure in four strains of rat: Difference due to, and separate from locomotor activity. Biol Rhythm Res 26:493–504
Mattes A, Lemmer B (1991) Effects of amlodipine on circadian rhythms in blood pressure, heart rate, and motility: a telemetric study in rats. Chronobiol Internat 8:526–538
Morimoto K, Morimoto A, Nakamori T, Tan N, Minagawa T, Murakami N (1992) Cardiovascular responses induced in free-moving rats by immune cytokines. J Physiol 448:307–320
Rubini R, Porta A, Baselli G, Cerutti S, Paro M (1993) Power spectrum analysis of cardiovascular variability monitored by telemetry in conscious unrestrained rats. J Autonom Nervous System 45:181–190
Sato K, Kandori H, Sato SH (1994) Evaluation of a new method using telemetry for monitoring the left ventricular pressure in free-moving rats. J Pharmacol Toxicol Meth 31:191–198
Sato K, Chatani F, Sato S (1995) Circadian and short-term variabilities in blood pressure and heart rate measured by telemetry in rabbits and rats. J Auton Nerv Syst 54:235–246
Savory CJ, Kostal L (1997) Application of a telemetric system for chronic measurement of blood pressure, heart rate, EEG and activity in the chicken. Physiol Behav 61:963–969
Schnell CR, Wood JM (1993) Measurement of blood pressure and heart rate by telemetry in conscious, unrestrained marmosets. Am J Physiol 264 (Heart Circ Physiol 33):H1509–H1516
Sgoifo A, Stilli D, deBoer SF, Koolhaas JM, Musso E (1998) Acute social stress and cardiac electrical activity in rats. Aggress Behav 24:287–296
Smith OA, Astley CA, Spelman FA, Golanov EV, Chalyan VG, Bowden DM, Taylor DJ (1993) Integrating behavior and cardiovascular responses: posture and locomotion. I. Static analysis. Am J Physiol 265 (Regul Integr Comp Physiol 34): R1458–R1568
Symons JD, Pitsillides KF, Longhurst CJ (1992) Chronic reduction of myocardial ischemia does not attenuate coronary collateral development in miniswine. Circulation 86:660–671
Tornatzky W, Miczek KA (1993) Long-term impairment of autonomic circadian rhythms after brief intermittent social stress. Physiol Behav 53:983–993
van den Buuse M (1994) Circadian rhythms of blood pressure, heart rate, and locomotor activity in spontaneously hypertensive rats as measured with radiotelemetry. Physiol Behav 55:783–786
van den Buuse M, Malpas SC (1997) 24-Hour recordings of blood pressure, heart rate and behavioural activity in rabbits by radiotelemetry: effects of feeding and hypertension. Physiol Behav 62:83–89
Webb RL, Navarrete AE, Davis S, de Gasparo M (1998) Synergistic effects of combined converting enzyme inhibition and angiotensin II antagonism on blood pressure in conscious telemetered spontaneously hypertensive rats. J Hypertens 16:843–852
Witte K, Schnecko A, Buijs R, van der Vliet J, Scalbert E, Delagrange P, Guardiola-Lemaître B, Lemmer B (1998) Effects of SCN lesions on circadian blood pressure rhythm in normotensive and transgenic hypertensive rats. Chronobiol Intern 15:135–145
Yonezawa Y, Caldwell WM, Schadt JC, Hahn AW (1989) A miniaturized ultrasonic flowmeter and telemetry transmitter for chronic animal blood flow measurements. Biomed Sci Instrument 25:197–111
Yonezawa Y, Nakayama T, Ninomija I, Caldwell WM (1992) Radiotelemetry directional ultrasonic blood flowmeter for use with unrestrained animals. Med Biol Eng Comput 30:659–665

A.1.3.17 Cardiovascular Effects After Intracerebroventricular Administration

PURPOSE AND RATIONALE

Several drugs, like α_2-adrenergic agonists, act primarily at central sites. Their effects can be most clearly demonstrated after injection into the cerebroventricular system. The first experiments have been performed in cats. The method has been adapted to rats.

PROCEDURE

Rats of either sex weighing 250–350 g are anesthetized with 100 mg/kg hexobarbital i.p. The scalp is cut in a sagittal line. With a dental drill a hole of 1–1.5 mm diameter is drilled through the cranial bone 1 mm lateral and 2 mm caudal of the bregma. A PVC-catheter is introduced perpendicular to the bone to a depth of 3 mm in order to reach the lateral cerebral ventricle. The catheter is fixed with dental cement and the wound closed. Test substances are administered through the catheter. To measure blood pressure one catheter is placed in one carotid artery and connected to a Statham transducer. Blood pressure and heart rate are recorded on a polygraph over a period of at least 30 min. For long acting drugs registration periods up to 2 h are necessary. After the experiment, the animal is sacrificed and the brain removed to confirm the site of injection.

EVALUATION

Systolic and diastolic blood pressure as well as heart rate after intracerebroventricular injection are expressed as percentage of pretreatment values. The response is compared with the standard clonidine which is effective in doses of 4–60 µg.

MODIFICATIONS OF THE METHOD

Based on the work of Feldberg et al. (1954) and Hayden et al. (1966), Mastrianni et al. (1986) developed an

intracerebroventricular perfusion system for the study of centrally acting antihypertensive drugs in the rat. The antihypertensive effect of clonidine could be observed over several hours.

Methods used to detect central hypotensive activity of drugs have been reviewed by Timmermans (1984).

REFERENCES AND FURTHER READING

Feldberg W, Sherwood SL (1954) Injections of drugs into the lateral ventricle of the cat. J Physiol 123:148–167

Hayden JF, Johnson LR, Maickel RP (1966) Construction and implantation of a permanent cannula for making injections into the lateral ventricle of the rat brain. Life Sci 5:1509–1515

Mastrianni JA, Harris TM, Ingenito AJ (1986) An intracerebroventricular perfusion system developed for the study of centrally acting antihypertensive drugs in the rat. J Pharmacol Meth 16:63–72

Timmermans PBMWM (1984). Centrally acting hypotensive drugs. In: van Zwieten (ed) Handbook of Hypertension, Vol 3, Pharmacology of Antihypertensive Drugs. Elsevier Amsterdam, pp 102–153

A.1.3.18 Influence on Orthostatic Hypotension

PURPOSE AND RATIONALE

Orthostatic hypotension with dizziness up to unconsciousness is a syndrome occurring in many human individuals. Moreover, several drugs are known to cause orthostatic hypotension. In several animal species, such as rabbit, cat and dog, this syndrome can be evoked by changing the usual horizontal position into a vertical position with the head upwards using a tilting table.

PROCEDURE

Cats of either sex weighing 2.0–3.0 kg are temporarily anesthetized with ether. Anesthesia is maintained by intravenous injection of 70 mg/kg chloralose. The animal is fixed with its legs on a heated operating table which can be tilted by 90 degrees. The carotid artery is cannulated for measuring blood pressure through a Statham P 23 Db transducer on a 6 channel Hellige recorder. The femoral vein is cannulated for injection of the test compound. After the blood pressure is stabilized for 30 min, the animal is quickly tilted to a vertical position for 1 minute. Due to the change of position and gravitational force, there is a rapid fall in blood pressure which recovers as soon as the animal is restored to its original position. After taking the control reading, the test compound is administered intravenously and the same procedure is repeated. The fall in blood pressure is recorded.

EVALUATION

A significant increase in postural hypotension with respect to the control would indicate that the test compound may produce orthostatic hypotension in human. Moreover, some compounds, like sympathomimetics, can reduce or prevent postural hypotension.

MODIFICATIONS OF THE METHOD

Sponer et al. (1981) described a method for evaluating postural hypotension in conscious **rabbits** placed on a tilting table whereby blood pressure was measured from the central artery of the ear.

Takata et al. (1999) reported a rabbit model for evaluation of chlorpromazine-induced orthostatic hypotension.

Humphrey and McCall (1982) described a model for predicting orthostatic hypotension during acute and chronic antihypertensive drug therapy in **rats** anesthetized with chloralose, urethane and pentobarbital using a heated tilting table.

Lee et al. (1982) evaluated postural hypotension induced by drugs in conscious restrained normotensive rats. Blood pressure was recorded after cannulation of the femoral artery under light ether anesthesia. A special tilting table was build for simultaneous studies in four rats.

Martel et al. (1996, 1998) studied the phenomenon of cardiovascular deconditioning observed in crew members of space flights in rats after tail suspension.

Socci et al. (2000) studied cardiovascular responses to simulated microgravity in Sprague-Dawley rats. Microgravity is known to induce orthostatic intolerance and baroreflex impairment in astronauts. The authors used 30° head-down tilt, 24-h whole-body suspension or 7-day tail suspension to mimic microgravity and to find treatment ameliorating the symptoms.

Baum et al. (1981) studied antihypertensive and orthostatic responses to drugs in conscious **dogs**. A catheter was placed in the subclavian artery for measurement of blood pressure and exteriorized at the back of the neck some days prior to the experiment. The animals were placed into a sling and tilted to the 90° upright position for periods of 60 s. every hour by lifting their forelimbs. Blood pressure response before and after treatment with test drugs was measured.

A none human **primate** model for evaluating the potential of antihypertensive drugs to cause orthostatic hypotension was described by Pals and Orley (1983) Polyvinyl catheters were implanted in the abdominal aorta and the vena cava via an external iliac artery and vein to **cynomolgus monkeys** during ketamine anesthesia. The catheters were routed subcutaneously from

the groin area to the top of the head and exteriorized. After recovery the animals were placed in restraining chairs allowing the change from vertical to horizontal position.

REFERENCES AND FURTHER READING

Baum Th, Vliet GV, Glennon JC, Novak PJ (1981) Antihypertensive and orthostatic responses to drugs in conscious dogs. J Pharmacol Meth 6:21–32

Boura ALA, Green AF (1959) The actions of bretylium: Adrenergic neuron blocking and other effects. Br J Pharmacol 14:536–548

Humphrey SJ, McCall RB (1982) A rat model for predicting orthostatic hypotension during acute and chronic antihypertensive drug therapy. J Pharmacol Meth 25:25–34

Lee CH, Strosber AM, Roszkowski AP, Warren LA (1982) A model for evaluation of postural hypotension induced by drugs in conscious restrained normotensive rats. J Pharmacol Meth 7:15–24

Martel E, Champeroux P, Lacolley P, Richard S, Safar M, Cuche JL (1996) Central hypervolemia in the conscious rat: a model of cardiovascular deconditioning. J Appl Physiol 80:1390–1396

Martel E, Ponchon P, Champeroux P, Elghozi JL, Renaud de la Faverie JF, Dabire H, Pannier B, Richard S, Safar M, Cuche JL (1998) Mechanism of cardiovascular deconditioning induced by tail suspension in the rat. Am J Physiol 274 (5 Pt2):H1667–H1673

Pals DT, Orley J (1983) A none human primate model for evaluating the potential of antihypertensive drugs to cause orthostatic hypotension. J Pharmacol Meth 9:183–192

Socci RR, Wang M, Thierry-Palmer M, Emmett N, Bayorh MA (2000) Cardiovascular responses to simulated microgravity in Sprague-Dawley rats. Clin Exp Hypertens 22:155–164

Sponer G, Mannesmann G, Bartsch W, Dietmann K (1981) A method for evaluating postural hypotension in conscious rabbits as a model to predict effects of drugs in man. J Pharmacol Meth 5:53–58

Takata Y, Kurihara J, Suzuki S, Okubo Y, Kato H (1999) A rabbit model for evaluation of chlorpromazine-induced orthostatic hypotension. Biol Pharm Bull 22:457–462

A.1.3.19 Bezold-Jarisch Reflex

PURPOSE AND RATIONALE

The circulatory collapse after intravenous injection of veratrine has been first described in cats and is known as BEZOLD-JARISCH-reflex (Bezold and Hirt 1867; Jarisch and Richter 1939a, b, Jarisch 1940; Aviado and Guavera-Aviado 2001). Fleckenstein et al. (1950) recommended this as a suitable animal model of shock.

The original observation was a triphasic blood pressure response in cats or dogs characterized by a short lasting fall in blood pressure accompanied by bradycardia, followed by a short lasting increase and than a long-lasting decrease of blood pressure after intravenous injection of veratridin or other veratrum alkaloids.

Kalkman et al. (1984) showed that three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin observed in the Bezold-Jarisch reflex.

The Bezold-Jarisch reflex has been studied in several species, such as **cats** (Takei et al. 1995; Vayssettes-Couchay et al. 1997), **dogs** (Zucker and Cornish 1981; Barron and Bishop 1982; Harron and Kobinger 1984; Giles and Sander 1986; Baugh et al. 1989; Watson et al. 1995), **ferrets** (Andrews and Bhandari 1993), **rabbits** (Chen 1979), guinea pigs, **rats** (Fozard 1984; Gylys et al. 1988; Cohen et al. 1989; Blower 1990; Miyata et al. 1991; Turconi et al. 1991; Matsumoto et al. 1992; Meller et al. 1992; Robertson et al. 1992; Kishibayashi et al. 1993; Geissler et al. 1993; Haga et al. 1994; Hegde et al. 1994; Eglen et al. 1995; Göthert et al. 1995; Delagrange et al. 1996; De Vries 1997; Malinowska et al. 2001; Godlewski et al. 2003) and **mice** (Eglen et al. 1994; Middlefell et al. 1996), whereby species differences have been observed (Yamono et al. 1995).

In cats and dogs, the Bezold-Jarisch reflex was elicited by veratrine and veratridine, but also by capsaicin and the 5-HT_3 receptor agonists 2-methyl-5-HT, phenylbiguanide, chlorophenylbiguanide and serotonin itself.

In rats, mostly 5-HT or 2-methyl-5-HT were used as stimuli to characterize 5-HT_3 receptor antagonists.

PROCEDURE

Male Sprague Dawley rats weighing 250–380 g are given food and water ad libitum, except those used for intraduodenal drug administration; these rats are deprived of food overnight. The animals are anesthetized by intraperitoneal injection of 1.5 g/kg urethane. Body temperature is maintained at 37°C by placing the animal on a heating pad. The left jugular vein or duodenum, trachea and left femoral vein are cannulated for drug administration (i.v. or i.d.), facilitation of respiration and injection of 2-methyl 5-HT, respectively. Heart rate is derived from a limb lead II ECG monitored via subdermal platinum electrodes and is recorded with amplifiers on a polygraph. A dose-response curve to 2-methyl 5-HT (5–100 µg/kg, i.v.) is constructed in each rat to establish a submaximal dose (usually 10 or 20 µg/kg, i.v.) which elicits a reproducible bradycardic response. Each rat receives then a single dose of test drug or standard and is then challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min post dosing. A separate group of rats receiving vehicle (saline for i.v.,

deionized water for i.d.) is similarly tested in each study.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls. Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's multiple comparison test.

MODIFICATIONS OF THE METHOD

Harron and Kobinger (1984) used capsaicin to elicit the Bezold-Jarisch reflex in anesthetized artificially respirated dogs pretreated with a beta-adrenoceptor antagonist to evaluate the activity of clonidine-like drugs on central alpha$_2$ adrenoceptors after intracisternal administration.

The Bezoold-Jarisch reflex in rats has been used for evaluation of 5-HT$_3$ receptor agonists (Rault et al. 1996; López-Tudanca et al. 2003).

Rocha et al. (2003) found an enhancement of the Bezold-Jarisch reflex in the acute phase of myocardial infarction of the anesthetized rabbit.

REFERENCES AND FURTHER READING

Andrews PLR, Bhandari P (1993) Resinferatoxin, an ultrapotent capsaicin analogue, has anti-emetic properties in the ferret. Neuropharmacology 32:799–806

Aviado DM, Guavera-Aviado D (2001) The Bezold-Jarisch reflex. A historical perspective of cardiopulmonary reflexes. Ann NY Acad Sci 940:48–58

Barron KW, Bishop VS (1982) Reflex cardiovascular changes with veratridine in the conscious dog. Am J Physiol, Heart Circ Physiol 11:H810–H817

Baugh L, Abraham W, Matthews E, Lahr P (1989) Pharmacological profile of MDL 26,024GO: A novel antiasthmatic agent. Agents Actions 27:431–434

Blower PR (1990) The role of specific 5-HT$_3$ receptor antagonism in the control of cytostatic drug induced emesis. Eur J Cancer 26, Suppl 1:S8–S11

Chen HI (1979) Interaction between the baroreceptor and Bezold-Jarisch reflexes. Am J Physiol 6:H655–H661

Cohen ML, Bloomquist W, Gidda JS, Lacefield W (1989) Comparison of the 5-HT$_3$ receptor antagonist properties of ICS 205–930, GR38032 and zacopride. J Pharmacol Exp Ther 248:197–201

Delagrange P, Emerit MB, Merahi N, Abraham C, Morain P, Rault S, Renard P, Pfeiffer B, Guardiola-Lemaitre P, Hamon M (1996) Interaction of S 21007 with 5-HT$_3$ receptors. *In vitro* and *in vivo* characterization. Eur J Pharmacol 316:195–203

De Vries P, Apaydin S, Villalon CM, Heiligers JPC, Saxena PR (1997) Interaction of GR127935, a 5-HT$_{1B/D}$ receptor ligand with functional 5-HT receptors. Naunyn-Schmiedeberg's Arch Pharmacol 355:423–430

Eglen RM, Lee CH, Khabbaz M, Fontana DJ, Daniels S, Kilfoil T, Wong EHF (1994) Comparison of potencies of 5-HT$_3$ receptor antagonists at inhibiting aversive behavior to illumination and the von Bezold-Jarisch reflex in the mouse. Neuropharmacol 33:227–234

Eglen RM, Lee CH, Smith WL, Johnson LG, Clark R, Whiting RL, Hedge SS (1995) Pharmacological characterization of RS 25259–197, a novel and selective 5-HT$_3$ receptor antagonist, *in vivo*. Br J Pharmacol 114:860–866

Fleckenstein A, Muschaweck R, Bohlinger F (1950) Weitere Untersuchungen über die pharmakologische Ausschaltung des BEZOLD-JARISCH-Reflexes. Naunyn Schmiedeberg's Arch exper Path Pharmakol 211:132–142

Fozard JR (1984) MDL 72222: A potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. Naunyn-Schmiedeberg's Arch Pharmacol 326:36–44

Geissler MA, Torrente JR, Elson AS, Gylys JA, Wright RN, Iben LG, Davis HH, Yocca FD (1993) Effects of BMY 33462, a selective and potent serotonin type-3 receptor antagonist, on mesolimbic dopamine mediated behavior. Drug Dev Res 29:16–24

Giles TD, Sander GE (1986) Comparative cardiovascular responses to intravenous capsaicin, phenyldiguanide, veratrum alkaloids and enkephalins in the conscious dogs. J Auton Pharmacol 6:1–7

Godlewski G, Göthert M, Malinowska B (2003) Cannabinoid receptor-independent inhibition of cannabinoid agonists of the peripheral 5-HT$_3$ receptor-mediated Bezold-Jarisch reflex. Br J Pharmacol 138:767–774

Göthert M, Hamon M, Barann M, Bönisch H, Gozlan H, Laguzzi R, Metzenauer P, Nickel B, Szelenyi I (1995) 5-HT3 antagonism by anpirtoline, a mixed 5-HT$_1$ receptor agonist/5-HT$_3$ receptor antagonist. Br J Pharmacol 114:269–274

Gylys JA, Wright RN, Nicolosi WD, Buyniski JP, Crenshaw RR (1988) BMY 25801, an anti-emetic agent free of D$_2$ dopamine antagonist properties. J Pharmacol Exp Ther 244:830–837

Haga K, Asano K, Inaba K, Morimoto Y, Setoguchi M (1994) Effect of Y-25130, a selective 5-hydroxytryptamine-3 receptor antagonist, on gastric emptying in mice. Arch Int Pharmacodyn Ther 328:344–355

Harron DWG, Kobinger W (1984) Facilitation of the Bezold-Jarisch reflex by central stimulation of alpha-2 adrenoceptors in dogs. Naunyn-Schmiedeberg's Arch Pharmacol 325:193–197

Hegde SS, Wong AG, Perry MR, Ku P, Moy TM, Loeb M, Eglen RM (1995) 5-HT$_4$ receptor mediated stimulation of gastric emptying in rats. Naunyn-Schmiedeberg's Arch Pharmacol 351:589–595

Jarisch A (1940) Vom Herzen ausgehende Kreislaufreflexe. Arch Kreislaufforsch 7:260–274

Jarisch A, Richter H (1939a) Die Kreislaufwirkung des Veratrins. Naunyn Schmiedeberg's Arch exp Path Pharmakol 193:347–354

Jarisch A, Richter H (1939b) Der Bezold-Effekt – Eine vergessene Kreislaufreaktion. Klin Wochschr 18:185–187

Kalkman HO, Engel G, Hoyer D (1984) Three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin in rats. J Hypertens 2, Suppl 3:143–145

Kishibayashi N, Ichikawa S, Yokoyama T, Ishii A, Karasawa A (1993) Pharmacological properties of KF19259, a novel 5-HT$_3$ receptor antagonist, in rats: Inhibition of the distal colonic function. Jpn J Pharmacol 63:495–502

López-Tudanca PL, Labeaga L, Innerárity A, Alonso-Cires L, Tapia I, Mosquera R, Orjales A (2003) Synthesis and pharmacological characterization of a new benzoxazole derivative as a potent 5-HT_3 receptor agonist. Bioorg Med Chem 11:2709–2714

Malinowska B, Kwolek G, Göthert M (2001) Anandamide and methanandamide induce both vanilloid VR_1 and cannabinoid CB_1 receptor mediated changes in heart rate and blood pressure in anesthetized rats. Naunyn-Schmiedebergs Arch Pharmacol 364:562–569

Matsumoto M, Yoshioka M, Togashi H, Saito H (1992) Effects of ondasentron on cardiovascular reflex to exogenous serotonin in anesthetized rats. Biog Amines 8:443–449

Meller ST, Lewis SJ, Brody MJ, Gebhart GF (1992) Vagal afferent-mediated inhibition of a nociceptive reflex by i.v. serotonin in the rat. II. Role of 5-HT receptor subtypes. Brain Res 585:71–86

Middlefell VC, Bill DJ, Brammer NT, Coleman J, Fletcher A, Hallett I, Rhodes KF, Wainwright TL, Ward TJ (1996) WAY-SEC-579: A novel 5-HT_3 receptor antagonist. CNS Drug Rev 2:269–293

Miyata K, Kamato T, Yamano M, Nishida A, Ito H, Katsuyama Y, Yuki H, Tsutsumi R, Ohta M, Takeda M, Honda K (1991) Serotonin 5-HT_3 receptor blocking activities of YM060, a novel 4,5,6,7-tetrahydrobenzimidazole derivative and its enantiomer in anesthetized rats. J Pharmacol Exp Ther 259:815–819

Rault S, Lancelot JC, Prunier H, Robba M, Renard P, Delagrange P, Pfeiffer B, Caignard DH, Guardiola-Lemaitre B, Hamon M (1996) Novel selective and partial agonists of 5-HT_3 receptors. Part 1. Synthesis and biological evaluation of piperazinopyrrolothienopyrazines. J Med Chem 39:2068–2080

Robertson DW, Bloomquist W, Wong DT, Cohen ML (1992) MCPP but not TFMPP is an antagonist at cardiac 5-HT_3 receptors. Life Sci 50:599–605

Rocha I, Rosario LB, de Oliviera EI, Barros MA, Silva-Carvallho L (2003) Enhancement of carotid chemoreceptor reflex and cardiac chemosensitive reflex in the acute phase of myocardial infarction of the anesthetized rabbit. Basic Res Cardiol 98:175–180

Takei N, Takei S, Tomomatsu S, Suzuki K, Yagi S (1995) Augmentation of clonidine on Bezold-Jarisch reflex control of renal sympathetic nerve activity in cats. Jpn J Nephrol 37:12–16

Turconi M, Donetti A, Schiavone A, Sagrada A, Montagna E, Nicola M, Cesana R, Rizzi C, Micheletti R (1991) Pharmacological properties of a novel class of 5-HT_3 receptor antagonists. Eur J Pharmacol 203:203–211

Vayssettes-Couchay C, Bouysset F, Laubie M, Verbeuren TJ (1997) Central integration of the Bezold-Jarisch reflex in the cat. Brain Res 74:272–278

von Bezold A, Hirt L (1867) Über die physiologischen Wirkungen des essigsauren Veratrin's. Untersuchungen aus dem physiologischen Laboratorium Würzburg 1:75–156

Watson JW, Gonsalves SF, Fossa AA, McLean S, Seeger T, Obach S, Andrews PLR (1995) The anti-emetic effects of CP-99,994 in the ferret and the dog: Role of the NK_1 receptor. Br J Pharmacol 115:84–94

Yamono M, Ito H, Kamoto T, Miyata K (1995) Species difference in the 5-hydroxytryptamine$_3$ receptor associated with the Bezold-Jarisch reflex. Arch Int Pharmacodyn Ther 330:177–189

Zucker IH, Cornish KG (1981) The Bezold-Jarisch reflex in the conscious dog. Circ Res 49:940–948

A.1.3.20 Endotoxin Induced Shock

PURPOSE AND RATIONALE

Many bacterial infections as well as allergic reactions are known to induce pathophysiological events that may lead to shock in man. When experimental animals are injected with endotoxin and galactosamine, shock and death occur in all untreated animals 5–7 h after injection. The endotoxin induced shock is marked by pulmonary embolism, bronchospasm and renal failure. Bacterial liposaccharides (endotoxins) play an important role in the pathogenicity of Gram-negative infections.

The reactivity of animals to endotoxin may be enhanced by simultaneous administration of galactosamine. Galactosamine is a specific hepatotoxic agent that leads to early metabolic alterations and consequent cellular liver damage. The following procedure is used to detect compounds that prevent the occurrence of endotoxin-induced shocks.

Cardiovascular parameters of endotoxin induced shock are greatly influenced by various anesthetics. For this reason, a model was proposed by Brackett et al. (1985) and Schaefer et al. (1987) to study the circulatory shock pattern after endotoxemia in conscious unrestrained rats.

PROCEDURE

Male Sprague-Dawley rats weighing 300 ± 10 g are anesthetized with 5% enflurane. A tracheal cannula is connected to a rodent respirator delivering 2% enflurane. Via the right jugular vein the tip of one catheter is placed just adjacent to the right atrium for injection of endotoxin, monitoring of central venous pressure, and rapid injection of room-temperature saline to produce thermodilution curves for calculation of cardiac output. The right carotid artery is cannulated with a thermistor-catheter combination for measurement of thermodilution cardiac output curves and aortic blood pressure. The thermistor tip is placed in the aortic arch just distal to the aortic valve. The catheters are guided under the skin exiting through the back of the neck just below the base of the skull.

The animals are allowed to regain consciousness and are then placed in cages that allow unrestrained movements about the cage at all times throughout the study with no further handling. The experimental animals receive a 20-sec infusion of 40 mg/kg endotoxin (*E. coli*, Difco) being paired with sham animals with identical catheters but receiving an equal volume of saline. Test compounds are injected intravenously

10 min prior to endotoxin injection. Cardiac outputs are measured using the thermodilution technique by rapidly injecting a volume calculated to deliver 100 µl of room temperature saline to the circulatory system. Central venous and aortic blood pressure and heart rate are continuously monitored for the following 4 h. Cardiac output measurements are made 5, 15, 30, 60, 120, 180, and 240 min after endotoxin. At the end of the study, the animals are sacrificed and the catheters checked visually to ensure proper placement.

EVALUATION

Central venous pressure, arterial pressure, and cardiac output of drug treated animals receiving endotoxin are compared with animals receiving endotoxin only and saline sham treated animals. Furthermore, cardiac index, total peripheral resistance, and stroke volume are calculated. The small intestines of all rats are examined for severity of hemorrhage using a five point scale. Repeated-measures analysis of variance is used to analyze the data.

MODIFICATIONS OF THE METHOD

Lindenbaum et al. (1990) studied the effect of *E. coli* endotoxin on cardiovascular parameters of anesthetized **dogs**. Inhibition of the detoriation of metabolic functions and improvement of cardiovascular parameters were found after cocarboxylase treatment.

Endotoxin induced shock has been tested in **mice** (Galanos et al. 1979). Groups of 10 male C57 BL/6 mice weighing 20–22 g are injected intravenously with a mixture of 0.01 µg of Salmonella abortus equi lipopolysaccharide and 7.5–15 mg galactosamine in 0.02 ml phosphate buffered saline. The test compound is administered either intravenously at the same time or orally 45 min prior challenge. Twenty-four hours later, the number of surviving mice is determined.

Metz and Sheagren (1990) reviewed the effects of ibuprofen in animal models of septic shock.

Von Asmuth et al. (1990), Luongo et al. (1998), and Cuzzocrea et al. (2004) described a zymosan-induced shock model in **mice**. Male CD mice (20–22 g) were treated intraperitoneally with zymosan (500 mg/kg, suspended in saline solution) or with zymosan and drug (rosiglitazone 3 mg/kg, intraperitoneally) at 1 and 6 h after zymosan. Eighteen hours after administration of zymosan, animals were assessed for non-septic shock. Clinical severity of systemic toxicity was scored for the whole experimental period (12 days) in the mice after zymosan or saline injection on a subjective scale ranging from 0 to 3: 0 = absence, 1 = mild, 2 = moderate, 3 = serious. The ranging scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhea, and lethargy) observed in the animals. The final score was the sum of the single evaluation (maximum value 12).

Overbergh et al. (2003) studied acute shock induced by antigen in NOD mice. The 8-week-old NOD, BALB/c, and C57BL/6 mice were immunized by injection of 100 µg antigen [Hen egg white lysozyme (HEL), GAD65 (p524–542) (SRLSKVAPVIKARMMEYGTT), bovine insulin B (ins-B) chain, heat shock protein (hsp)-65, PLP peptide (amino acids 135–151), ovalbumin, keyhole limpet hemocyanine (KLH), and tetanus toxin] emulsified at a 1:1 concentration in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich., USA) in the hind footpads. NOD-*scid* mice were injected with 100 µg HEL antigen suspended in CFA. All mice were reinjected with the same antigen 3 weeks later in a similar manner. Clinical evolution and survival rate after sensitization with various peptides were monitored in different mouse strains. Shock was characterized by pilo-erection, prostration, erythema of the tail, ears, and footpads, and dyspnea with shallow breathing. Serum for antibody measurement and spleens for quantification of mRNA levels were collected before immunization and again before booster administration.

Baldwin et al. (1991) tested the effect of Polymyxin B on experimental shock from meningococcal lipooligosaccharide and *Escherichia coli* lipopolysaccharide endotoxins in anesthetized **rabbits**.

Muacevic and Heuer (1992) tested the effect of platelet-activating factor antagonists in anesthetized rats.

Otterbein et al. (1993) tested the effects of peptides on survival of mice injected with 50 mg/kg lipopolysaccharide endotoxin in mice and on survival of rats with fecal peritonitis.

Mountz et al. (1995) reported an increased susceptibility of fas mutant **MRL-Ipr/Ipr mice** to Staphylococcal enterotoxin B-induced septic shock.

REFERENCES AND FURTHER READING

Baldwin G, Alpert G, Caputo GL, Baskin M, Parsonnet J, Gillis ZA, Thompson C, Silber GR, Fleisher GR (1991) Effect of Polymyxin B on experimental shock from meningococcal and *Escherichia coli* endotoxins. J Infect Dis 164:542–549

Brackett DJ, Schaefer CF, Tompkins P, Fagraeus L, Peters LJ, Wilson MF (1985) Evaluation of cardiac output, total peripheral vascular resistance, and plasma concentrations of vasopressin in the conscious, unrestrained rat during endotoxemia. Circulat Shock 17:273–284

Cuzzocrea S, Pisano B, Dugo L, Ianaro A, Patel NSA, di Paola R, Genovese T, Chatterjee PK, Fulia F, Cuzzocrea E, di Rosa M, Caputi AP, Thiemermann C (2004) Rosiglitazone, a ligand of peroxisome proliferator-activated receptor-γ, reduces development of nonseptic shock in mice. Crit Care Med 32:457–466

Galanos C, Freudenberg MA, Reutter W (1979) Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci USA 76:5939–5943

Lindenbaum GA, Lerrieu AJ, Carrol SF, Kapusnick RA (1990) Efectos de la cocarboxilasa en perros sometidos a choque septico experimental. Compend Invest Clin Latinoam 10:18–26

Luongo C, Imperatore F, Cuzzocrea S, Filippelli A, Scafuro MA, Mangoni G, Portolano F, Rossi F (1998) Effects of hyperbaric oxygen exposure on a zymosan-induced shock model. Crit Care Med 26:1972–1976

Metz CA, Sheagren JN (1990) Ibuprofen in animal models of septic shock. J Crit Care 5:206–212

Mountz JD, Baker TJ, Borcherding DR, Bluethmann H, Zhou T, Edwards CK (1995) Increased susceptibility of fas mutant MRL-Ipr/Ipr mice to staphylococcal enterotoxin B-induced septic shock. J Immunol 155:4829–4837

Muacevic G, Heuer HO (1992) Platelet-activating factor antagonists in experimental shock. Arzneim Forsch/Drug Res 42:1001–1004

Otterbein L, Lowe VC, Kyle DJ, Noronha-Blob L (1993) Additive effects of a bradykinin antagonist, NPC 17761, and a leumedin, NPC 15669, on survival in animal models. Agents Actions 39, Special Conference Issue: C125–C127

Overbergh L, Decallonne B, Branisteanu DD, Valckx D, Kasran A, Bouillon R, Mathieu C (2003) Acute shock induced by antigen in NOD mice. Diabetes 52:335–342

Schäfer CF, Biber B, Brackett DJ, Schmidt DD, Fagraeus L, Wilson MF (1987) Choice of anesthetic alters the circulatory shock as gauged by conscious rat endotoxemia. Acta Anaesthesiol Scand 31:550–556

Von Asmuth EJ, Maessen JG, van der Linden CJ, Buurman WA (1990) Tumor necrosis factor alpha (TNF-α) and interleukin 6 in a zymosan-induced shock model. Scand J Immunol 32:313–319

A.1.3.21 Hemorrhagic Shock

PURPOSE AND RATIONALE

Hemorrhagic shock is one of the most severe consequences of accidents. Several animal models in various species have been developed to resemble the conditions in man and to test therapeutic or prophylactic measures (Lamson and de Turk 1945; Selkurt and Rothe 1961; Mills 1976). A method for hemorrhagic shock in anesthetized as well as in unanesthetized rats has been described by van der Meer et al. (1987). Experimental hemorrhagic shock is defined as a situation in which the cardiovascular system, after a period of hypovolemia followed by complete re-infusion of the shed blood, gradually detoriates ending in the death of the animal.

PROCEDURE

Female rats weighing 170–190 g are anesthetized by i.p. injection of sodium pentobarbital, 25 m/kg, followed after 20 min by 20 mg/kg, and kept in a chamber at 30°C and relative humidity over 80%. The left femoral vein is cannulated for application of the test drug. The right common iliac artery is cannulated and the cannula (polyvinyl chloride, 14 cm long, inner diameter 2 mm) is filled with heparin and exteriorized in the neck. After intraarterial injection of 0.2 ml heparin 500 IU/ml, the cannula is connected to a siliconized calibrated glass reservoir (inner diameter 18 mm), the height of which can be changed to adjust the surface of the shed blood to a fixed level.

The test drug is injected i.v. 5 min prior to bleeding. Bleeding is performed against (at heart level) 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, 30 mm Hg for 1 h, 25 mm Hg for 0.5 h, and finally 30 mm Hg for 1 h. The shed blood is partially taken up again spontaneously. After 4 h, re-infusion is started by increasing the pressure to 60 mm Hg for 5 min, to 80 mm Hg for 5 min, and (if necessary) to 100 mm Hg. During the hypovolemic phase respiration becomes gradually slower. If respiration arrest is imminent 0.5 ml 5% glucose are injected intra-arterially, thus avoiding death during the period of hypovolemia. Practically all rats die at an average of 4 h after complete re-infusion.

EVALUATION

Survival time is taken as the time between complete re-infusion and death. Average survival time of treated animals is compared with that of controls. Furthermore, after autopsy the number of gastrointestinal lesions, subendocardial hemorrhage, kidney tubular necrosis and liver cell necrosis are registered by histological examination.

CRITICAL ASSESSMENT OF THE METHOD

In spite of the fact that hemorrhagic shock does not reflect the situation of traumatic shock in man in every aspect, the condition is close enough to use the model for testing compounds which potentially inhibit or ameliorate shock in man.

MODIFICATIONS OF THE METHOD

A method to study hemorrhagic shock in dogs has been described in detail by Mills (1967). Large dogs weighing 20–30 kg are anesthetized by an i.v. injection of 25 mg/kg sodium pentobarbital. The animals are respirated by means of a Harvard respirator set at a stroke volume of 400 ml and a rate of 20 respiration/min. Blood pH is regulated between 7.37 and 7.42

by varying the gas flow between 100% O_2 and a mixture of 95% O_2 and 5% CO_2. Central arterial blood pressure is recorded by inserting a catheter through one femoral artery to the aortic arch. Pulmonary artery pressure is measured by inserting a PE 50 catheter through a small neck vein, reaching the right ventricle and allowing to float into the pulmonary artery. The right atrial catheter is also inserted through a small neck vein. After the chest is opened, the left atrial catheter is tied in place through a small opening in the left atrial appendage. Blood flow is measured in the ascending aorta (cardiac output), carotid, superior mesenteric, renal and femoral arteries using electromagnetic flowmeters. Furthermore, pulse rate is monitored from the electrocardiogram.

The test drug is injected i.v. 10 min prior bleeding. Blood is removed either at a specific volume or until a selected reduction of blood pressure has occurred. The cardiovascular parameters of treated animals are compared with those of controls.

Shock associated with hemoconcentration was produced in dogs by Davis (1941) by bleeding from the carotid artery and injections of 25% sodium chloride solution subcutaneously in doses of 25 ml.

The effect of insulin on glucose uptake in the soleus muscle of rats during hemorrhagic shock was studied by Chaudry et al. (1975).

Bauer et al. (1995) used hemorrhagic shock in rats to evaluate the influence of interleukin-1 on leukocyte-endothelial cell interactions and the microcirculation in the liver by means of intravital microscopy after application of an interleukin-1 receptor antagonist.

Kitajima et al. (1995) studied gastric mucosal injury induced by hemorrhagic shock in rats.

REFERENCES AND FURTHER READING

Bauer C, Marci I, Bauer M, Fellger H, Larsen R (1995) Interleukin-1 receptor antagonist attenuates leukocyte-endothelial interactions in the liver after hemorrhagic shock in the rat. Crit Care Med 23:1099–1105

Chaudry IH, Sayeed MM, Baue AE (1975) The effect of insulin on glucose uptake in soleus muscle during hemorrhagic shock. Can J Physiol Pharmacol 53:67–73

Davis HA (1941) Physiologic effects of high concentrations of oxygen in experimental secondary shock. Arch Surg 43:1–13

Kitajima T, Tani K, Yamaguchi T, Kubota Y, Okuhira M, Mizuno T, Inoue K (1995) Role of endogenous endothelin in gastric mucosal injury by hemorrhagic shock in rats. Digestion 56:111–116

Lamson PD, de Turk (1945) Studies on shock induced by hemorrhage. XI. A method for the accurate control of blood pressure. J Pharmacol Exp Ther 83:250–252

Mills LC (1967) Animal and clinical techniques for evaluating drugs in various types of shock. In: Siegler PF, Moyer JH (eds) Animal and clinical pharmacologic techniques in drug evaluation. Year Book Medical Publ. Inc., Chicago, pp 478–492

Selkurt EE, Rothe CF (1961) Critical analysis of experimental shock models. In: Seeley SF, Weisiger JR (eds) Recent progress and present problems in the field of shock. Fed Proc 20, Suppl 9, part III, pp 30–37

Van der Meer C, Valkenburg PW, Snijders PM, Wijnans M, van Eck P (1987) A method for hemorrhagic shock in the rat. J Pharmacol Meth 17:75–82

A.1.3.22 Tourniquet Shock

PURPOSE AND RATIONALE

Compression of extremities in man by heavy objects for periods of several hours results in the so-called crush syndrome. The rescued individual shows immediately a favorable response to therapy, but within a few hours symptoms of shock develop followed by signs of progressive renal damage leading to death (Duncan and Blalock 1942). Moreover, arterial bleeding after accidents needs the applications of tourniquets. During surgical procedures on extremities a tourniquet may be necessary (Wilgis 1971), the time of which has to be limited in order to avoid fatal consequences. The pathophysiological mechanisms of tourniquet induced shock remain still to be elucidated. Nevertheless, animal models in rats (Chandra and Dave 1970), rabbits (Little 1974), and dogs (Goto et al. 1988) had to be developed to evaluate drugs capable to inhibit the fatal consequences of crush and tourniquet shock.

PROCEDURE

Wistar rats of either sex weighing 250–280 g are anesthetized with phenobarbital. The tourniquets consist of rubber tubes (internal diameter 4 mm, external diameter 5.8 mm). Both tights are fastened by the rubber tubes and the pressure which is monitored by a miniature pressure sensor and an amplifier (e. g. Kyowa Electronic Instruments Co, Tokyo) is adjusted to 1.5 kg/cm^2. The rubber tubes are knotted and the sensor removed. After 3 h the animals are treated with the test compound or the control solution. The tourniquet is left in place for 6 h while the animals remain under pentobarbital anesthesia. Then, the rubber tubes are removed, and the rats are returned to their cages. Within a few min, the reperfused hind limbs, which have been pale blue, turn pink. The animals are then allowed free access to food and water. Blood is withdrawn at different intervals during the tourniquet and afterwards for measurement of hematocrit, transaminases, urea nitrogen and total protein. Time to death is registered.

EVALUATION

Statistical evaluation of the survival intervals is performed with the log rank test according to Peto et al. (1976). Blood chemical data are analyzed using the Kruskal-Wallis (1952) rank sum test. Multiple comparisons are corrected by the Bonferroni's method (1980).

CRITICAL EVALUATION OF THE METHOD

These methods are valuable to find drugs effective in this life-threatening situation.

MODIFICATIONS OF THE METHOD

Ghussen et al. (1979) studied the effect of methylprednisolone on the experimental tourniquet shock in **dogs**.

Haugan and Kirkebo (1984) used a model in anesthetized **rats** with tourniquet shock by bilateral hindlimb occlusion for 3 1/2 h, and burn shock by scalding the hind 50% of the body surface for 30 s in 90°C water.

Horl and Horl (1985) investigated the effect of tourniquet ischemia on carbohydrate metabolism in **dog** skeletal muscle

Sáez et al. (1982) followed the time course of appearance of lactic dehydrogenase enzymes in the serum of **rats** after different periods of ischemia by bilateral application of rubber band tourniquets to the hind legs.

Sáez et al. (1986) studied the effects of allopurinol on biochemical changes of the gastrocnemius muscle in rats subjected to tourniquet shock followed by reperfusion.

REFERENCES AND FURTHER READING

Aoki Y, Nata M, Odaira T, Sagisaka K (1992) Suppression of ischemia-reperfusion injury by liposomal superoxide dismutase in rats subjected to tourniquet shock. Int J Leg Med 105:5–9

Chandra P, Dave PK (1970) Effect of dipyrone on tourniquet shock. Arzneim Forsch (Drug Res) 20:409–412

Duncan GW, Blalock A (1942) The uniform production of experimental shock by crush injury: possible relationship to clinical crush syndrome. Ann Surg 115:684–694

Ghussen F, Stock W, Bongartz R (1979) The effect of methylprednisolone on the experimental tourniquet shock in dogs. Res Exp Med 176:87–95

Goto H, Benson KT, Katayama H, Tonooka M, Tilzer LL, Arakawa K (1988) Effect of high-dose of methylprednisolone on tourniquet ischaemia. Can J Anaesth 35:484–488

Haugan A, Kirkebo A (1984) Local blood flow changes in the renal cortex during tourniquet and burn shock in rats. Circ Shock 14:147–157

Horl M, Horl WH (1985) Effect of tournique ischemia on carbohydrate metabolism in dog skeletal muscle. Eur Surg Res 17:53–60

Kruskal JB, Wallis WA (1952) Use of ranks in one-criterion variance analysis. J Am Stat Assoc 47:583–621

Little RA (1974) The compensation of post-traumatic oedema in the rabbit at different ages. J Physiol 238:207–221

Paletta FX, Willman V, Ship AG (1960) Prolonged tourniquet ischemia of extremities. An experimental study on dogs. J Bone Joint Surg 42:945–950

Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG (1976) Design and analysis of randomized clinical trials requiring prolonged observation of the patient. I. Introduction and design. II. Analysis and examples. Br J Cancer 585–612

Sáez JC, Vivaldi E, Günther B (1982) Tourniquet shock in rats: Appearance of lactic dehydrogenase isoenzymes in serum. IRCS Med Sci 10:191–192

Sáez JC, Cifuentes F, Ward PH, Günther B, Vivaldi E (1986) Tourniquet shock in rats: Effects of allopurinol on biochemical changes of the gastrocnemius muscle subjected to ischemia followed by reperfusion. Biochem Med Metabol Biol 35:199–209

Sen PK (1980) Nonparametric simultaneous inference for some MANOVA models. In: Krishnaiah PR (ed) Handbook of Statistics. Vol 1; North-Holland, Amsterdam, New York, pp 673–702

Van der Meer C, Valkenburg PW, Ariëns AT, van Benthem MJ (1966) Cause of death in tourniquet shock in rats. Am J Physiol 210:513–525

Vujnov St, Prostran M, Savic JD, Varagic VM, Lovric M (1992) Beta-adrenergic receptors and catecholamines in the rat heart during tourniquet trauma. Circulat Shock 36:38–44

Wilgis EFS (1971) Observations on the effects of tourniquet ischemia. J Bone Joint Surg 53 A:1343–1346

A.1.3.23 Heat Stroke

PURPOSE AND RATIONALE

Heat stroke is a medical emergency where quick diagnosis and treatment of victims are essential for positive prognosis. Several animal models have been established by investigators in heat related studies. Rats (Francesconi and Mager 1978; Hubbard et al. 1977, 1979; Kielblock et al. 1982), rabbits (Shih et al. 1984), dogs (Bynum et al. 1977) and sheep (Tayeb and Marzouki 1990) are considered to be the most suitable models because of their similarity to man in response to high temperature.

PROCEDURE

Male Sprague Dawley rats weighing 450 to 550 g are fasted 18–24 h before the experiment. For prevention studies the animals are treated subcutaneously 1 h before either being restrained in an appropriate wire cage which is placed into an environmental chamber set at 41.5°C ambient temperature or being exercised in a motor-driven treadmill. Core temperature (rectal probe inserted 6.5 cm) are measured using copper/constantan thermocouples in conjunction with a thermocouple reference oven and a 10-channel data acquisition system with a teletype printout. After

reaching exhaustion or a predetermined core temperature, all rats are monitored at 26°C ambient temperature while resting in plastic cages lined with wood shavings. After recovery, animals are returned to their cages and allowed water but no food for 24 h.

EVALUATION

LD_{50} values are determined in treated and control animals.

MODIFICATIONS OF THE METHOD

Kielblock et al. (1982) analyzed cardiovascular function by direct recording of arterial blood pressure and ECG-analysis.

Francesconi and Mager (1978) studied pathochemical indices, such as serum lactate concentration, potassium levels and plasma creatine phosphokinase.

Kregel et al. (1988) investigated peripheral vascular responses to hyperthermia in the rat by implantation of Doppler flow probes on the superior mesenteric, left iliac or left renal, and external caudal arteries. They concluded that a selective loss of compensatory vasoconstriction triggers the cascade of events that characterize heat stroke.

Shido and Nagasaka (1990) studied thermoregulatory responses to acute body heating in rats acclimated to continuous heat exposure. Indirect external warming was performed by raising the jacket water temperature surrounding the calorimeter from 24 to 39°C. Intraperitoneal heating was made through an electric heater implanted chronically in the peritoneal cavity.

Chiu et al. (1995) reported an increased survival in rat heatstroke by reducing hypothalamic serotonin release after administration of interleukin-1 receptor antagonist.

REFERENCES AND FURTHER READING

Bynum G, Patton J, Bowers W, Leav I, Wolfe D, Hamlet M, Marsili M (1977) An anesthetized dog heat stroke model.J Appl Physiol: Resp Environ Exercise Physiol 43:292–296

Chiu WT, Kao TY, Lin MT (1995) Interleukin-1 receptor antagonist increases survival in rat heatstroke by reducing hypothalamic serotonin release. Neurosci Lett 202:33–36

Damanhouri ZA, Tayeb OS (1992) Animal models for heat stroke studies. J Pharmacol Toxicol Meth 28:119–127

Francesconi RP, Mager M (1978) Heat-injured rats: pathochemical indices and survival time. J Appl Physiol: Resp Environ Exercise Physiol 45:1–6

Hubbard RW, Bowers WD, Matthew WT, Curtis FC, Criss REL, Sheldon GM, Ratteree JW (1977) Rat model of acute heatstroke mortality. J Appl Physiol: Resp Environ Exercise Physiol 42:809–816

Hubbard RW, Criss REL, Elliott LP, Kelly C, Matthew WT, Bowers WD, Mager M (1979) Diagnostic significance of selected serum enzymes in a rat heatstroke model. J Appl Physiol: Respirat Environ Exercise Physiol 46:334–339

Kielblock AJ, Strydom NB, Burger FJ, Pretorius PJ, Manjoo M (1982) Cardiovascular origins of heatstroke pathophysiology: an anesthetized rat model. Aviat Space Environ Med 53:171–178

Kregel KC, Wall PT, Gisolfi CV (1988) Peripheral vascular responses to hyperthermia in the rat. J Appl Physiol 64:2582–2588

Shido O, Nagasaka T (1990) Thermoregulatory responses to acute body heating in rats acclimated to continuous heat exposure. J Appl Physiol 68:59–65

Shih CJ, Lin MS, Tsai SH (1984) Experimental study on the pathogenesis of heat stroke. J Neurosurg 60:1252–1264

Tayeb OS, Marzouki ZM (1990) Effect of dantrolone pretreatment on heat stroke in sheep. Pharmacol Res 22:565–572

A.1.3.24
α- and β-Adrenoreceptors in the Mouse Iris

PURPOSE AND RATIONALE

A simple method to test mydriatic substances is the test on the mouse pupil as described by Pulewka (1932). The diameter of the pupil is narrowed by intensive light illumination. A dose-dependent increase of pupil diameter can be achieved by intraperitoneal application of atropine and synthetic mydriatics (Ing et al. 1950; Burn et al. 1950). The mydriatic effect of hexamethonium analogues has been measured by Blackman et al. (1956). Mydriasis is induced by norepinephrine, epinephrine and isoproterenol and can be antagonized by α- or β-blockers (Freundt 1965).

PROCEDURE

Male mice weighing 15–20 g are used. They are kept for at least 30 min in separate beakers under bright illumination before the pupil diameters are measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice are placed beneath long low-power fluorescent tubes and on top of glossy white paper. The pupil diameter is measured in mm before and at various time intervals after treatment. Groups of 5–10 mice are used for each dose of compound and for vehicle control.

To test sympatholytic activity, various doses of the α- or β-blocker are injected subcutaneously 30 min prior to intravenous injection of 0.1 mg/kg norepinephrine, or 0.05 mg/kg epinephrine, or 20 mg/kg isoproterenol. The effect of norepinephrine is blocked by α-blockers, but not by β-blockers, the effect of epinephrine by both α- and β-blockers, and the effect of isoproterenol by β-blockers, but not by α-blockers.

EVALUATION

The mean values of diameters in the groups treated with α- or β-blockers are compared with those of an-

imals treated with norepinephrine, epinephrine or isoproterenol only.

MODIFICATIONS OF THE METHOD

Edge (1953) used mydriasis in the mouse as a quantitative method of estimating parasympathetic ganglion block.

Håkanson et al. (1987) used the isolated iris sphincter of pigmented rabbits to test multiple tachykinin pools in sensory nerve fibres. The eyes were taken out within 1 min after sacrifice and opened by an incision 2–3 mm posterior to the limbus, followed by excision of the iris from the ciliary margin. The iris sphincter muscle was then opened, cut in half and mounted vertically on a Perspex holder in a 7 ml tissue bath maintained at 35°C. The mechanical activity after electrical stimulation was recorded isometrically using a force displacement transducer and a polygraph.

Kern (1970) used isolated sphincter and dilator muscles from human eyes obtained at autopsy for studies on sympathomimetics and adrenergic blocking agents. Cholinotropic and α- and β-adrenergic receptors were identified.

Responses to bradykinin and or capsaicin of the isolated iris sphincter were considered to be mediated by substance P released from the trigeminal nerve (Ueda et al. 1984).

Pupillary dilatation can be used as an index for central nervous system α_2-adrenoreceptor activation (Koss 1986).

Clonidine induces mydriasis which is mediated by α_2-adrenoreceptors located in the brain (Berridge et al. 1983; Hey et al. 1985). Blockade of presynaptically located α_2-adrenoreceptors is considered as a possible mechanism for antidepressant drugs. Mianserin was able to antagonize clonidine-induced mydriasis in the rat.

Gower at al (1988) studied a large number of psychotropic drugs in this model with the aim to reveal *in vivo* α_2-adrenoreceptor blocking effects of new compounds.

Male Wistar rats weighing 230–300 g were anesthetized with pentobarbital, 60 mg/kg i.p., and a polyethylene catheter was inserted into the femoral vein for drug administration. The rat's head rested on the base platform of a binocular Olympus microscope positioned so that the pupil diameter of the right eye could be measured by means of a micrometer inserted into one eyepiece of the microscope. A constant light intensity was maintained throughout the experiment. Rats were first injected with saline 25 min after anesthesia induction. The pupil diameter was measured 1 min after injection. Five min after measurement, mydriasis was induced by clonidine (0.1 mg/kg, i.v.) and the diameter was measured 1 min after injection. This was followed by the test compound, injected at 6 min intervals at increasing doses. The pupil was measured at 1 min after each injection. The dose inhibiting 50% of the clonidine-induced mydriasis (ID_{50}) was determined per rat from the cumulative dose-response curve.

Savontaus et al. (1997) studied the effect of an imidazoline derivative against detomidine-induced mydriasis in anesthetized rats.

REFERENCES AND FURTHER READING

Berridge TL, Sadie B, Roach AG, Tulloch IF (1983) α_2-adrenoceptor agonists induce mydriasis in the rat by an action within the CNS. Br J Pharmacol 78:507–515

Blackman JG, Fastier FN, Patel CM, Wong LCK (1956) Assessment of depressor activity and mydriatic activity of hexamethonium analogues. Br J Pharmacol 11:282–288

Burn JH, Finney DJ, Goodwin LG (1950) Biological Standardization. Oxford University Press, London, pp 320–324

Bynke G, Håkanson R, Hörig J, Leander S (1983) Bradykinin contracts the pupillary sphincter and evokes ocular inflammation through release of neuronal substance P. Eur J Pharmacol 91:469–475

Edge ND (1953) Mydriasis in the mouse: a quantitative method of estimating parasympathetic ganglion block. Br J Pharmacol Chemother 8:10–14

Freundt KJ (1965) Adrenergic alpha- and beta-receptors in the mouse iris. Nature 206:725–726

Gower AJ, Broekkamp CLE, Rijk HW, van Delft AME (1988) Pharmacological evaluation of *in vivo* tests for α_2-adrenoceptor blockade in the central nervous system and the effects of mianserin and its aza-analog ORG 3770. Arch Int Pharmacodyn 291:185–201

Håkanson R, Beding B, Erkman R, Heilig M, Wahlestedt C, Sundler F (1987) Multiple tachykinin pools in sensory nerve fibres in the rabbit iris. Neurosci 21:943–950

Hey JA, Gherezghiher T, Koss MC (1985) Studies on the mechanism of clonidine-induced mydriasis in the rat. Naunyn-Schmiedeberg's Arch Pharmacol328:258–263

Ing HR, Dawes GS, Wajda I (1945) Synthetic substitutes for atropine. J Pharm Exp Ther 85:85–105

Kern R (1970) Die adrenergischen Receptoren der intraoculären Muskeln des Menschen. Graefes Arch klin exp Ophthal 180:231–248

Koss MC (1986) Pupil dilatation as an index of central nervous system α_2-adrenoceptor activation. J Pharmacol Meth 15:1–19

Pulewka P (1932) Das Auge der weißen Maus als pharmakologisches Testobjekt. I. Mitteilung: Eine Methode zur quantitativen Bestimmung kleinster Mengen Atropin und anderer Mydriatika. Naunyn Schmiedeberg's Arch exp Path Pharmak 168:307–315

Savontaus E, Raasmaja A, Rouru J, Koulu M, Pesonen U, Virtanen R, Savola JM, Huupponen R (1997) Anti-obesity effect of MPV-1743 A III, a novel imidazoline derivative, in genetic obesity. Eur J Pharmacol 328:207–215

Ueda N, Muramatsu I, Fujiwara M (1984) Capsaicin and bradykinin-induced substance P-ergic Koss MC (1986) Pupillary dilatation as an index for central nervous system α_2-adrenoreceptor activation. J Pharmacol Meth 15:1–19

A.1.3.25
α_2-Adrenoreceptor Blockade Measured in Vivo by Clonidine-Induced Sleep in Chicks

PURPOSE AND RATIONALE

In young chicks, clonidine causes a loss of righting reflex which is antagonized by mianserin (Pommier et al. 1982). This phenomenon was used to measure α_2-adrenoceptor blockade *in vivo* by Gower et al. (1988).

PROCEDURE

Male white Leghorn chicks are used either a few hours after hatching or 1 or 2 days later. Clonidine-induced loss of righting reflex (sleep) is determined with 8 animals at a time. Two animals are treated with placebo and 2 with each of 3 dose levels of the test compound. Tests with groups of 8 animals are continued until 10 animals are tested per dose level or placebo treatment. The chicks are marked with ink and injected intraperitoneally with placebo or the test compound. Ten min later, 1.2 mg/kg clonidine is injected into a leg muscle and the animals are placed individually in small Macrolon cages. The beginning of sleep time is defined as the moment at which the animals can be placed on their back and remain in this position. Sleep time is recorded until they return to their feet spontaneously or another attempt to put them on their back fails. Sleep time is recorded for a maximum period of 30 min.

EVALUATION

Statistical evaluations of differences in median sleeping times are done with the Mann-Whitney *U*-test. Dose-response relations for various drugs can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

Compounds with known α_2-adrenoceptor blocking activity antagonize clonidine-induced sleep in chicks dose-dependently. Yohimbine is one of the most active compounds. However, also other centrally active compounds of which their main effect is not α_2-blockade, reduce clonidine-induced sleeping time. One of the most potent is apomorphine acting on dopamine D_2-receptors. Therefore, the clonidine-induced sleeping test in chicks can not be regarded as highly specific for α_2-adrenoceptors.

REFERENCES AND FURTHER READING

Gower AJ, Broekkamp CLE, Rijk HW, van Delft AME (1988) Pharmacological evaluation of *in vivo* tests for α_2-adrenoceptor blockade in the central nervous system and the effects of mianserin and its aza-analog ORG 3770. Arch Int Pharmacodyn 291:185–201

Pommier Y, Andréjak M, Mouillé P, Dabiré H, Lucet B, Schmitt H (1982) Interaction between mianserin and clonidine at α_2-adrenoceptors. Naunyn-Schmiedeberg's Arch Pharmacol 318:288–294

A.1.3.26
Activity at β_1- and β_2-Adrenoreceptors in the Rat

PURPOSE AND RATIONALE

The relative potency of catecholamines as stimulants of β-adrenoreceptor mediated responses vary in different tissues indicating the existence of two subtypes of β-receptors (β_1 and β_2) (Lands et al. 1967). β-adrenoreceptors in the heart have been classified as being of the β_1-subtype. β-adrenoreceptors in the uterus, diaphragm, bronchioles and small intestine have been classified as being of the β_2-subtype, since in these tissues, epinephrine is more potent than norepinephrine. These observations led to the development of selective agonists and antagonists. Isolated organs (see below) having predominantly one receptor subtype, such as the isolated heart and the isolated atrium for β_1, and the isolated uterus or the isolated tracheal chain for β_2, are used to test compounds for selective activity. Assessing both activities in the same animal *in vivo* results in the advantage that pharmacokinetic and metabolic influences of the drug being tested are the same for both parameters.

PROCEDURE

Female Sprague-Dawley rats (200–220 g) are anesthetized with 60 mg/kg pentobarbital i.p. prior to pithing (Gillespie and Muir 1967). The animals are artificially respired with room air using a Harvard small animal ventilator (90 strokes/min at a pressure of 7 cm H_2O). Body temperature is maintained by placing the animals on a heated operating table. The left carotid artery is cannulated for continuous monitoring of blood pressure via a Statham p23Id pressure transducer. The blood pressure signal is used to trigger an instantaneous rate meter for continuous monitoring of heart rate. A femoral vein is cannulated for intravenous administration of drugs.

A midline incision is made to expose one horn of the uterus. The ovarian artery is cut, tied and one horn dissected free from the ovary leaving the myometrial blood supply intact. A cotton thread is attached to the free end of the uterine horn, passed through a glass-jacketed organ bath and connected to an isometric (Pioden UF1) transducer for measurement of spontaneous contractions. A cannula is inserted into the peritoneal

cavity for administration of drugs by the i.p. route. The organ bath is positioned such that it surrounds the uterine horn without touching it. The tissue is perifused with Krebs-Henseleit solution being gassed with 95% O_2/5% CO_2 and maintained at 37°C. A resting tension of 0.2 g is applied to the tissue, which is allowed to stabilize until spontaneous contractions are constant over a period of 5–10 min. All recordings are made on a polygraph.

EVALUATION OF AGONISTS

Dose-response curves after i.v. injection are established for isoprenaline (nonselective between β_1- and β_2-adrenoreceptors), salbutamol (selective for β_2-adrenoreceptors), and noradrenaline (selective for β_1-adrenoreceptors) in increasing heart rate (beats/min) and decreasing the height of uterine contraction (calculated as percentage of the original amplitude). Animals given noradrenaline are pretreated with phenoxybenzamine (3.3 mmol/kg i.v.) in order to antagonize irreversibly the α-adrenoreceptors. Agonist dose-response curves ($n > 4$) on heart rate and uterine relaxation are carried out by assessing the activity of at least 3 doses of each agonist. New synthetic compounds can be tested after intraperitoneal administration additionally.

EVALUATION OF ANTAGONISTS

The ability of a non-selective β-blocker, such as propranolol (1 mmol/kg i.v.), a β_1-selective β-blocker, such as atenolol, and a β_2-selective β-blocker to inhibit responses to isoprenaline on both heart rate and uterine relaxation is assessed by comparing the log linear portion of the dose-response curve to isoprenaline in the absence and in the presence of the β-adrenoreceptor antagonist in the same animal. Dose ratios for each antagonist are calculated.

CRITICAL ASSESSMENT OF THE METHOD

The method described by Piercy (1988) has the advantage to measure both agonistic and antagonistic activity and to differentiate between effects on β_1- and β_2-adrenoreceptors. Compared to tests in isolated organs, *in vivo* activity can be determined after intraperitoneal or intraduodenal administration.

MODIFICATIONS OF THE METHOD

Härtfelder et al. (1958) studied the influence of various agents on the contractions of electrically stimulated **isolated uteri of rabbits and guinea pigs**.

Nathason (1985) evaluated the activity of beta-blockers to inhibit the cardio-acceleratory effect of systemically administered isoproterenol in **unanesthetized, restrained albino rabbits** together with the effect on membrane bound adenylate cyclase in homogenized ciliary process villi in order to find compounds selectively lowering intraocular pressure.

REFERENCES AND FURTHER READING

Gillespie JS, Muir TC (1967) A method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat. Br J Pharmacol Chemother 30:78–87

Härtfelder G, Kuschinsky G, Mosler KH (1958) Über pharmakologische Wirkungen an elektrisch gereizten glatten Muskeln. Naunyn-Schmiedeberg's Arch exp Path Pharmak 234:66–78

Lands AM, Arnold A, McAuliff JP, Ludena FP, Brown TG (1967a) Differentiation of receptor systems activated by sympathetic amines. Nature 214:597–598

Lands AM, Ludena FP, Buzzo HD (1967b) Differentiation of receptors responsive to isoproterenol. Life Sci 6:2241–2249

Lish PM, Weikel JH, Dungan KW (1965) Pharmacological and toxicological properties of two new β-adrenergic receptor antagonists. J Pharmacol Exp Ther 149:161–173

Nathason JA (1985) Differential inhibition of beta adrenergic receptors in human and rabbit ciliary process and heart. J Pharmacol Exper Ther 232:119–126

Piercy V (1988a) Method for assessing the activity of drugs at β_1- and β_2-adrenoreceptors in the same animal. J Pharmacol Meth 20:125–133

Piercy V (1988b) The β-adrenoreceptors mediating uterine relaxation throughout the oestrus cycle of the rat are predominantly of the β_2-subtype. J Autonom Pharmacol 8:11–18

A.1.3.27
β_1- and β_2-Sympatholytic Activity in Dogs

PURPOSE AND RATIONALE

Intravenous administration of isoprenaline (isoproterenol) stimulates β_1-receptors of the heart which can be detected as an increase in contractility (dp/dt max). Intraarterial injection of isoprenaline stimulates β_2-receptors of peripheral blood vessels leading to an increased peripheral blood flow. Therefore, a β_1- or β_2-blocking activity of a compound is revealed by the inhibition of the effects of isoprenaline. The following tests are used to evaluate β-blocking activity of drugs. A β-blocker screening is done in anesthetized dogs (a); in addition, the test allows a differentiation between β_1- and β_2-receptor activity and the determination of ED_{50} values (b).

PROCEDURE

Male or female Beagle dogs weighing about 20 kg are used. Animals are premedicated with 1 g Inactin(i.v.) and anesthetized by intravenous administration of 20 mg/kg chloralose and 250 mg/kg urethane. In addition, they receive a subcutaneous injection of 2 mg/kg morphine 1 h after the start of anesthesia. Animals are

heparinized. Respiration is maintained through a tracheal tube using a positive pressure respirator. End-expiratory CO_2 content is measured continuously; respiratory rate and depth of respiration are adjusted to 4.5–6 vol% end-expiratory CO_2. For administration of isoprenaline, a peripheral vein is cannulated.

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a cannula inserted into a femoral artery is connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted via the left arteria carotis communis. LVEDP is measured from a high-sensitivity scale. From the pressure curve, d*p*/d*t* max is differentiated and heart rate is counted. Peripheral blood flow in the femoral artery is measured with an electromagnetic flow probe.

Screening for β-Blocking Effects in Anesthetized Dogs

Following a steady-state period of 30–60 min, isoprenaline is administered intravenously 2–3 times to the anesthetized animal and hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses (final concentrations of 0.01, 0.05 and 0.15 mg/kg). For each dose, 10 min "drug effects" are monitored by measuring hemodynamic parameters. Then the effect of isoprenaline is tested again (3 times).

In other experiments, a single dose of the drug is administered to determine the duration of action.

If a test compound does not show an inhibitory influence on isoprenaline effects, a second test compound is administered.

All hemodynamic parameters are registered continuously during the whole experiment.

Testing for β_1- and β_2-Blocking Effects; Determination of ED_{50}

Following a steady-state period of 30–60 min, isoprenaline is administered for i.v. administration (β_1-test) twice at a dose of 0.5 µg/kg and for intraarterial administration (β_2-test) twice at a dose of 0.05 µg/kg. Hemodynamic parameters are recorded (control values = 100%). Then, the test substance is injected intravenously at cumulative doses. Consecutively increasing doses are given at 15-min intervals. For each dose, 10 min "drug effects" are monitored by measuring hemodynamic parameters. Thereafter isoprenaline is given intravenously and 5 min later intra-arterially.

All hemodynamic parameters are registered continuously during the whole experiment.

Characteristics:

- blood pressure
  - systolic, BPs
  - diastolic, BPd
- heart rate
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- d*p*/d*t* max
- peripheral flow, A. femoralis
- ECG, lead II

Standard compounds:

- propranolol HCl
- practolol
- metoprolol tartrate

EVALUATION

β_1-receptor antagonism is measured as a decrease in contractility (d*p*/d*t* max).

Inhibition of the isoprenaline-induced elevation of heart rate is considered as an indicator for non-selective β-blockade. For cardioselective β-receptor blockers the increase in d*p*/d*t* max is inhibited with lower doses of test drug than the rise in heart rate.

β_2-receptor blockade by a test drug is measured as inhibition of the isoprenaline-induced increase in peripheral blood flow.

The different hemodynamic parameters are determined.

Percent inhibition of the isoprenaline-induced effects by a test compound is calculated and compared to the isoprenaline effects before drug administration (= 100%).

ED_{50} values for β_1- and β_2-antagonism are calculated by log-probit analyses. ED_{50} is defined as the dose of drug leading to a 50% inhibition of the isoprenaline effects.

An ED_{50} β_1/ED_{50} β_2-ratio of < 1 indicates that a β-blocking agent predominantly influences β_1-receptors (cardioselectivity).

REFERENCES AND FURTHER READING

Turner RA (1971) β-adrenergic blocking agents. In: Turner RA, Hebborn P (eds) Screening methods in pharmacology. Vol II. pp 21–40. Academic Press, New York and London

A.1.3.28 Intrinsic β-Sympathomimetic Activity in Reserpine-Pretreated Dogs

PURPOSE AND RATIONALE

β-blocking agents can be classified as

- β-blocking agents with intrinsic sympathomimetic activity (ISA),
- β-blocking agents with membrane stabilizing activity (MSA),
- β-blocking agents with organ selectivity (high affinity to heart β_1-receptors).

In the following procedure with reserpine-pretreated dogs, β-blocking agents with intrinsic sympathomimetic activity can be identified. Reserpine administration 24 h before the start of the experiment leads to a depletion of catecholamine depots. Thus, it is possible to differentiate between indirectly acting sympathomimetics such as tyramine and directly acting ones such as noradrenaline.

This test is used to identify β-blocking drugs with intrinsic sympathomimetic activity.

PROCEDURE

Male or female Beagle dogs weighing about 15 kg are used. Twenty-four h before the test, dogs receive an intramuscular injection of 0.3 mg/kg reserpine. At the day of the experiment, the animals are anesthetized by intravenous administration of 10–20 mg/kg pentobarbital sodium. Respiration through a tracheal tube using a positive pressure respirator is controlled by measuring end-expiratory CO_2 concentrations (4–5 vol%).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, a femoral artery is cannulated and connected to a pressure transducer (Statham p 23 DB). For determination of LVP, a Millar microtip catheter (PC 350) is inserted into the left ventricle via the left common carotid artery. LVEDP is measured from a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate is counted.

Experimental Course

The test substance is administered by continuous intravenous infusion of 0.02 mg/kg (1 ml/min) until a cumulative dose of 3 mg/kg is achieved (within approximately 150 min). Thereafter, the velocity of infusion is doubled (0.04 mg/kg, 2 ml/min). The test is finished when a cumulative dose of 7 mg/kg is achieved (after a total time of approximately 250 min).

Hemodynamic parameters are registered continuously during the entire experiment.

Characteristics:

- blood pressure
  - systolic blood pressure
  - diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVED
- dp/dt max
- heart rate, HR

EVALUATION

The different hemodynamic parameters are determined. As a measure for intrinsic sympathomimetic activity (ISA), the increase in dp/dt max and in heart rate are evaluated. Absolute and relative differences of these parameters in drug-treated animals are compared to vehicle control values.

Statistical evaluations are performed by means of the Student's t-test if $n > 4$.

Scores are allotted relative to the efficacy of standard compounds for intensity as well as for duration of the effect.

REFERENCES AND FURTHER READING

DiPalma (1964) Animal techniques for evaluating sympathomimetic and parasympathomimetic drugs. In: Nodine JH, Siegler PE (eds) Animal and pharmacologic techniques in drug evaluation. Vol I, pp 105–110. Year Book Medical Publ., Inc. Chicago

Green AF, Boura ALA (1964) Depressants of peripheral sympathetic nerve function. In: Laurence DR, Bacharach AL (eds) Evaluation of Drug Activities: Pharmacometrics. Academic Press, London and New York, 369–430

A.1.3.29 Cat Nictitating Membrane Preparation (Ganglion Blocking Activity)

PURPOSE AND RATIONALE

Nicotinic acetylcholine receptors are involved in the ganglionic neurotransmission. Various subtypes are described for nicotinic acetylcholine receptors (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

The nictitating membrane of the cat has been used extensively in pharmacological studies to evaluate ganglion blocking activity because of the ease with which its movements can be recorded, because of the simplicity of its innervation (the purely adrenergic fibres have their cell bodies in the easily accessible superior cervical ganglion of the same site) and because its blood supply (via the external carotid artery) is accessible for intraarterial injections. Preganglionic and postganglionic stimulation allow the interpretation of the mode of action of vasoactive drugs.

PROCEDURE

The animal is anesthetized with 35 mg/kg pentobarbitalsodium i.p. Tracheostomy is performed and a tra-

cheal cannula is inserted. On one side, the sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic and postganglionic stimulation. Preferably, the vagus nerve at this site is severed at the central end. The head of the animal is fixed in a head holder to prevent head movements. A linear transducer is fixed at the mid of the border of the nictitating membrane allowing the registration of the contractions on a polygraph. Preganglionic and postganglionic stimuli are exerted by a square wave stimulator, with a pulse width of 0.3 to 0.5 ms, an amplitude of 1–3 V, and a frequency of 20/min. The amplitude and pulse width varies from animal to animal. The sympathetic nerve is stimulated before and after the administration of the compound and the changes in the contraction of the nictitating membrane are noted. Furthermore, the response of the nictitating membrane to exogenous adrenaline is registered.

EVALUATION

The decrease of the response after drug application is expressed as percentage of the control before drug. Ganglionic blockers decrease the response to preganglionic stimulation but have no influence on postganglionic stimulation or exogenous adrenaline. Neuronal blockers decrease the response to both preganglionic and postganglionic stimulation but do not affect the response to exogenous adrenaline which may even be enhanced. α-receptor blockers decrease the response to both preganglionic and postganglionic stimulation as well as decrease the effect of exogenous adrenaline. Catecholamine uptake inhibitors increase the response to both preganglionic and postganglionic stimulation as well as enhance the response to exogenous adrenaline.

CRITICAL ASSESSMENT OF THE METHOD

The nictitating membrane preparation has been widely used for differentiation of cardiovascular effects. Since the use of higher animals such as cats has been limited to a great extent, this model is now being used only exceptionally.

As alternative, the contraction of the inferior eyelid of anesthetized **rats** after preganglionic electrical stimulation of the superior cervical ganglion has been recommended (Gertner 1956; Steinbrecher and Schmid-Wand 1986). In the modification used by Steinbrecher and Schmid-Wand (1986) the method is suitable for testing compounds with potential adrenergic and antiadrenergic activity but not for testing ganglion blocking activities.

Male Sprague Dawley rats are anesthetized with 100 mg/kg thiobutabarbital i.p. and kept on a heated operation table at a rectal temperature of 37°C. One femoral vein is cannulated and filled with 4% heparin solution. One femoral artery is cannulated for registration of blood pressure. Tracheotomy is performed and a polyethylene catheter of 5 cm length inserted. The head of the animal is fixed carefully. The vibrissae at the lower eyelid on the right side are cut, a thread attached at the margin of this eyelid and attached to a strain-gauge. To immobilize the musculature of the face, the mouth of the animal is sutured and the head support attached. The right sympathetic nerve is exposed, separated from the vagus nerve and prepared in order to place electrodes for preganglionic stimulation. For calibration, stimulation is performed twice with an interval until contraction is back to baseline. Furthermore, a dose of 0.001 mg/kg adrenaline is given as bolus injection. Eyelid contraction and blood pressure increase are recorded. Then the putative adrenergic blocker or the standard 1.0 mg/mg phentolamine are injected intravenously. Eyelid contraction after electrical stimulation or after adrenaline is reduced dose-dependently.

MODIFICATIONS OF THE METHOD

Quilliam and Shand (1964) assessed the selectivity of drugs by comparing the effects on ganglionic transmission and on the pre- and post-ganglionic nerves in the isolated superior cervical ganglion preparation of the rat.

Langer and Trendelenburg (1969) performed experiments with normal nictitating membranes of pithed cats as well as with isolated normal nictitating membranes.

Koss and Hey (1992) used frequency-dependent nictitating membrane responses by sympathetic nerve stimulation in anesthetized cats to determine the potential role of prejunctional histamine H_3 receptors.

Gurtu et al. (1992) used contractions of the cat nictitating membrane to explore the effects of calcium channel blockers on neurotransmission *in vivo*, by comparing the effects of verapamil and nifedipine on contractions of nictitating membrane following either electrical stimulation of the superior cervical ganglion or intravenous injection of phenylephrine.

Koss (1992) compared the peripheral and central nervous system sympatholytic actions of prazosin using the cat nictitating membrane. Submaximal contractions of the nictitating membranes were evoked by electrical stimulation of the preganglionic cervical

sympathetic nerve trunk and by stimulation of the posterior hypothalamus in anesthetized cats.

Badio et al. (1996) evaluated spiropyrrolizidines, a new structural class of blockers of nicotinic receptor channels with selectivity for ganglionic type receptors in rat pheochromocytoma PC12 cells (with an $\alpha_3\beta_{4(5)}$-nicotinic receptor) and human medulloblastoma TE671 cells (with an $\alpha_1\beta_1\gamma\delta$-nicotinic receptor).

REFERENCES AND FURTHER READING

Alexander S, Peters J, Mathie A, MacKenzie G, Smith A (2001) TiPS nomenclature Supplement 2001, pp 7–12

Badio B, Shi D, Shin Y, Hutchinson KD, Padgett WL, Daly JW (1996) Spiropyrrolizidines, a new class of blockers of nicotinic receptors. Biochem Pharmacol 52:933–939

Boura AL, Green AF (1959) The actions of brethylium: Adrenergic neuron blocking and other effects. Br J Pharmacol 14:536–548

Bowman WC, Webb SN (1972) Neuromuscular blocking and ganglion blocking activities of some acetylcholine antagonists in the cat. J Pharm Pharmacol 24:762–772

Claßen HG, Marquardt P, Späth M (1968) Sympathicomimetische Wirkungen von Cyclohexylamin. Arzneim Forsch/Drug Res 18:590–594

Fleckenstein A, Burn JH (1953) The effect of denervation on the action of sympathicomimetic amines on the nictitating membrane. Br J Pharmacol 8:69–78

Gertner SB (1956) Pharmacological studies on the inferior eyelid of the anaesthetized rat. Br J Pharmacol 11:147

Green AF, Boura ALA (1964) Depressants of peripheral sympathetic nerve function. I Sympathetic nerve blockade. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press, London and New York, pp 370–430

Gurtu S, Seth S, Roychoudhary AK (1992) Evidence of verapamil-induced functional inhibition of noradrenergic neurotransmission *in vivo*. Naunyn-Schmiedeberg's Arch Pharmacol 345:172–175

Isola W, Bacq ZM (1946) Innervation symapthique adrénergique de la musculature lisse des paupières. Arch Internat Physiol 54:30–48

Karlin A, Akabas MH (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. Neuron 15:1231–1244

Koss MC (1992) Comparison of peripheral and central nervous system sympatholytic actions of prazosin using the cat nictitating membrane. Eur J Pharmacol 211:61–67

Koss MC, Hey JA (1992) Activation of histamine H_3 receptors produces presynaptic inhibition of neurally evoked cat nictitating membrane responses *in vivo*. Naunyn-Schmiedeberg's Arch Pharmacol 346:208–212

Langer SZ, Trendelenburg U (1969) The effect of a saturable uptake mechanism on the slopes of dose-response curves for sympathomimetic amines and on the shifts of dose-response curves produced by a competitive antagonist. J Pharm Exp Ther 167:117–142

McGehee DS, Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521–546

Quilliam JP, Shand DG (1964) The selectivity of drugs blocking ganglionic transmission in the rat. Br J Pharmacol 23:273–284

Sargent PB (1995) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403–443

Steinbrecher W, Schmid-Wand M (1986) Das elektrisch gereizte Unterlid der narkotisierten Ratte. Eine alternative Methode zur elektrisch gereizten Nickhaut der narkotisierten Katze. Personal Commun 2000

Trendelenburg U, Haeusler G (1975) Nerve-muscle preparations of the nictitating membrane. In: Daniel EE, Paton DM (eds) Methods in Pharmacology, Vol 3, Smooth muscle, Plenum Press New York and London pp 457–468

A.1.3.30 Assessment of Ganglion-Blocking Activity in the Isolated Bovine Retractor Penis Muscle

PURPOSE AND RATIONALE

The use of the bovine retractor penis muscle for the assessment of ganglion-blocking activity of neuromuscular blocking drugs has been recommended by Alaranta et al. (1990) and Klinge et al. (1993). Klinge and Sjöstrand (1974) performed not only extensive studies on the physiology and pharmacology of the retractor penis in the bull, but also discussed the various hypotheses on inhibitory and excitatory innervation of this muscle, which is present in many vertebrates such as horses, cats, dogs and rats, but not in men and rabbits. They also found that the effects on the isolated retractor penis muscle and on penile arteries are rather similar. The excitatory innervation was found to be predominantly α-adrenergic (Klinge et al. 1970; Klinge and Sjöstrand 1977) whereas other transmitters such as histamine and bradykinin were effective only in some species. Relaxation of the isolated retractor penis muscle could be elicited by nicotine and other nicotinic agonists (Klinge et al. 1988). In the studies on ganglion-blocking activity, strips of the retractor penis muscle are precontracted by 5-hydroxytryptamine. Relaxation induced by nicotine is antagonized by ganglion-blockers.

PROCEDURE

Retractor penis muscles are obtained from bulls of different breeds weighing 250–500 kg. Samples are dissected 10–30 cm distal to the points where the paired muscle bundles pass the anal orifice. Immediately after slaughter, the samples are freed from fat and other surrounding tissue and placed into Tyrode solution at 2–4°C. Strips, 15–25 mm in length and 2–3 mm wide, are prepared and mounted in 20-ml organ baths containing Tyrode solution at 35°C aerated with 95% O_2 and 5% CO_2. An equilibrium time of 2 to 4 h is allowed. During the equilibrium period washed are performed at about 60-min intervals. Changes in tension are recorded by means of Grass FT 03 force displacement transducers coupled to a polygraph.

A high-enough tone for studying the nicotine-induced relaxation, usually 8–15 g, is generated by adding 5-HP in a concentration between 0.1 and 6 µM to the organ bath. Washing is performed 2 min after application of nicotine; 60–80 min later the tone is again raised and the application of nicotine is repeated. The effect of a neuromuscular blocking drug is studied only if the relaxations caused by nicotine in two consecutive controls are equal in size.

EVALUATION

The blocking activity of a certain concentration of a drug is expressed as % reduction in the relaxation of the muscle strip, according to the following equation:

$$\frac{A - B}{A} \times 100$$

Where A is the size of the control relaxation in millimeters, and B is the size of the relaxation of the blocking drug. In order to construct regression lines, the activity of four or five dose levels from the assumed linear part of the concentration-effect curve is studied. The activity of each dose level is studied in at least 5 strips obtained from different animals. IC_{50} values are calculated from the regression lines. The parallelism of the regression lines is tested by covariance analysis.

CRITICAL ASSESSMENT OF THE METHOD

Molar potency ratios of known ganglion-blocking agents obtained with this method were compared with the results of other methods, such as inhibition of contraction of cat nictitating membrane evoked by preganglionic sympathetic stimulation (Bowman and Webb 1972; see 1.3.29), inhibition of nicotine-induced contraction of the isolated guinea pig ileum (Feldberg 1951), inhibition of contraction of guinea pig vas deferens evoked by preganglionic stimulation of the hypogastric nerve *in vitro* (Birmingham and Hussain 1980), depression of postganglionic action potentials evoked by preganglionic stimulation of the superior cervical ganglion of the rat *in vitro* (Quilliam and Shand 1964), induction of mydriasis in mouse by blocking the ciliary ganglion (Edge 1953). A fair but not a complete agreement between the results obtained with various methods was found.

MODIFICATIONS OF THE METHOD

Gillespie and Sheng (1990) studied the effects of pyrogallol and hydroquinone on the response to non-adrenergic, non-cholinergic nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles.

Parkkisenniemi and Klinge (1996) used samples of retractor penis muscles and penile arteries from bulls for functional characterization of endothelin receptors.

La et al. (1997) studied the inhibition of nitrergic nerve-induced relaxations in rat anococcygeus and bovine retractor penis muscles by hydroxycobalamin.

REFERENCES AND FURTHER READING

Alaranta S, Klinge E, Pätsi T, Sjöstrand NO (1990) Inhibition of nicotine-induced relaxation of the bovine retractor penis muscle by compounds known to have ganglion-blocking activity. Br J Pharmacol 101:472–476

Birmingham AT, Hussain SZ (1980) A comparison of the skeletal neuromuscular and autonomic ganglion-blocking potencies of five non-depolarizing relaxants. Br J Pharmacol 70:501–506

Bowman WC, Webb SN (1972) Neuromuscular blocking and ganglion blocking activities of some acetylcholine antagonists in the cat. J Pharm Pharmacol 24:762–772

Edge ND (1953) Mydriasis in the mouse: a quantitative method of estimating parasympathetic ganglion block. Br J Pharmacol Chemother 8:10–14

Feldberg W (1951) Effects of ganglion-blocking substances on the small intestine. J Physiol (Lond) 113:483–505

Gillespie JS, Sheng H (1990) The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles. Br J Pharmacol 99:194–196

Klinge E, Sjöstrand NO (1974) Contraction and relaxation of the retractor penis muscle and the penile artery in the bull. Acta Physiol Scand, Suppl 420:5–88

Klinge E, Sjöstrand NO (1977) Comparative study of some isolated mammalian smooth muscle effectors of penile erection. Acta Physiol Scand 100:354–365

Klinge E, Potho P, Solatunturi E (1970) Adrenergic innervation and structure of the bull retractor penis muscle. Acta Physiol Scand 78:110–116

Klinge E, Alaranta S, Sjöstrand NO (1988) Pharmacological analysis of nicotinic relaxation of bovine retractor penis muscle. J Pharmacol Exp Ther 245:280–286

Klinge E, Alaranta S, Parkkisenniemi UM, Kostiainen E, Sjöstrand NO (1993) The use of the bovine retractor penis muscle for the assessment of ganglion-blocking activity of neuromuscular blocking and other drugs. J Pharmacol Toxicol Meth 30:197–202

La M, Paisley K, Martin W, Rand MJ (1997) Effects of hydroxycobalamin on nitrergic transmission in rat anococcygeus and bovine retractor penis muscles: sensitivity to light. Eur J Pharmacol 321:R5–R6

Parkkisenniemi UM, Klinge E (1996) Functional characterization of endothelin receptors in the bovine retractor penis muscle and penile artery. Pharmacol Toxicol 79:73–79

Quilliam JP, Shand DG (1964) The selectivity of drugs blocking ganglion transmission in the rat. Br J Pharmacol 23:273–284

A.1.3.31 Angiotensin II Antagonism

PURPOSE AND RATIONALE

Angiotensin II antagonists can be tested in rats after elimination of cardiovascular reflexes by vagotomy and ganglionic blockade. Several angiotensin II

antagonists possess intrinsic agonistic activity. This can be tested by injection of various doses to the vagotomized, ganglion-blocked animal. The antagonistic activity of the angiotensin II antagonist can be evaluated by antagonism against graded doses of angiotensin II. The duration of activity can be tested during continuous infusion of angiotensin II.

PROCEDURE

Male Sprague-Dawley rats weighing about 300 g are used. They are anesthetized with 60 mg/kg pentobarbital sodium i.v. One carotid artery is cannulated and connected with a Statham transducer P 23 Db. Blood pressure is recorded on a polygraph. Both jugular veins are cannulated for application of test compounds and for infusion. Both vagal nerves are cut 3 mm dorsal of the larynx. For ganglionic blockade, 10 mg/kg pentolinium tartrate are injected intravenously. At least 5 animals are used for evaluation of one test drug.

Intrinsic Agonistic Activity

After the blood pressure has reached a constant value, doses of 1, 2, 4 and 16 µg/kg of the test compound are injected via the jugular vein. Blood pressure is recorded.

Antagonistic Activity

In 10 min intervals doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected to establish dose-response curves. After 10 min, continuous infusion is started of the potential angiotensin II blocker in a dosage of 10 µg/kg/0.1 ml/min. Ten min after beginning of the infusion, again doses of 0.5; 1.0; and 2.0 µg/kg angiotensin II are injected.

Duration of Activity

In this set-up, angiotensin II is administered as continuous infusion at a dosage of 1 µg/kg/0.02 ml/min. When blood pressure has reached an elevated steady state level, 0.1 mg/kg of the angiotensin II antagonist is administered.

Intensity and duration of the fall of blood pressure are recorded.

EVALUATION

Intrinsic Agonistic Activity

An increase of blood pressure indicates the intrinsic agonistic activity.

Antagonistic Activity

Increases of blood pressure after graduated doses of angiotensin II during the infusion is expressed as percentage of the increase before infusion. The results are compared with known angiotensin II antagonists.

CRITICAL ASSESSMENT OF THE METHOD

In this test not only potency and duration of activity but also the intrinsic agonistic activity of an angiotensin II antagonist can be tested.

MODIFICATIONS OF THE METHOD

Various other pharmacological models have been used to test angiotensin II antagonists:

Blood pressure in conscious unrestrained rats with chronically implanted catheters with normal blood pressure, spontaneous hypertension and chronic renal hypertension (Vogel et al. 1976; Chiu et al. 1989; Brooks et al. 1992; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Nagura et al. 1995; Nozawa et al. 1995; Renzetti et al. 1995; Wong 1995; Junggren et al. 1996),

Blood pressure in conscious spontaneously hypertensive and in anesthetized ganglion-blocked rats (Olins et al. 1993),

Blood pressure in pithed and in conscious renovascular hypertensive rats (Criscone et al. 1993; Wienen et al. 1993; Deprez et al. 1995; Kivlighn et al. 1995a; Kushida et al. 1995),

Blood pressure in rats after intracerebroventricularly injected angiotensin II (Vogel et al. 1976; Batt et al. 1988),

Blood pressure in conscious angiotensin I-infused and renin-dependent **hypertensive dogs** (Brooks et al. 1992; Cazaubon et al. 1993; Aiyar et al. 1995; Deprez et al. 1995; Gabel et al. 1995; Keiser et al. 1995; Wong et al. 1995),

Blood pressure and heart rate in conscious sodium-depleted and sodium-repleted **cynomolgus monkeys** (Lacour et al. 1993; Cazaubon et al. 1993; Keiser et al. 1995),

Angiotensin II induced pressor responses in **marmosets** (Nagura et al. 1995),

Blood pressure and heart rate in conscious **rhesus monkeys** and anesthetized **chimpanzees** (Gabel et al. 1995; Kivlighn et al. 1995b; Kivlighn et al. 1995c).

Inhibition of angiotensin II-induced contraction in isolated **aorta** rings or strips from **rabbits** (Chui et al. 1989, 1990; Criscione et al. 1993; Cazaubon et al. 1993; Olins et al. 1993; Wienen et al. 1993; Aiyar et al. 1995; Caussade et al. 1995; Hilditch et al. 1995; Keiser et al. 1995; Kushida et al. 1995; Nagura et al. 1995; Renzetti et al. 1995; Wong et al. 1995), from **rats** (Nozawa et al. 1997), from **neonatal rats** (Keiser et al. 1993), from **guinea pigs** (Mizuno et al. 1995),

Inhibition of angiotensin II-induced contraction in isolated rat pulmonary artery (Chang et al. 1995),

Antagonism against angiotensin II in isolated strips of rabbit aorta, rabbit jugular vein, rabbit pulmonary artery, rat portal vein, rat stomach, rat urinary bladder, human urinary bladder, human colon, human ileum (Rhaleb et al. 1991),

Contractions of **guinea pig ileum** *in situ* (Khairallah and Page 1961),

Antagonism against angiotensin II in the **isolated rat uterus** (Wahhab et al. 1993),

Contractile force and prostaglandin E synthesis in electrically stimulated **rabbit isolated vas deferens** (Trachte et al. 1990),

Antagonism against angiotensin II-induced aldosterone release in **bovine adrenal glomerulosa cells**. (Criscione et al. 1993), and in rat dispersed adrenal capsular cells (Chang et al. 1995),

Antagonism against angiotensin II-induced inhibition of guanylate cyclase activity in the **rat pheochromocytoma cell line** PC12W (Brechler et al. 1993).

Brooks et al. (1995) compared the cardiovascular and renal effects of an angiotensin II receptor antagonist and captopril in **rats with chronic renal failure** induced by 5/6 nephrectomy. Under sodium pentobarbital anesthesia the right kidney was removed and approximately two thirds of the left kidney was infarcted by ligating two or three branches of the left renal artery.

Kim et al. (1997) studied the effects of an angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. An abdominal aortacaval shunt was prepared in 9-weeks old male Wistar rats under sodium pentobarbital anesthesia. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two thirds caudal to the renal artery and one third cephalic to the aortic bifurcation with a 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating the vena cava. After the aorta was clamped, the needle was withdrawn, and a drop of cyanoacrylate glue was used to seal the aortic puncture point. The patency of the shunt was verified visually by swelling of the vena cava and mixing of arterial and venous blood. The rats were treated either with vehicle or the angiotensin antagonist. Four days after the preparation of the AC shunt, 24 h-urine volume, electrolytes and aldosterone were measured. Six days after the AC shunt blood was collected by puncture of a tail vein and plasma renin activity and aldosterone were measured. Seven days after AC shunt, hemodynamic studies were performed in pentobarbital anesthesia. Afterwards, the heart was rapidly excised, left and right atria and ventricles were separated and frozen in liquid nitrogen for the extraction and measurement of cardiac tissue RNA.

Shibasaki et al. (1997) tested the effect on the renin-angiotensin-aldosterone system in **conscious rats** after cannulation of the abdominal aorta under anesthesia 3–4 days before the experiment. After oral dosing of the angiotensin II receptor antagonist blood samples were withdrawn and plasma renin and aldosterone determined by radioimmunoassay.

Similar to the effects of ACE inhibitors, lifespan of hypertensive rats could be doubled by long-term treatment with an angiotensin II type 1 receptor blocker (Linz et al. 2000).

Ledingham and Laverty (1996) treated **genetically hypertensive New Zealand rats** with a specific AT_1 receptor antagonist via osmotic minipumps for several weeks and measured the effects on blood pressure, cardiac hypertrophy and the structure of resistance arteries.

Transgenic animals were recommended for further studies to influence the human renin-angiotensin system (Müller et al. 1995; Wagner et al. 1995; Bohlender et al. 1996).

REFERENCES AND FURTHER READING

Aiyar N, Baker E, Vickery-Clark L, Ohlstein EH, Gellai M, Fredrickson TA, Brooks DP, Weinstock J, Weidley EF, Edwards RM (1995) Pharmacology of a potent long-acting imidazole-5-acrylic acid angiotensin AT_1 receptor antagonist. Eur J Pharmacol 283:63–72

Batt CM, Klein EW, Harding JW, Wright JW (1988) Pressor responses to amastatin, bestatin and Plummer's inhibitors are suppressed by pretreatment with the angiotensin receptor antagonist Sarthran. Brain Res Bull 21:731–735

Bohlender J, Ménard J, Wagner J, Luft FC, Ganten D (1996) Hypertension 27:535–540

Brechler V, Jones PW, Levens NR, de Gasparo M, Bottari SP (1993) Agonistic and antagonistic properties of angiotensin analogs at the AT_2 receptor in PC12W cells. Regul Pept 44:207–213

Brooks DP, Fredrickson TA, Weinstock J, Ruffolo RR Jr, Edwards RM, Gellai M (1992) Antihypertensive activity of the nonpeptide angiotensin II receptor antagonist, SK&F 108566, in rats and dogs. Naunyn Schmiedeberg's Arch Pharmacol 345:673–678

Brooks DP, Contino LC, Short BG, Gowan Ch, Trizna W, Edwards RM (1995) SB 203220: A novel angiotensin II receptor antagonist and renoprotective agent. J Pharmacol Exp Ther 274:1222–1227

Caussade F, Virone-Oddos A, Delchambre C, Cazes M, Versigny A, Cloarec A (1995) *In vitro* pharmacological characterization of UP 269–6, a novel nonpeptide angiotensin II receptor antagonist. Fundam Clin Pharmacol 9:119–128

Cazaubon C, Gougat J, Bousquet F, Guiraudou P, Gayraud R, Lacour C, Roccon A, Galindo G, Barthelmy G, Gautret B,

Bernhart C, Perreaut P, Breliere JC, le Fur G, Nisato D (1993) Pharmacological characterization of SR 47436, a new non-peptide AT_1 subtype angiotensin II receptor antagonist. J Pharmacol Exp Ther 265:826–834

Chang RSL, Lotti VJ, Chen TB, O'Malley SS, Bedensky RJ, Kling PJ, Kivlighn SD, Siegl PKS, Ondeyka D, Greenlee WJ, Mantlo NB (1995) *In vitro* pharmacology of an angiotensin AT_1 receptor antagonist with balanced affinity for AT_2 receptors. Eur J Pharmacol 294:429–437

Chiu AT, Duncia JV, McCall DE, Wong PC, Price WA, Thoolen MJMC, Carini DJ, Johnson AL, PBMWM Timmermans (1989) Nonapeptide angiotensin II receptor antagonists. III. Structure-function studies. J Pharmacol Exp Ther 250:867–874

Chui AT, McCall DE, Price WA, Wong PC, Carini DJ, Duncia JV, Wexler RR, Yoo SE, Johnson AL, PBMWM Timmermans (1990) Nonapeptide angiotensin II receptor antagonists: Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. J Pharm Exp Ther 252:711–718

Criscione L, de Gasparo M, Bühlmayer P, Whitebread S, Ramjoué HPR, Wood J (1993) Pharmacological profile of valsartan: a potent, orally active, nonpeptide antagonist of angiotensin II AT_1-receptor subtype. Br J Pharmacol 110:761–771

Deprez P, Guillaume J, Becker R, Corbier A, Didierlaurent S, Fortin M, Frechet D, Hamon G, Heckmann B, Heitsch H, Kleemann HW, Vevert JP, Vincent JC, Wagner A, Zhang J (1995) Sulfonylureas and sulfonylcarbamates as new non-tetrazole angiotensin II receptor antagonists. Discovery of the highly potent orally active (imidazolylbiphenyl)sulfonylurea (HR 720). J Med Chem 38:2357–2377

Gabel RA, Kivlighn SD, Zingaro GJ, Schorn TW, Schaffer LW, Ashton WT, Chang LL, Flanagan K, Greenlee WJ, Siegl PKS (1995) *In vivo* pharmacology of L-159,913, a new highly potent and selective nonpeptide angiotensin II receptor antagonist. Clin Exp Hypertens 17:931–953

Hilditch A, Hunt AAE, Travers A, Polley J, Drew GM, Middlemiss D, Judd DB, Ross BC, Robertson MJ (1995) Pharmacological effects of GR138950, a novel angiotensin AT_1 receptor antagonist. J Pharm Exp Ther 272:750–757

Junggren IL, Zhao X, Sun X, Hedner Th (1996) Comparative cardiovascular effects of the angiotensin II type 1 receptor antagonists ZD 7155 and losartan in the rat. J Pharm Pharmacol 48:829–833

Keiser JA, Major TC, Lu GH, Davis LS, Panek RL (1993) Is there a functional cardiovascular role for the AT_2 receptors? Drug Dev Res 29:94–99

Keiser JA, Ryan MJ, Panek RL, Hedges JC, Sircar I (1995) Pharmacologic characterization of CI-996, a new angiotensin receptor antagonist. J Pharm Exp Ther 272:963–969

Khairallah PA, Page IH (1961) Mechanism of action of angiotensin and bradykinin on smooth muscle *in situ*. Am J Physiol 200:51–54

Kim S, Sada T, Mizuno M, Ikeda M, Yano M, Miura K, Yamanaka S, Koike H, Iwao H (1997) Effects of angiotensin AT_1 receptor antagonist on volume overload-induced cardiac gene expression in rats. Hypertens Res 20:133–142

Kivlighn SD, Huckle WR, Zingaro GJ, Rivero RA, Lotti VJ, Chang RSL, Schorn TW, Kevin N, Johnson RG Jr, Greenlee WJ, Siegl PKS (1995a) Discovery of L-162,313: a nonpeptide that mimics the biological actions of angiotensin II. Am J Physiol 268 (Regul Integr Comp Physiol 37): R820–R823

Kivlighn SD, Zingaro GJ, Gabel RA, Broten TB, Schorn TW, Schaffer LW, Naylor EM, Chakravarty PK, Patchett AA, Greenlee WJ, Siegl PKS (1995b) *In vivo* pharmacology of a novel AT_1 selective angiotensin II receptor antagonist, MK-996. Am J Hypertension 8:58–66

Kivlighn SD, Zingaro GJ, Gabel RA, Broten TB, Chang RSL, Ondeyka DL, Mantlo NB, Gibson RE, Greenlee WJ, Siegl PKS (1995c) *In vivo* pharmacology of an angiotensin AT_1 receptor antagonist with balanced affinity for angiotensin AT_2 receptors. Eur J Pharmacol 294:439–450

Kushida H, Nomura S, Morita O, Harasawa Y, Suzuki M, Nakano M, Ozawa K, Kunihara M (1995) Pharmacological characterization of the nonpeptide angiotensin II receptor antagonist, U-97018. J Pharmacol Exp Ther 274:1042–1053

Lacour C, Roccon A, Cazaubon C, Segondy D, Nisato D (1993) Pharmacological study of SR 47436, a non-peptide angiotensin II AT_1-receptor antagonist, in conscious monkeys. J Hypertens 11:1187–1194

Ledingham JM, Laverty R (1996) Remodelling of resistance arteries in genetically hypertensive rats by treatment with valsartan, an angiotensin II receptor antagonist. Clin Exper Pharmacol Physiol 23:576–578

Linz W, Heitsch H, Schölkens BA, Wiemer G (2000) Lonterm angiotensin II type 1 receptor blockade with fonsartan doubles lifespan of hypertensive rats. Hypertension 35:908–913

Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanagisawa H, Koike H (1995) Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. Eur J Pharmacol 285:181–188

Müller D, Hilgers K, Bohlender J, Lippoldt A, Wagner J, Fischli W, Ganten D, Mann JFE, Luft FC (1995) Effects of human renin in the vasculature of rats transgenic for human angiotensinogen. Hypertension 26:272–278

Nagura J, Yasuda S, Fujishima K, Yamamoto M, Chui C, Kawano KI, Katano K, Ogino H, Hachisu M, Konno F (1995) Pharmacological profile of ME3221, a novel angiotensin II receptor antagonist. Eur J Pharmacol 274:210–221

Nozawa Y, Haruno A, Oda N, Yamasaki Y, Matsuura N, Miyake H, Yamada S, Kimura R (1997) Pharmacological profile of TH-142177, a novel orally active AT_1-receptor antagonist. Fundam Clin Pharmacol 11:395–401

Olins GM, Corpus VM, Chen ST, McMahon EG, Paloma MA, McGraw DE, Smits GJ, Null CL, Brown MA, Bittner SE, Koepke JB, Blehm DJ, Schuh JR, Baierl CS, Schmidt RE, Cook CS, Reitz DB, Penick MA, Manning RE, Blaine EH (1993) Pharmacology of SC-52458, an orally active, nonpeptide angiotensin AT_1 receptor antagonist. J Cardiovasc Pharmacol 22:617–625

Renzetti AR, Cucchi P, Guelfi M, Cirillo R, Salimbeni A, Subissi A, Giachetti A (1995) Pharmacology of LR-B/057, a novel active AT_1 receptor antagonist. J Cardiovasc Pharmacol 25:354–360

Rhaleb NE, Rouissi N, Nantel F, D'Orléans-Juste P, Regoli D (1991) DuP 753 is a specific antagonist for the angiotensin receptor. Hypertens 17:480–484

Siegl PKS (1993) Discovery of losartan, the first specific nonpeptide angiotensin II receptor antagonist. J Hypertension 11 (Suppl 3):S19–S22

Shibasaki M, Fujimori A, Kusayama T, Tokioka T, Satoh Y, Okazaki T, Uchida W, Inagaki O, Yangisawa I (1997) Antihypertensive activity of a nonpeptide angiotensin II receptor antagonist, YM358, in rats and dogs. Eur J Pharmacol 335:175–184

Smits GJ, Koepke JP, Blaine EH (1991) Reversal of low dose angiotensin hypertension by angiotensin receptor antagonists. Hypertension 18:17–21

Trachte GJ, Ferrario CM, Khosla MC (1990) Selective blockade of angiotensin responses in the rabbit isolated vas deferens by angiotensin receptor antagonists. J Pharm Exp Ther 255:929–934

Vogel HG, Jung W, Schoelkens BA (1976) Hypotensive action of central injection of angiotensin II antagonist in conscious rats with experimental hypertension. Abstr. No. 286, p 117, V. Intern Congr Endocin, Hamburg

Wagner J, Thiele F, Ganten D (1996) The renin-angiotensin system in transgenic rats. Pediatr Nephrol 10:108–112

Wahhab A, Smith JR, Ganter RC, Moore DM, Hondrelis J, Matsoukas J, Moore GJ (1993) Imidazole based non-peptide angiotensin II receptor antagonists. Investigation of the effects of the orientation of the imidazole ring on biological activity. Arzneim Forsch/Drug Res 43:1157–1168

Wienen W, Hauel N, van Meel JCA, Narr B, Ries U, Entzeroth M (1993) Pharmacological characterization of the novel non-peptide angiotensin II receptor antagonist, BIBR 277. Br J Pharmacol 110:245–252

Wong PC, Price WA Jr, Chiu AT, Thoolen MJMC, Duncia JV, Johnson AL, Timmermans PBMWM (1989) Nonapeptide angiotensin II receptor antagonists. IV. EXP6155 and EXP6803. Hypertension 13:489–497

Wong PC, Hart SH, Zaspel AM, Chiu AT, Ardecky RJ, Smith RD, Timmermans PBMWM (1990) Functional studies on nonpeptide angiotensin II receptor subtype-specific ligands: DuP 753 (A ll-1) and PD 123177 (A ll-2) J Pharmac Exp Ther 255:584–592

Wong PC, Hart SD, Chiu AT, Herblin WF, Carini DJ, Smith RD, Wexler RR, Timmermans PBMWM (1991a) Pharmacology of DuP 5323, a selective and noncompetitive AT1 receptor antagonist. J Pharm Exp Ther 259:861–870

Wong PC, Price WA, Chiu AT, Duncia JV, Carini DJ, Wexler RR, Johnson AL, Timmermans PBMWM (1991b) *In vivo* pharmacology of DuP 753. Am J Hypertens 4:288S–298S

Wong PC, Quan ML, Saye JAM, Bernard R, Crain EJ Jr, NcCall DE, Watson CA, Zaspel AM, Smith RD, Wexler RR, Timmermans PBMWM, Chiu AT (1995) Pharmacology of XR510, a potent orally active nonpeptide angiotensin II AT_1 receptor antagonist with high affinity to the AT_2 receptor subtype. J Cardiovasc Pharmacol 26:354–362

A.1.3.32 ACE Inhibition Measured in Vivo in the Rat

PURPOSE AND RATIONALE

The angiotensin-converting enzyme (ACE) is responsible for the cleavage of the almost inactive angiotensin I to the active angiotensin II. The same enzyme (kininase II) is responsible for the degradation of the active peptide bradykinin to inactive products. ACE activity can therefore be measured in two ways: activity of the newly formed angiotensin II and diminution of the activity of bradykinin. ACE inhibition results in decreased activity of the precursor angiotensin I and potentiation of the bradykinin effect. The cardiovascular system is sensitive to both peptides, reacting with an increase of blood pressure to angiotensin II and with a decrease to bradykinin. These reactions can be used for quantitative determination of ACE inhibiting activity.

PROCEDURE

Male Sprague-Dawley rats weighing 300–400 g are used. The animals are anesthetized by i.p. injection of 70 mg/hg pentobarbital. After intubation of the trachea they are artificially respirated with 30 strokes/min and a stroke volume of 6–8 ml. The right carotid artery is cannulated and blood pressure registered with a Statham-element (P 23 Db) and a polygraph. One jugular vein is cannulated for i.v. injections. After laparotomy a catheter is inserted into the duodenum for enteral administration and the wound closed again. Blood pressure is stabilized 30% below the normal level by i.m. injection of 5 mg/kg pentolinium. In order to prevent excessive mucus production in the bronchial system, 40 µg/kg atropine sulfate are injected intramuscularly.

Inhibition of Angiotensin I Cleavage

After stabilization of blood pressure, 310 ng/kg angiotensin I is injected intravenously in 0.1 ml saline. The injection is repeated in 5-min intervals until an identical pressure reaction occurs. The test compounds are administered at doses of 1 and 10 mg/kg intravenously or 25 mg/kg intraduodenally. 3 min after iv. injection or 10 min after i.d. administration, again 310 ng/kg angiotensin I is injected. Standards are ramipril, enalapril or captopril.

Potentiation of Bradykinin-Induced Vasodepression

A low dose of bradykinin has to be chosen in order to visualize the bradykinin potentiation. One µg/kg, eventually 3 µg/kg bradykinin are injected intravenously at 5 min intervals until a stable reaction is achieved. Three min after i.v. injection or 10 min after intraduodenal administration of the test substance, the bradykinin injection is repeated.

EVALUATION

Inhibition of Angiotensin I Cleavage

The diminution of the pressure reaction to angiotensin I after administration of a potential ACE inhibitor is the parameter for the activity of the new compound. The inhibition is calculated as percent of controls. Using various doses of the ACE inhibitor, dose-response curves can be established and ID_{50} values be calculated.

Potentiation of Bradykinin-Induced Vasodepression

Potentiation of bradykinin induced vasodepression is expressed as percentage of controls. Using various doses of the test compound and the standard, dose-response curves can be established and potency ratios calculated.

CRITICAL ASSESSMENT OF THE METHOD

Both parameters, inhibition of angiotensin I response and potentiation of bradykinin-induced vasodepression have been proven as reliable parameters for evaluation of ACE inhibitors.

MODIFICATIONS OF THE METHOD

Natoff et al. (1981) used the ratio of responses to angiotensin I and angiotensin II in spontaneously hypertensive rats, either pithed or anesthetized with urethane, to determine the degree and the duration of effect of captopril.

Blood levels of angiotensin II can also be measured by radioimmunoassay.

Several studies in rats showed the beneficial effects of prolonged treatment with ACE inhibitors. Postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction was decreased by ACE inhibition (Linz et al. 1996).

Inhibition of angiotensin I-induced pressure response by administration of ACE-inhibitors can be measured not only in anesthetized rats, but also in anesthetized dogs, conscious rats and conscious dogs (Becker et al. 1984).

Life-long ACE inhibition doubles lifespan of hypertensive rats not only if the treatment is started at the age of one month (Linz et al. 1997), but ramipril also increases survival in old spontaneously hypertensive rats if treatment is started at the age of 15 months (Linz et al. 1999).

Panzenbeck et al. (1995) reported that captopril-induced hypotension is inhibited by the bradykinin blocker HOE 140 in Na^+ depleted **marmosets.**

REFERENCES AND FURTHER READING

Becker RHA, Schölkens BA, Metzger M, Schulze KJ (1984) Pharmacological activities of the new orally active angiotensin converting enzyme inhibitor 2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (Hoe 498). Arzneim Forsch/Drug Res 34:1411–1416

Linz W, Wiemer G, Schmidts HL, Ulmer W, Ruppert D, Schölkens BA (1996) ACE inhibition decreases postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction. Clin Exper Hypertension 18:691–712

Linz W, Jessen T, Becker RHA, Schölkens BA, Wiemer G (1997) Long-term ACE inhibition doubles lifespan of hypertensive rats. Circulation 96:3164–3172

Linz W, Wohlfart P, Schoelkens BA, Becker RHA, Malinski T, Wiemer G (1999) Late treatment with ramipril increases survival in old spontaneously hypertensive rats. Hypertension 34:291–295

Natoff IL, Brewster M, Patel AT (1981) Method for measuring the duration of inhibition of angiotensin I-converting enzyme *in vivo*. J Pharmacol Meth 5:305–312

Panzenbeck M, Loughnan CL, Madwed JB, Winquist RJ, Fogal SE (1995) Captopril-induced hypotension is inhibited by the bradykinin blocker HOE 140 in Na^+ depleted marmosets. Am J Physiol 269(4 Pt 2): H1221–H1228

Pettinger W, Sheppard H, Palkoski Z, Renyi E (1973) Angiotensin antagonism and antihypertensive activity of phosphodiesterase inhibiting agents. Life Sci 12:49–62

Rubin B, Laffan RJ, Kotler DG, O'Keefe EH, Demaio DA, Goldberg ME (1978) SQ 14,225 (D-3-mercapto-2-methylpropanoyl-L-proline), a novel orally active inhibitor of angiotensin I-converting enzyme. J Pharmacol Exp Ther 204:271–280

A.1.3.33
Evaluation of Renin Inhibitors in Dogs

PURPOSE AND RATIONALE

Highly specific inhibitors of the enzyme renin are considered to be potential antihypertensive agents. These agents cause a fall in blood pressure of sodium-deficient dogs and decrease plasma renin activity as well as angiotensin II level.

PROCEDURE

Animal Experiment

Adult mongrel dogs (8–14 kg) of either sex are given water ad libitum and maintained on a low sodium diet for 1–2 weeks before the experiment. A single intramuscular injection of 5 mg/kg furosemide is given 48 h before the experiment. On the day of the experiment the dogs are anesthetized with sodium pentobarbital (30 mg/kg i.v.) and a cuffed endotracheal tube is positioned to allow artificial respiration. To measure arterial blood pressure a femoral artery is catheterized with polyethylene tubing. The right and left femoral veins are catheterized for drug administration and delivery of a maintenance infusion of sodium pentobarbital (5 mg/kg/h). Blood pressure is measured directly through the catheter, which is connected to a Gould-Statham pressure transducer. Blood samples are collected from the arterial catheter.

Increasing doses of the potential renin inhibitor are infused over 30 min followed by a 30 min recovery period. Immediately after the last recovery period, the dogs are given an i.v. infusion of the angiotensin receptor antagonist saralasin (20 µg/kg/min) for 30 min. For measurement of plasma renin activity and an-

giotensin II levels, the dogs are infused over a period of 30 min with the test compound and blood is withdrawn at 0, 15, 30, 60, 90, 120, 180, and 240 min after the start of the infusion. After the final blood drawing, 20 µg/kg/min saralasin is infused for 30 min.

Analytical Procedures

The antibody-trapping method is preferred to measure plasma renin activity (PRA). In this procedure PRA is determined at pH 7.4 by RIA quantification of angiotensin I (ANG I) generated and then trapped by excess anti-ANG I antibody (Poulsen and Jorgensen 1973; Nussberger et al. 1987). In tubes coated with rabbit anti-ANG I antibody (Gamma Coat™ ^{125}I Plasma renin activity RIA kit; Baxter Travenol Diagnostics) and incubated in an ice-water bath, 75 µl plasma are mixed with 7 µl 3 M TRIS base buffer (pH 7.2) containing 200 mM EDTA, and 3 µl 0.2 M TRIS base (pH 7.5) containing 3 g/L human serum albumin (fraction V, Sigma). Tubes are vortexed and incubated at 37°C for 60 min. The incubation is terminated by placing the tubes in an ice-water bath. Next, 75 µl of the TRIS albumin buffer are added, followed by 1 ml phosphate RIA buffer (Gamma Coat™) containing 15,000 cpm of ^{125}I ANG I. Standard ANG I (0.2–50 ng/ml) is also incubated at 37°C for 60 min with 10 µl TRIS/albumin buffer. In an ice-water bath, low renin plasma (75 µl) is added to the standards before the addition of a 1 ml tracer solution. Samples and standards are incubated for 24 h at 4°C. Tubes are then aspirated and counted in a gamma counter.

Levels of immunoreactive angiotensin II (ir-ANG II) are measured using a procedure described by Nussberger et al. (1985). Two–three ml of whole blood are collected in prechilled glass tubes containing 125 µl of the following "inhibitor" solution: 2% ethanol, 25 mM phenanthroline, 125 mM EDTA, 0.5 mM pepstatin A, 0.1 mM captopril, 2 g/l neomycin sulfate, and 0.1 mM of the renin inhibitor CGP 38560. The tubes are then centrifuged and the plasma quickly frozen in liquid nitrogen and stored at –70°C. For extraction of angiotensin peptides, Bond-Elut cartridges (Bond-Elut-pH) containing 100 mg phenylsilica are used, along with a Vac Elut SPS24 vacuum manifold (Analytichem; Harbor City, CA). Each cartridge is preconditioned with 1.0 ml methanol (HPLC grade) followed by 1.0 ml of water (HPLC grade) at a vacuum pressure of 5 mm Hg. One ml of the thawed sample is then applied to the cartridge and washed with 3 ml HPLC grade water. The angiotensin peptides retained at the columns are eluted with 0.5 ml methanol (HPLC grade, vacuum pressure less than 5 mm Hg) into polypropylene tubes coated with a buffer containing 0.2 M TRIS, 0.02% NaN_3, and 2.5 mg/ml fatty acid-free bovine serum albumin (pH 7.4 with glacial acetic acid). The methanol is evaporated at 40°C and ir-ANG II measured using an antibody (IgG Corp., Nashville, TN) with greater than 1000-fold selectivity for ANG II.

EVALUATION

All data are expressed as mean ± SEM. The hypotensive responses after various doses of the renin antagonist are compared with the inhibition of plasma renin activity and the decrease of immunoreactive angiotensin II.

CRITICAL ASSESSMENT OF THE METHOD

The antibody-trapping method, reported here, gives a better correlation with the blood pressure lowering effect in dogs than the conventional method based on RIA for generated ANG I (Palmer et al. 1993).

MODIFICATIONS OF THE METHOD

Pals et al. (1990) described a rat model for evaluating inhibitors of human renin using anesthetized, nephrectomized, ganglion-blocked rats. The blood pressure rise induced by sustained infusion of renin was dose-dependently decreased by a renin inhibitor.

REFERENCES AND FURTHER READING

Nussberger J, Brunner DB, Waeber B, Brunner HR (1985) True versus immunoreactive angiotensin II in human plasma. Hypertension 7 (Suppl I):I1–I17

Nussberger J, d'Amore TF, Porchet M, Waeber B, Brunner DB, Brunner HR, Kler L, Brown AN, Francis RJ (1987) Repeated administration of the converting enzyme inhibitor Cilazapril to normal volunteers. J Cardiovasc Pharmacol 9:39–44

Palmer RK, Rapundalo ST, Batley BL, Barnes AE, Singh S, Ryan MJ, Taylor DG (1993) Disparity between blood pressure and PRA inhibition after administration of a renin inhibitor to anesthetized dogs: Methodological considerations. Clin Exper Hypertens 15:663–681

Pals DT, Lawson JA, Couch SJ (1990) Rat model for evaluating inhibitors of human renin. J Pharmacol Meth 23:230–245

Poulsen K, Jørgensen J (1973) An easy radioimmunological assay of renin activity, concentration and substrate in human and animal plasma and tissue based on angiotensin I trapping by antibody. J Clin Endocrin Metab 39:816–825

Sealey JE, Laragh JH (1975) Radioimmunoassay of plasma renin activity. Semin Nucl Med 5:189–202

A.1.3.34 Evaluation of Renin Inhibitors in Monkeys

PURPOSE AND RATIONALE

The renin-angiotensin-system as the main regulator of blood pressure can be influenced in several ways.

One approach involves the inhibition of renin. Renin is an aspartyl protease that hydrolyzes angiotensinogen to release the dekapeptide angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting-enzyme. Sequencing of renin and angiotensinogen from various species revealed marked species differences for both the enzyme and the substrate. Inhibitors developed for human renin show a high specificity for primate renin and show only weak inhibition of renin from subprimate species. This means that the most common laboratory animals, such as rats and dogs, are not suitable for the *in vivo* evaluation of renin inhibitors. The marmoset was chosen by Wood et al. (1985, 1989) as a primate model.

PROCEDURE

Marmosets (*Callithrix jacchus*) of both sexes weighing between 300 and 400 g are fed a pellet diet supplemented with fruit. Two days prior to the experiment the animals are anesthetized and catheters are implanted in a femoral artery for measurement of blood pressure and in a lateral tail vein for injection or infusion of test substances. Thirty min before the experiment, the animals receive an intravenous injection of 5 mg/kg furosemide in order to stimulate renin release. During the experiment, the marmosets are sedated with diazepam (0.3 mg/kg i.p.) and kept in restraining boxes. Mean blood pressure is recorded continuously, and heart rate is measured at fixed intervals. The test compound or the standard are injected at various doses by intravenous infusion or administered orally.

EVALUATION

Blood pressure is recorded after 30 min of intravenous infusion and 30 min after stopping the infusion. Comparing the changes from pretreatment values after various doses, dose-response curves can be established.

MODIFICATIONS OF THE METHOD

Fischli et al. (1991) monitored arterial pressure in conscious and chronically instrumented monkeys using a telemetry system. One week before the experiment, the animals were anesthetized, and a 3F high fidelity pressure tip transducer (Millar Instruments, Inc.) was inserted into the abdominal aorta through the right femoral artery. Then the catheter was tunneled subcutaneously to the back of the monkey in the interscapular region. The proximal part of the catheter was connected to a transmitter located in a jacket worn by the monkey. The blood pressure was transmitted continuously to a receiver, which transformed the signal to an analogue value of blood pressure.

Linz et al. (1994) reported on the effects of renin inhibitors in anesthetized rhesus monkeys weighing between 5 and 13 kg. The animals are sodium-depleted by administration of 10 mg/kg/day furosemide-Na for 6 consecutive days. At day 7, 10 mg/kg furosemide is given i.v. 30 min before the start of the experiment. Anesthesia is induced with 20 mg/kg ketamine-hydrochloride i.m. and continued with 40 mg/kg pentobarbitone-Na, slow i.v. drip. After completion of surgical procedures and after insertion of catheters under fluoroscopic control, the following hemodynamic parameters are measured: Pulse rate, and systolic and diastolic blood pressures are registered with a transducer (Statham P23 ID) in one femoral artery. A catheter tip manometer (Millar Instruments, Houston, Texas, USA) is introduced into the left ventricular cavity for the determination of left ventricular pressure. Contractility is electronically deduced from left ventricular pressure with appropriate amplifiers (Hellige GmbH, Freiburg, Germany). The electrocardiogram (ECG) from conventional lead II is taken using an ECG transducer (Hellige GmbH). Heart rate is measured from QRS-peaks using a biotachometer (Hellige GmbH). Cardiac output is determined using the thermodilution method. Thermodilution is integrated and converted to cardiac output readings by commercially available equipment (HMV 7905, Hoyer, Bremen). To determine cardiac output, 2 ml chilled 0–5°C isotonic glucose solution (5%) is injected rapidly into the right ventricle by a catheter via the right jugular vein. A thermistor is placed into the aortic arch via the right carotid artery.

Hemodynamics are monitored for 30 min following i.v. injection of various doses of the potential renin inhibitor. At the end of the experiments the ACE inhibitor ramiprilat 100 µg/kg is given i.v. to probe for an additional blood pressure lowering effect. Blood samples for the determination of ANG II concentration, renin inhibition and plasma drug levels are withdrawn at 10, 30 and 60 min after i.v. injection of the renin inhibitor. The volume is replaced by i.v. injections of isotonic glucose solution (5%). After all data and blood samples have been obtained, animals are sacrificed by an overdose of pentobarbitone-Na.

For experiments after intraduodenal administration sodium depletion and anesthesia are done as described above. A small side branch of the femoral or radial artery is surgically exposed and cannulated for blood pressure measurements using a pressure transducer (P23 ID). Heart rate is determined from a conventional

ECG lead by a biotachometer. Blood samples are withdrawn via a catheter placed into the saphenous vein. A gastric fiberscope (Olympus XP 10) is introduced into the duodenum under visual control and the renin inhibitor is administered intraduodenally through the service channel of the fiberscope in a volume of 5 ml. Blood samples are withdrawn before and at 15, 30, 45, 60, 90 and 120 min after intraduodenal administration.

Wood et al. (2005) tested an orally effective renin inhibitor (aliskiren) in marmosets. Blood pressure and heart rate were measured by telemetry in conscious animals moving freely in their home cages. Pressure transmitters (AM Unit, model TA11PA-C40Data Sciences, USA) were implanted into the peritoneal cavity under aseptic conditions and light anesthesia. The sensor catheter was placed in the aorta below the renal artery pointing upstream.

CRITICAL ASSESSMENT OF THE METHOD

Due to the high species specificity of renin and its substrate, angiotensinogen, renin inhibitors for treatment of hypertension have to be tested in primate models. The marmoset as well as the rhesus monkey have been proven to be suitable models.

REFERENCES AND FURTHER READING

Evans DB, Cornette JC, Sawyer TK, Staples DJ, de Vaux AE, Sharma SK (1990) Substrate specificity and inhibitor structure-activity relationships of recombinant human renin: implications in the *in vivo* evaluation of renin inhibitors. Biotechnol Appl Biochem 12:161–175

Fischli W, Clozel JP, Amrami KE, Wostl W, Neidhart W, Stadler H, Branca Q (1991) RO 42–5892 is a potent orally active renin inhibitor in primates. Hypertension 18:22–31

Greenlee WJ (1990) Renin inhibitors. Med Res Rev 10:173–236

Hiwada K, Kobuko T, Murakami E, Muneta S, Morisawa Y, Yabe Y, Koike H, Iijima Y (1988) A highly potent and long-acting oral inhibitor of human renin. Hypertension 11:707–712

Linz W, Heitsch H, Henning R, Jung W, Kleemann HW, Nickel WU, Ruppert D, Urbach H, Wagner A, Schölkens BA (1994) Effects of the renin inhibitor *N*-[*N*-(3-(4-amino-1-piperidinylcarbonyl)-2(*R*)-benzylpropionyl)-L-histidinyl]-(2*S*,3*R*,4*S*)-1-cyclohexyl-3,4-dihydroxy-6(2-pyridyl)-hexane-2-amide acetate (S 2864) in anesthetized rhesus monkeys. Arzneim Forsch/Drug Res 44:815–820

Wood JM, Gulati N, Forgiarini P, Fuhrer W, Hofbauer KG (1985) Effects of a specific and long-acting renin inhibitor in the marmoset. Hypertension 7:797–803

Wood JM, Baum HP, Forgiarini P, Gulati N, Jobber RA, Neisius D, Hofbauer KG (1989a) Haemodynamic effects of acute and chronic renin inhibition in marmosets. J Hypertension 7, Suppl 2:S37–S42

Wood JM, Criscione L, de Gasparo M, Bühlmayer P, Rüeger H, Stanton JL, Jupp RA, Kay J (1989b) CGP 38 560: orally active, low-molecular-weight renin inhibitor with high potency and specificity. J Cardiovasc Pharmacol 14:221–226

Wood JM, Schnell CR, Cumin F, Menard J, Webb RL (2005) Aliskiren, a novel, orally effective renin inhibitor, lowers blood pressure in marmosets and spontaneously hypertensive rats. J Hypertens 23:417–426

A.1.3.35 Penile Erection in Rabbits

PURPOSE AND RATIONALE

The discovery of inhibitors of phosphodiesterase as effective drugs for patients with erectile dysfunction (Klotz et al. 2001; Porst et al. 2001) has stimulated the use of appropriate *in vivo* animal models. In particular, rabbits have been recommended as models for impotence research (Bischoff and Schneider 2000, 2001; Bischoff 2001; Bischoff et al. 2001; Saenz de Tejada et al. 2001) confirming earlier work in this area (Thielen et al. 1969; Sjöstrand and Kline 1979; Naganuma et al. 1993; Lin and Lin 1996).

PROCEDURE

Adult male Chinchilla rabbits weighing 3.5–4.5 kg are housed in individual cages for at least 1 week after arrival, at room temperature with water and food ad libitum.

For the study, an indwelling catheter filled with saline is inserted into a marginal ear vein and taped in position. The drugs are injected into the ear vein, followed by a small volume of saline. The time is noted and at appropriate times the animal gently removed from the cage and held by one research worker. The rabbit penis is not visible when it is not erect (Naganuma et al. 1993). However, when erection occurs, it is possible to examine the pudendal area and measure the length of the uncovered penile mucosa with sliding calipers.

EVALUATION

Penile erection is evaluated by measuring the length to the nearest millimeter of the uncovered penile mucosa with a sliding caliper at 5, 10, 15, 30, 50, 60, 90, and 120 min after administration of the test compounds and continued hourly for up to 5 h. Mean values are calculated and results expressed as means ± SEM. The area under the curve is calculated by an integration program.

MODIFICATIONS OF THE METHOD

Choi et al. (2002) compared the efficacy of verdenafil and sildenafil in facilitating penile erection in **anesthetized rabbits**. Penile erections were elicited by submaximal pelvic nerve stimulation every 5 min for 30 min. Response was assessed by continuously

recording intracavernosal pressure and systemic arterial pressure.

Min et al. (2000) tested the augmentation of pelvic-nerve-mediated sexual arousal in anesthetized **female rabbits** by sildenafil. The following parameters were measured before, during and after pelvic nerve stimulation at 4, 16, and 32 Hz: (1) hemoglobin concentration and oxygen saturation in female genital (vaginal, labial, clitoral) tissues by laser oximetry; (2) clitoral blood flow by laser Doppler flowmetry; (3) vaginal luminal pressure by a balloon catheter pressure transducer; (4) vaginal lubrication by tampon.

Carter et al. (1998) tested the effect of the selective phosphodiesterase type 5 inhibitor sildenafil on erectile dysfunction in **pentobarbital-anesthetized dogs**. Increases in intracavernosal pressure in the corpus cavernosum and penile blood flow were induced by pelvic nerve stimulation over a frequency range of 1–16 Hz. The effects of increasing doses of sildenafil on electric-stimulated intracavernosal pressure, penile blood flow, blood pressure, and heart rate were evaluated.

REFERENCES AND FURTHER READING

Bischoff E (2001) Rabbits as models for impotence research. Int J Impot Res 13:146–148

Bischoff E, Niewoehner U, Haning H, Es Sayed M, Schenke T, Schlemmer KH (2001) The oral efficacy of verdenafil hydrochloride for inducing penile erection in a conscious rabbit model. J Urol 165:1316–1318

Bischoff E, Schneider K (2000) A conscious rabbit model is able to demonstrate the efficacy of verdenafil and sildenafil on penile erection. Int J Impot Res Suppl 12:65

Bischoff E, Schneider K (2001) A conscious-rabbit model to study verdenafil hydrochloride and other agents that induce penile erection. Int J Impot Res 13:230–235

Carter AJ, Ballard SA, Naylor AM (1998) Effect of the selective phosphodiesterase type 5 inhibitor sildenafil on erectile dysfunction in the anaesthetized dog. J Urol 160:242–246

Choi S, O'Connell L, Min K, Kim NN, Munarriz R, Goldstein I, Bischoff E, Traisch AM (2002) Efficacy of verdenafil and sildenafil in facilitating penile erection in an animal model. J Androl 23:332–337

Klotz T, Sachse R, Heidrich A, Jockenhövel F, Rhode G, Wensing G, Horstmann R, Engelmann R (2001) Verdenafil increases penile rigidity and tumescence in erectile dysfunction patients: a RigiScan and pharmacokinetic study. World J Urol 19:32–39

Lin YM, Lin JS (1996) The rabbit as an intracavernous injection study model. Urol Res 24:27–32

Min K, Kim NN, McAuley I, Stankowicz M, Goldstein I, Traisch AM (2000) Sildenafil augments pelvic nerve-mediated female sexual arousal in the anesthetized rabbit. Int J Impot Res 12 [Suppl 3]:S32–S39

Naganuma H, Egashira T, Fujii I (1993) Neuroleptics induce penile erection in the rabbit. Clin Exp Pharmacol Physiol 20:177–183

Porst H, Rosen R, Padma-Nathan H, Goldstein I, Giuliano F, Ulbrich E, Bandel T (2001) The efficacy and tolerability of vardenafil, a new, oral, selective phosphodiesterase type 5 inhibitor, in patients with erectile dysfunction: the first at-home clinical trial. Int J Impot Res 13(4):192–199

Saenz de Tejada I, Angulo J, Cuevas P, Fernández A, Moncada I, Allona A, Lledó E, Körschen HG, Niewöhner U, Haning H, Pages E, Bischoff E (2001) The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor verdenafil. Int J Impot Res 13:282–290

Sjöstrand NO, Klinge E (1979) Principal mechanisms controlling penile retraction and protrusion in rabbits. Acta Physiol Scand 106:199–214

Thielen P, Renders M, Rectem D (1969) Medullary regions controlling the erection and retraction of the penis in rabbits. Arch Int Physiol Biochim 77:340–342

A.1.4 Cardiovascular Safety Studies

See:
Brian D. Guth
Methods in Cardiovascular Safety Pharmacology
Chapter I. D.
In:
Drug Discovery an Evaluation
Safety and Pharmacokinetic Assays
By: H.G: Vogel (Ed), F.J. Hock, J. Maas, D. Mayer (CoEds)
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A.2 Methods to Induce Experimental Hypertension

A.2.0.1 Acute Renal Hypertension in Rats

PURPOSE AND RATIONALE

Since the classical experiments of Goldblatt et al. (1934) there is clear evidence that the ischemia of the kidneys causes elevation of blood pressure by activation of the renin-angiotensin system. The principle can be used both for acute and chronic hypertension. In rats acute renal hypertension is induced by clamping the left renal artery for 4 h. After reopening of the vessel, accumulated renin is released into circulation. The protease renin catalyzes the first and rate-limiting step in the formation of angiotensin II leading to acute hypertension. The test is used to evaluate antihypertensive activities of drugs.

PROCEDURE

Male Sprague-Dawley rats weighing 300 g are used. The animals are anesthetized by intraperitoneal injection of 100 mg/kg hexobarbital sodium. A PVC-coated Dieffenbach clip is placed onto the left hilum of the kidney and fixed to the back muscles. The renal artery is occluded for 3.5–4 h.

3.5 h following the surgery, the animals are anesthetized by intraperitoneal injection of 30–40 mg/kg pentobarbital sodium. The trachea is cannulated to facilitate spontaneous respiration. To measure systolic and diastolic blood pressure, the cannula in the carotid artery is connected to a pressure transducer (Statham P 23 Db).

For administration of the test compound, a jugular vein is cannulated.

Following a stable blood pressure state, ganglionic blockade is performed with pentolinium (10 mg/kg i.v.). After obtaining stable reduced blood pressure values, the renal arterial clip is removed. This leads to a rise in blood pressure as a consequence of elevated plasma renin level. Within 15 min a stable hypertension is achieved (control = 100%).

The test substance is then administered by intravenous injection at doses of 10 and 100 µg/kg.

Blood pressure is monitored continuously until a renewed increase to the starting level is obtained.

Ten–twelve animals are used per compound.

EVALUATION

Increase in blood pressure after reopening of the renal artery and reduction in blood pressure after administration of the test drug are determined [mm Hg]. Percent inhibition of hypertensive blood pressure values under drug treatment are calculated as compared to pretreatment hypertension values. Duration of the effect is determined [min]. Statistical significance is assessed by the paired *t*-test.

MODIFICATIONS OF THE METHOD

A sharp and transient in systemic arterial blood pressure associated with reflex bradycardia can be elicited by injection of 5-hydroxytryptamine, cyanide, nicotine or lobeline into the coronary artery blood stream of dogs (Berthold et al. 1989). The phenomenon is named the cardiogenic hypertensive chemoreflex and 5-HT proved to be the most powerful agent for its initiation (James et al. 1975).

REFERENCES AND FURTHER READING

Berthold H, Scholtysik G, Engel G (1989) Inhibition of the 5-HT-induced cardiogenic hypertensive chemoreflex by the selective 5-HT_3 receptor antagonist ICS 205–930. Naunyn-Schmiedeberg's Arch Pharmacol 339:259–262

Boura ALA, Green AF (1964) Antihypertensive agents. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press London and New York pp 431–456

Goldblatt H, Lynch J, Hanzal RF, Summerville WW (1934) Studies on experimental hypertension. I. The production of persistent evaluation of systolic blood pressure by means of renal ischemia. J exper Med 59:347–379

James TN, Isobe JH, Urthaler F (1975) Analysis of components in a cardiogenic hypertensive chemoreflex. Circulation 52:179–192

A.2.0.2 Chronic Renal Hypertension in Rats

PURPOSE AND RATIONALE

On the basis of the findings of Goldblatt et al. (1934) that ischemia of the kidneys induces hypertension, various modifications of the technique have been described for several animal species. One of the most effective modifications in rats is the so called 1-kidney-1-clip method.

PROCEDURE

Male Sprague-Dawley rats weighing 200–250 g are anaesthetized with 50 mg/kg i.p. pentobarbital. The fur on the back is shaved and the skin disinfected. In the left lumbar area a flank incision is made parallel to the long axis of the rat. The renal pedicel is exposed with the kidney retracted to the abdomen. The renal

artery is dissected clean and a U-shaped silver clip is slipped around it near the aorta. Using a special forceps (Schaffenburg 1959) the size of the clip is adjusted so that the internal gap ranges from 0.25–0.38 mm. The right kidney is removed through a flank incision after tying off the renal pedicle. The skin incisions are closed by wound clips.

4–5 weeks after clipping blood pressure is measured and rats with values higher than 150 mm Hg selected for the experiments. Blood pressure readings are taken on each of 3 days prior to drug treatment. Drugs are administered orally in volumes of 10 ml/kg. The rats are divided into 4 animals per dose and each animal is used as his own control. Compounds are administered for 3 days and predrug and 2 h postdrug blood pressure readings are taken.

EVALUATION

Changes in systolic blood pressure are expressed in mm Hg. Activity is determined by comparing treatment blood pressure values with the control blood pressure value (Day 1, predrug blood pressure). Comparisons are made using the paired *t*-test for evaluation of statistical significance.

MODIFICATIONS OF THE METHOD

Duan et al. (1996) induced renal hypertension in male Hartley **guinea pigs** by a two-step procedure consisting of ligation of the left caudal renal artery and right nephrectomy. Arterial blood pressure and heart rate were monitored in conscious animals. ACE-inhibitors reduced blood pressure in sham-operated and in renal hypertensive guinea pigs, whereas renin inhibitors were effective only in renal hypertensive animals.

REFERENCES AND FURTHER READING

Boura ALA, Green AF (1964) Antihypertensive agents. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press London and New York pp 431–456

Duan J, Jamarillo J, Jung GL, McLeod AL, Fernades BH (1996) A novel renal hypertensive guinea pig model for comparing different inhibitors of the renin-angiotensin system. J Pharmacol Toxicol Meth 35:83–89

Goldblatt H, Lynch J, Hanzal RF, Summerville WW (1934) Studies on experimental hypertension. I. The production of persistent evaluation of systolic blood pressure by means of renal ischemia. J exper Med 59:347–379

Leite R, Salgado MCO (1992) Increased vascular formation of angiotensin II in one-kidney, one clip hypertension. Hypertension 19:575–581

Schaffenburg CA (1959) Device to control constriction of main renal artery for production of hypertension in small animals. Proc Soc Exp Biol Med 101:676–677

Zandberg P (1984) Animal models in experimental hypertension: relevance to drug testing and discovery. In: van Zwieten (ed) Handbook of Hypertension, Vol 3, Pharmacology of Antihypertensive Drugs. Elsevier Amsterdam, pp 102–153

A.2.0.3 Chronic Renal Hypertension in Dogs

PURPOSE AND RATIONALE

Production of hypertension by clamping renal arteries has been first described by Goldblatt et al. (1934) in dogs. Later on, the method has been modified, e. g., as the "wrapping" technique (Abram and Sobin 1947).

PROCEDURE

Dogs weighing 8–12 kg are anesthetized with i.v. injection of 15 mg/kg thiopental. Anesthesia is maintained with a halothane-oxygen mixture. Under aseptic conditions, a midline abdominal incision is made. One kidney is exposed and wrapped in cellophane and then replaced. The contralateral kidney is exposed. The artery, vein and ureter are ligated and the kidney is removed. The abdomen is closed by sutures and clips. On the day of surgery and for 3 days following, the dogs are given antibiotics. Body temperature is measured twice daily for 4 days following surgery.

Six weeks following surgery, blood pressure is measured using a tail-cuff method. For recording, the tail-cuff is attached to a polygraph. Only animals with a systolic blood pressure higher than 150 mm Hg are considered to be hypertensive and can participate in studies evaluating potential antihypertensive compounds.

For the experiment, blood pressure is recorded either by the indirect tail-cuff method or by direct measurement via an implanted arterial cannula. On day 1 readings are made every 2 h, just before, and 2 and 4 h after oral treatment with the potential antihypertensive compound. Drug administration is repeated for 5 days. On days 3 and 5 blood pressure readings are taken before and 2 and 4 h after treatment. At least 3 dogs are used per dose and compound.

EVALUATION

The starting value is the average of the 2 readings before application of the drug. Each of the following readings is subtracted from this value and recorded as fall of blood pressure at the various recording times.

MODIFICATIONS OF THE METHOD

Renal hypertension in rats has been achieved by many modifications of the method (Stanton 1971) such as the

technique according to Grollman (1944). The kidney is exposed through a lumbar incision, the renal capsule is removed by gentle traction, and a figure-8 ligature is applied being tight enough to deform the kidney but not tight enough to cut the tissue.

Renal hypertension may be induced in the **rat** by encapsulating both kidneys with latex rubber capsules (Abrams and Sobin 1947). Moulds are formed from plastic using a rat kidney as a model. The capsules are prepared by dipping the moulds in liquid latex allowing them to dry in the air. Three applications of latex are applied before the capsules are toughened by placing them under warm running tap water. The kidney is exposed by lumbar incision, the renal capsule gently removed and the capsule applied.

REFERENCES AND FURTHER READING

Abrams M, Sobin S (1947) Latex rubber capsule for producing hypertension in rats by perinephritis. Proc Soc Exp Biol Med 64:412–416

Goldblatt H, Lynch J, Hanzal RF, Summerville WW (1934) Studies on experimental hypertension. I. The production of persistent evaluation of systolic blood pressure by means of renal ischemia. J exper Med 59:347–379

Grollman A (1944) A simplified procedure for inducing chronic renal hypertension in the mammal. Proc Soc Exp Biol Med 57:102–104

Schaffenburg CA (1959) Device to control constriction of main renal artery for production of hypertension in small animals. Proc Soc exper Biol Med 101:676–677

Sen S, Tarazi RC, Bumpus FM (1981) Reversal of cardiac hypertrophy in renal hypertensive rats: medical vs. surgical therapy. Am J Physiol 240:H408–H412

Stanton HC (1971) Experimental hypertension. In: Schwartz A (ed) Methods in Pharmacology, Vol 1, pp 125–150. Appleton-Century-Crofts, Meredith Corporation. New York

A.2.0.4 Neurogenic Hypertension in Dogs

PURPOSE AND RATIONALE

Vasodilator and depressor reflexes, originating in the baroreceptor areas of the carotid sinus and aortic arch, play an important part in the regulation of blood pressure. Stimulation of the afferent buffer fibres exerts an inhibitory influence on the vasomotor center, and their sectioning leads to a persistent rise in blood pressure. In this way, acute neurogenic hypertension can be induced in dogs.

PROCEDURE

Adult dogs of either sex weighing 10–15 kg are anesthetized using 15 mg/kg sodium thiopental, 200 mg/kg sodium barbital and 60 mg/kg sodium pentobarbital i.v. A femoral vein and artery are cannulated using polyethylene tubing to administer compounds i.v. and record arterial pressure and heart rate, respectively. Left ventricular pressure and dp/dt are recorded via the left common carotid artery (post-deafferentiation) using a Millar microtip pressure transducer. P_{max} is recorded by speeding up the chart paper. Cardiac output is determined by introducing a Swan-Ganz catheter into the right heart and pulmonary artery via a jugular vein. Five ml of cold 5% dextrose is injected into the right atrium and an Edwards Cardiac output computer is used to calculate the cardiac output from the temperature change in the pulmonary artery. All recordings are made with a polygraph.

Both of the carotid arteries are cleared up to the bifurcation of the internal and external carotid arteries. The carotid sinus nerves are isolated, ligated and sectioned and a bilateral vagotomy is performed to produce neurogenic hypertension (mean arterial pressure more than 150 mm Hg). The dog is allowed to equilibrate for approximately 30 min and a bolus of the test compound is administered by intravenous injection. Heart rate, arterial pressure, left ventricular pressure, Pmax and dp/dt are monitored for 90 min. A minimum of 3 dogs are used for each compound.

EVALUATION

Changes of the cardiovascular parameters are expressed as percentage of the values before administration of the drug.

MODIFICATIONS OF THE METHOD

Neurogenic hypertension through baroreceptor denervation has also been described in **rabbits** (Angell-James 1984) and in rats (Krieger 1984).

CRITICAL ASSESSMENT OF THE METHOD

The neurogenic hypertension is useful for acute experiments. However, it is less useful for chronic experiments since the elevated blood pressure caused by buffer nerve section is more labile than that caused by renal ischemia.

REFERENCES AND FURTHER READING

Angell-James JE (1984) Neurogenic hypertension in the rabbit. In: de Jong (ed) Handbook of Hypertension, Vol. 4: Experimental and Genetic Models of Hypertension. Elsevier Science Publ, pp 364–397

Boura ALA, Green AF (1964) Antihypertensive agents. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press London and New York pp 431–456

Grimson KS (1941) The sympathetic nervous system in neurogenic and renal hypertension. Arch Surg, Chicago, 43:284–305
Krieger EM (1984) Neurogenic hypertension in the rat. In: de Jong (ed) Handbook of Hypertension, Vol. 4: Experimental and Genetic Models of Hypertension. Elsevier Science Publ, pp 350–363
Maxwell RA, Plummer AJ, Schneider F, Povalski H, Daniel AI (1960) Pharmacology of [2-(octahydro-1-azocinyl)-ethyl]-guanidine sulfate (SU-5864). J Pharmacol 128:22–29

A.2.0.5 DOCA-salt Induced Hypertension in Rats

PURPOSE AND RATIONALE

Mineralocorticoid-induced hypertension is thought to be due to the sodium retaining properties of the steroid causing increases in plasma and extracellular volume. The hypertensive effect is increased by salt loading and unilateral nephrectomy in rats.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized with ether. Through a flank incision the left kidney is removed. The rats are injected twice weekly with 20 mg/kg s.c. desoxycorticosteroneacetate in olive oil for 4 weeks. Drinking water is replaced with a 1% NaCl solution. Blood pressure starts to rise after one week and reaches systolic values between 160 and 180 mm Hg after 4 weeks.

MODIFICATIONS OF THE METHOD

The regimen to induce DOCA-salt hypertension has been modified by many authors (Stanton 1971).

DOCA pellets (Peterfalvi and Jequier 1960; Passmore and Jimenez 1990) or implants in silastic devices (Ormsbee and Ryan 1973; King and Webb 1988) were used instead of repeated injections.

DOCA-salt hypertension can also be achieved without nephrectomy (Bockman et al. 1992).

Using kininogen-deficient Brown Norway Katholiek (BN-Ka) rats, Majima et al. (1991, 1993) showed suppression of rat desoxycorticosterone-salt hypertension by the kallikrein-kinin system.

Li et al. (1996) examined small-artery structure on a wire myograph and quantified endothelin-1 messenger RNA by Northern blot analysis in DOCA-salt hypertensive rats after administration of an ACE-inhibitor, a calcium channel antagonist and a nitric oxide synthase inhibitor.

Ullian (1997) described the **Wistar–Furth rat** as a model of mineralocorticoid resistance. These rats developed two-kidney, one-clip hypertension to the same degree as did Wistar rats and reacted to glucocorticoid treatment with a rapid onset of hypertension, but were resistant to the development of DOCA-NaCl hypertension.

Studies in DOCA-salt hypertensive **mice** were reported by Gross et al. (1998, 1999), Honeck et al. (2000), Peng et al. (2001).

REFERENCES AND FURTHER READING

Bockman ChS, Jeffries WB, Pettinger WA, Abel PW (1992) Enhanced release of endothelium-derived relaxing factor in mineralocorticoid hypertension. Hypertension 20:304–313
Codde JP, Croft KD, Beilin LJ (1987) Dietary suppression of prostaglandin synthesis does not accelerate DOCA/salt hypertension in rats. Clin Exp Pharm Physiol 14:513–523
Dardik BN, Di Bello PM, Chatelain RE (1988) Elevated arterial cyclic AMP levels during the development of one kidney, one clip and DOCA hypertension in rats. Eur J Pharmacol 158:139–143
Friedman SM, McIndoe RA, Tanaka M (1988) The relation of cellular sodium to the onset of hypertension induced by DOCA-saline in the rat. J Hypertension 6:63–69
Gross V, Lippoldt A, Bohlender J, Bader M, Hansson A, Luft FC (1998) Cortical and medullary hemodynamics in desoxycorticosterone acetate-salt hypertensive mice. J Am Soc Nephrol 9:346–354
Gross V, Schneider W, Schunk WH, Mervaala E, Luft FC (1999) Chronic effects of lovastatin and bezafibrate on cortical and medullary hemodynamics in desoxycorticosterone acetate-salt hypertensive mice. J Am Soc Nephrol 10:1430–1439
Hasnain Q, MacDonald G (1993) Metabolic studies of uridine in rats with DOCA-salt hypertension and on high sodium diet. Clin Exper Pharm Physiol 20:384–387
Honeck H, Gross V, Erdmann B, Kärgel E, Neunaber R, Milia AF, Schneider W, Luft FC, Schunk WH (2000) Cytochrome P450-dependent renal arachidonic acid metabolism in desoxyxcorticosterone acetate-salt hypertensive mice. Hypertension 36:610
King CM, Webb RC (1988) The endothelium partially obscures enhanced microvessel reactivity in DOCA hypertensive rats. Hypertension 12:420–427
Li JS, Sventek P, Schiffrin EL (1996) Effect of antihypertensive treatment and N^{ω}-nitro-L-arginine methyl ester on cardiovascular structure in deoxycorticosterone acetate-salt hypertensive rats. J Hypertension 14:1331–1339
Majima M, Katori M, Hanazuka M, Mizogami S, Nakano T, Nakao Y, Mikami R, Uryu H, Okamura R, Mohsin SSJ, Oh-Ishi S (1991) Suppression of rat desoxycorticosterone-salt hypertension by the kallikrein-kinin system. Hypertension 17:806–813
Majima M, Yoshida O, Mihara H, Muto T, Mitsogami S, Kuribayashi Y, Katori M, Oh-Ishi S (1993) High sensitivity to salt in kininogen-deficient Brown Norway Katholiek rats. Hypertension 22:705–714
Opoku J, Kalimi M (1992) Role of the antiglucocorticoid RU 486 in the prevention of steroid-induced hypertension. Acta Endocrin 127:258–261
Ormsbee HS, Ryan CF (1973) Production of hypertension with desoxycorticosterone acetate-impregnated silicone rubber implants. J Pharmacol Sci 62:255–257
Passmore JC, Jimenez AE (1990) Separate hemodynamic roles for chloride and sodium in deoxycorticosterone acetate-salt hypertension. Proc Soc Exp Biol Med 194:283–288

Peng H, Carretero OA, Alfie ME, Masura JA, Rhaleb NE (2001) Effects of angiotensin-converting enzyme inhibitor and angiotensin type 1 receptor antagonist in deoxycorticosterone acetate-salt hypertensive mice lacking *Ren-2 gene*. Hypertension 37:974

Peterfalvi M, Jequier R (1960) La 10-methoxy deserpidine. Étude pharmacologique. Arch Int Pharmacodyn 124:237–254

Schenk J, McNeill JH (1992) The pathogenesis of DOCA-salt hypertension. J Pharm Toxicol Meth 27:161–170

Stanton HC (1971) Experimental hypertension. In: Schwartz A (ed) Methods in Pharmacology, Vol 1, pp 125–150. Appleton-Century-Crofts, Meredith Corporation. New York

Ullian ME (1997) The Wistar–Furth rat as a model of mineralocorticoid resistance. Kidney Internat 52: Suppl 61:S10–S13

A.2.0.6 Fructose Induced Hypertension in Rats

PURPOSE AND RATIONALE

Increases in dietary carbohydrate intake can raise blood pressure in experimental animals. The increased intake of either sucrose or glucose was shown to enhance the development of either spontaneous hypertension or salt hypertension in rats (Hall and Hall 1966; Preuss and Preuss 1980; Young and Landsberg 1981). Hwang et al. (1987) first reported that hypertension could be induced in normal rats by feeding a high-fructose diet. Fructose feeding was also found to cause insulin resistance, hyperinsulinemia, and hypertriglyceridemia in normal rats (Zavaroni et al. 1980; Tobey et al. 1982). Dai and McNeill (1995) studied the concentration- and duration-dependence of fructose-induced hypertension in rats.

PROCEDURE

Groups of 8 male Wistar rats weighing 210–250 g are used. The are housed two per cage on a 12-h light 12-h dark cycle and are allowed free access to standard laboratory diet (Purina rat chow) and drinking fluid. Drinking fluid consists either of tap water or 10%-fructose solution. Body weight, food intake and fluid intake of each rat are measured every week during treatment. Using the tail-cuff method, systolic blood pressure and pulse rate is measured before and every week during treatment. Blood samples are collected before and every second week during treatment for determination of plasma glucose, insulin, and triglycerides.

EVALUATION

Since maximum effects on the chosen parameters are achieved after 6 weeks, the duration of treatment can be limited to this time. Statistical analysis is performed using a one-way or two-way analysis of variance, followed by the Newman-Keuls test.

MODIFICATIONS OF THE METHOD

Reaven et al. (1988, 1989) found an attenuation of fructose-induced hypertension by exercise training and an inhibition by somatostatin treatment.

Brands et al. (1991, 1992) found an increase of arterial pressure during chronic hyperinsulinemia in conscious rats.

Hall et al. (1995) reported the effects of 6 weeks of a high-fat diet on cardiovascular, renal, and endocrine functions in chronically instrumented conscious **dogs**. Body weight increased by approximately 16.9 kg, whereas MAP, cardiac output, and heart rate increased by 28%, 77%, and 68%, respectively.

REFERENCES AND FURTHER READING

Brands MW, Hildebrandt DA, Mizelle HL, Hall JE (1991) Sustained hyperinsulinemia increases arterial pressure in conscious rats. Am J Physiol 260:R764–R768

Brands MW, Hildebrandt DA, Mizelle HL, Hall JE (1992) Hypertension during chronic hyperinsulinemia in rats is not salt sensitive. Hypertension 19 (Suppl I):I83–I89

Dai S, McNeil JH (1995) Fructose-induced hypertension in rats is concentration- and duration-dependent. J Pharmacol Toxicol Meth 33:101–107

Hall CE, Hall O (1966) Comparative effectiveness of glucose and sucrose in enhancement of hyperalimentation and salt hypertension. Proc Soc Exp Biol Med 123:370–374

Hall JE, Brands MW, Zappe DH, Dixon WN, Mizelle HL, Reinhart GA, Hildebrandt DA (1995) Hemodynamic and renal responses to chronic hyperinsulinemia in obese, insulin-resistant dogs. Hypertension 25:994–1002

Hwang IS, Ho H, Hoffman BB, Reaven GM (1987) Fructose-induced insulin resistance and hypertension in rats. Hypertension 10:512–516

Hwang IS, Huang WC, Wu JN, Reaven GM (1989) Effect of fructose-induced hypertension on the renin-angiotensin-aldosterone system and atrial natriuretic factor. Am J Hypertens 2:424–427

Preuss MB, Preuss HG (1980) The effects of sucrose and sodium on blood pressures in various substrains of Wistar rats. Labor Invest 43:101–107

Reaven GM, Ho H, Hoffman BB (1988) Attenuation of fructose-induced hypertension in rats by exercise training. Hypertension 12:129–132

Reaven GM, Ho H, Hoffman BB (1989) Somatostatin inhibition of fructose-induced hypertension. Hypertension 14:117–120

Tobey TA, Mondon CE, Zavaroni I, Reaven GM (1982) Mechanism of insulin resistance in fructose-fed rats. Metabolism 31:608–612

Young JB, Landsberg L (1981) Effect of sucrose on blood pressure in the spontaneously hypertensive rat. Metabolism 30:421–424

Zavaroni I, Sander S, Scott A, Reaven GM (1980) Effect of fructose feeding on insulin secretion and insulin action in the rat. Metabolism 29:970–973

A.2.0.7 Genetic Hypertension in Rats

SURVEY

Inherited hypertension in rats has been described by Smik and Hall 1958; Phelan and Smirk 1960; Laverty and Smirk 1961; Phelan 1968 as **genetically hypertensive (GH) rats** (Simpson and Phelan 1984).

Okamoto et al. (1963, 1966) reported the development of a strain of spontaneously hypertensive rats from mating one Wistar male rat with spontaneously occurring high blood pressure with a female with slightly elevated blood pressure. By inbreeding over several generations a high incidence of hypertension with blood pressure values of 200 mm Hg or more was achieved. These strains were called "**Spontaneously hypertensive rats (Akamoto-Aoki)**" = **SHR** or **"Wistar–Kyoto rats**" = **WKY**. Hypertension in these rats is clearly hereditary and genetically determined, thus comparable to primary hypertension in humans. Cardiac hypertrophy (Sen et al. 1974) and cellular ionic transport abnormalities have been observed (Yamori 1984).

Inbred strains being **salt-hypertension-sensitivey** and **salt-hypertension-resistant (RD)** have been developed by Dahl et al. (1962, 1963), Rapp (1984), Cicila et al. (1993). Inoko et al. (1994) reported the transition from compensatory hypertrophy to dilated, failing left ventricles in Dahl salt-sensitive rats.

Two strains of rats with inbred dissimilar sensitivity to DOCA-salt hypertension (**"Sabra strain"**) have been separated by Ben-Ishay et al. (1972, 1984).

Another hypertensive strain derived from Wistar rats was produced by brother-sister mating in the group of Bianchi et al. (1974, 1986) at the University of Milan called **"Milan hypertensive strain**" = **MHS**. These rats show a cell membrane defect resulting in abnormal kidney function. Salvati et al. (1990) studied the diuretic effect of bumetanide in isolated perfused kidneys of Milan hypertensive rats.

Furthermore, the **"Lyon"** strains of hypertensive, normotensive and low-blood-pressure rats were developed (Dupont et al. 1973; Vincent et al. 1984; Dubay et al. 1993). These rats show a genetically determined defect in central nervous function.

Spontaneously hypertensive rats which develop failure before 18 months have been selectively bred. Several substrains of spontaneous hypertensive rats were separated by the group of Okamoto et al. (1974) including the **stroke-prone strain SHR = SHRSP**. These rats have an increased sympathetic tone and show a high incidence of hemorrhagic lesions of the brain with motor disturbances followed by death (Yamori 1984; Feron et al. 1996).

A strain of obese spontaneously hypertensive rats has been described by Koletsky (1975), Ernsberger et al. (1993).

With new techniques of genetic engineering, **transgenic rats with hypertension** could be created. Increase of blood pressure of spontaneously hypertensive rat is determined by multiple genetic loci (Deng and Rapp 1992; Dubay et al. 1993). With new technology not only these loci could be defined but also new models in hypertension research and models to detect antihypertensive drugs could be established (Bohlender et al. 1997; Pinto et al. 1998).

Mullins et al. (1990) reported fulminant hypertension in transgenic rats harboring the mouse Re-2 gene.

A **rat strain TGR(mREN2)27** as a monogenetic model in hypertension research was described by Peters et al. (1993), Lee et al. (1996), Langheinrich et al. (1996), and Ohta et al. (1996).

Bohlender et al. (1996) reconstructed the human renin-angiotensin system in transgenic rats overexpressing the human angiotensin gene TGR(hOGEN) 1623 by chronically injecting human recombinant renin intravenously using Alzet pumps.

Zolk et al. (1998) described the effects of quinapril, losartan and hydralazine on cardiac hypertrophy and β-adrenergic neuroeffector mechanisms in transgenic TGR(mREN2)27 rats.

CRITICAL ASSESSMENT OF THE METHOD

The use of spontaneously hypertensive rats to detect potential antihypertensive compounds is well established. On the basis of available data no preference can be given to a particular strain. The most abundant experience has been gained with the Wistar–Kyoto strain. Transgenic rats with well defined genomes are gaining more importance.

MODIFICATIONS OF THE METHOD

Pijl et al. (1994) described streptozotocin-induced diabetes mellitus in spontaneously hypertensive rats as a pathophysiological model for the combined effects of hypertension and diabetes.

Rosenthal et al. (1997) used rats of the Cohen-Rosenthal diabetic hypertensive strain to examine the effects of an ACE-inhibitor, an ATII antagonist and a calcium antagonist on systolic pressure and spontaneous blood glucose levels.

Holycross et al. (1997) used hypertensive SHHF/Mcc-facp rats to study plasma renin activity during development of heart failure.

Linz et al. (1997) compared the outcome of lifelong treatment with the ACE inhibitor ramipril in young prehypertensive stroke-prone spontaneously hypertensive rats and age-matched normotensive Wistar–Kyoto rats. Lifelong ACE inhibition doubled the lifespan in hypertensive rats matching that of normotensive rats.

Studies in **genetically hypertensive mice** were reported by Rosenberg et al. (1985), Hamet et al. (1990) Meneton et al. (2000). Ohkubo et al. (1990) generated transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes.

REFERENCES AND FURTHER READING

Ben-Ishay D (1984) The Sabra hypertension-prone and -resistant strain. In: de Jong W (ed) Handbook of Hypertension. Vol 4. Experimental and Genetic Models of Hypertension. Elsevier Science Publ., New York, pp 296–313

Ben-Ishay D, Saliternik R, Welner A (1972) Separation of two strains of rats with inbred dissimilar sensitivity to DOCA-salt hypertension. Experientia 28:1321–1322

Berthelot A (1991) Hypertension models and screening of antihypertensive drugs. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany, pp 106–109

Bianchi G, Fox U, Imbasciati E (1974) The development of a new strain of spontaneously hypertensive rats. Life Sci 14:339–347

Bianchi G, Ferrari P, Barber BR (1984) The Milan hypertensive strain. In: de Jong W (ed) Handbook of Hypertension, Vol 4: Experimental and Genetic Models of Hypertension. pp 328–340

Bianchi G, Ferrari P, Cusi D, Salardi S, Giudi E, Niutta E, Tripodi G (1986) Genetic and experimental hypertension in the animal model – Similarities and dissimilarities to the development of human hypertension. J Cardiovasc Pharmacol 8 (Suppl 5) S64–S70

Bohlender J, Ménard J, Wagner J, Luft FC, Ganten D (1996) Développement chez le rat d'un modèle d'hypertension à la rénine humaine. Arch Mal Cœur 89:1009–1011

Bohlener J, Fukamizu, Lippoldt A, Nomura T, Dietz R, Ménard J, Muarakami K, Luft FC, Ganten D (1997) High human renin hypertension in transgenic rats. Hypertension 29(part 2):428–434

Cicila GT, Rapp JP, Wang JM, Lezin ES, Ng SC, Kurtz TW (1993) Linkage of 11β-hydroxylase mutations with altered steroid biosynthesis and blood pressure in the Dahl rat. Nature Genetics 3:346–353

Dahl LK, Heine M, Tassinari L (1962) Role of genetic factors in susceptibility to experimental hypertension due to chronic salt ingestion. Nature 194:480–482

Dahl LK, Heine M, Tassinari L (1962a) Effects of chronic salt ingestion. Evidence that genetic factors play an important role in susceptibility to experimental hypertension. J Exper Med 115:1173–1190

Dahl LK, Heine M, Tassinari L (1963b) Effects of chronic excess salt ingestion: role of genetic factors in both DOCA-salt and renal hypertension. J Exper Med 118:605

Deng Y, Rapp JP (1992) Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic receptor genes using Dahl salt-sensitive rats. Nature Genetics 1:267–272

Dubay Ch, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi JP, Kotelevtsev Y, Beckmann JS, Soubrier F, Sassard J, Lathorp GM (1993) Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. Nature Genetics 3:354–357

Dupont J, Dupont JC, Fromnet A, Milon H, Vincent M (1973) Selection of three strains of rats with spontaneously different levels of blood pressure. Biomedicine 19:36–41

Ernsberger P, Koletsky RJ, Collins LA, Douglas HC (1993) Renal angiotensin receptor mapping in obese spontaneously hypertensive rats. Hypertension 21:1039–1045

Feron O, Salomone S, Godfraind T (1996) Action of the calcium channel blocker lacidipine on cardiac hypertrophy and endothelin-1 gene expression in stroke-prone hypertensive rats. Br J Pharmacol 118:659–664

Ganten D (1987) Role of animal models in hypertension research. Hypertension, Suppl. 9:1:I2–I4

Gouyon B, Julier C, Takahashi S, Vincent M, Ganten D, Georges M, Lathrop GM (1991) Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. Nature 353:521–529

Hamet P, Malo D, Tremblay J (1990) Increased transcription of a major stress gene in spontaneously hypertensive mice. Hypertension 15:904–908

Hilbert P, Lindpaintner K, Beckmann JS, Serikawa T, Soubrier F, Dubay C, Cartwright P, Ganten D, Lindpainter K, Ganten U, Peters J, Zimmermann F, Bader M,. Mullins J (1991) Transgenic animals: New animal models in hypertension research. Hypertension 17:843–855

Holycross BJ, Summers BM, Dunn RB, McCune SA (1997) Plasma renin activity in heart failure-prone SHHF/Mcc-facp rats. Am J Physiol 273(1 Pt2):H228–H233

Inoko M, Kihara Y, Morii I, Fujiwara H, Saayama S (1994) Transition from compensatory hypertrophy to dilated, failing left ventricles in Dahl salt-sensitive rats. Am J Physiol 267:H2471–H2482

Jacob HJ, Lindpainter K, Lincoln SE, Kusumi K, Bunker RK, Mao YP, Ganten D, Dzau VJ, Lander ES (1991) Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. Cell 67:213–224

Koletsky S (1975) Pathologic findings and laboratory data in a new strain of obese hypertensive rats. Am J Pathol 80:129–140

Langheinrich M, Jee MA, Böhm M, Pinto YM, Ganten D, Paul M (1996) The hypertensive *Ren-2* transgenic rat TGR(mREN2)27 in hypertension research. Characteristics and functional aspects. Am J Hypertens 9:506–512

Laverty R, Smirk FH (1961) Observations on the pathogenesis of spontaneous inherited hypertension and constricted renal-artery hypertension in rats. Circ Res 9:455–464

Lee MA, Böhm M, Paul M, Bader M, Ganten U, Ganten D (1996) Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. Am J Physiol 270(6 Pt 1):E919–E929

Linz W, Ganten D (1992) Contributions of animal models to understanding hypertension. In: Zipes DP,. Rowlands DJ (eds) Progress in Cardiology, pp 25–36, Lea and Febiger, Philadelphia

Linz W, Jessen T, Becker RHA, Schölkens BA, Wiemer G (1997) Long-term ACE inhibition doubles lifespan of hypertensive rats. Circulation 96:3164–3172

Meneton P, Ichikawa I, Inagami T, Schnermann J (2000) Renal physiology of the mouse. Am J Physiol 278:339–351

Mullins JJ, Ganten D (1990) Transgenic animals: new approaches to hypertension research. J Hypertension 8 (Suppl 7):S35–S37

Mullins JJ, Peters J, Ganten D (1990) Fulminant hypertension in transgenic rats harboring the mouse Re-2 gene. Nature 344:541–544

Ohkubo H, Kawakami H, Kakehi Y, Takumi T, Arai H, Yokota Y, Iwai M, Tanabe Y, Masu M, Hata J, Iwao H, Okamoto H, Yokoyama M, Nomura T, Katsuki M, Nakanishi S (1990) Generation of transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes. Proc Natl Acad Sci USA 87:5153–5156

Ohta K, Kim S, Wanibuchi H, Ganten D, Iwao K (1996) Contribution of local renin-angiotensin system to cardiac hypertrophy, phenotypic modulation, and remodelling in TGR(mREN2)27 transgenic rats. Circulation 94:785–791

Okamoto K, Aoki K (1963) Development of a strain of spontaneously hypertensive rats. Jap Circulat J 27:282–293

Okamoto K, Tabei R, Fukushima M, Nosaka S, Yamori Y, Ichijima K, Haebara H, Matsumoto M, Maruyama T, Suzuki Y, Tamegai M (1966) Further observations of the development of a strain of spontaneously hypertensive rats. Jap Circulat J 30:703–716

Okamoto K, Yamori Y, Nagaoka A (1974) Establishment of the stroke-prone spontaneously hypertensive rat (SHR) Circ Res 34/35 Suppl: I143–I153

Peters J, Münter K, Bader M, Hackenthal E, Mullins JJ, Ganten D (1993) Increased adrenal renin in transgenic hypertensive rats, TGR(mREN2)27, and its regulation by cAMP, angiotensin II, and calcium. J Clin Invest 91:742–747

Phelan EL (1968) The New Zealand strain of rats with genetic hypertension. NZ Med J 67:334–344

Phelan EL, Smirk FH (1960) Cardiac hypertrophy in genetically hypertensive rats. J Path Bact 80:445–448

Pijl AJ, van der Wal AC, Mathy MJ, Kam KL, Hendriks MGC, Pfaffendorf M, van Zwieten PA (1994) Streptozotocin-induced diabetes mellitus in spontaneously hypertensive rats: a pathophysiological model for the combined effects of hypertension and diabetes. J Pharmacol Toxicol Meth 32:225–233

Pinto YM, Paul M, Ganten D (1998) Lessons from rat models of hypertension: from Goldblatt to genetic engineering. Cardiovasc Res 39:77–88

Pravenec M, Klír P, Kren V, Zicha J, Kuneš J (1989) An analysis of spontaneous hypertension in spontaneously hypertensive rats by means of new recombinant inbred strains. J Hypertension 7:217–222

Rapp JP (1984) Characteristics of Dahl salt-susceptible and salt-resistant rats. In: de Jong W (ed) Handbook of Hypertension. Vol 4. Experimental and Genetic Models of Hypertension. Elsevier Science Publ., New York, pp 286–295

Rapp JP, Wang SM, Dene H (1989) A genetic polymorphism in the renin gene of Dahl rats cosegregates with blood pressure. Science 242:542–544

Rosenberg WL, Schlager G, Gennaro JF Jr (1985) Glomerular filtration and fluid balance in genetically hypertensive mice. Proc Soc Exp Biol Med 178:629–634

Rosenthal T, Erlich Y, Rosenmann E, Cohen A (1997) Effects of enalapril, losartan, and verapamil on blood pressure and glucose metabolism in the Cohen-Rosenthal diabetic hypertensive rat. Hypertension 29:1260–1264

Salvati P, Ferrario RG, Bianchi G (1990) Diuretic effect of bumetanide in isolated perfused kidneys of Milan hypertensive rats. Kidney Internat 37:1084–1089

Samani NJ, Brammar WJ, Swales JD (1989) A major structural abnormality in the renin gene of the spontaneously hypertensive rat. J Hypertension 7:249–254

Sen S, Tarazi RC, Khairallah PA, Bumpus FM (1974) Cardiac hypertrophy in spontaneously hypertensive rats. Circ Res 35:775–781

Simpson FO, Phelan EL (1984) Hypertension in the genetically hypertensive rat strain. In: de Jong W (ed) Handbook of Hypertension, Vol 4: Experimental and Genetic Models of Hypertension. pp 200–223. Elsevier Science Publ., New York

Smirk FH, Hall WH (1958) Inherited hypertension in rats. Nature 182:727–728

Vincent M, Sacquet J, Sassard J (1984) The Lyon strains of hypertensive, normotensive and low-blood-pressure rats. In: de Jong W (ed) Handbook of Hypertension, Vol 4: Experimental and Genetic Models of Hypertension. pp 314–327. Elsevier Science Publ., New York

Yamori Y (1984a) Development of the spontaneously hypertensive rat (SHR) and of various spontaneous rat models, and their implications. In: de Jong W (ed) Handbook of Hypertension, Vol 4: Experimental and Genetic Models of Hypertension. pp 224–239. Elsevier Science Publ., New York

Yamori Y (1984b) The stroke-prone spontaneously hypertensive rat: contributions to risk factor analysis and prevention of hypertensive diseases. In: de Jong W (ed) Handbook of Hypertension. Vol 4. Experimental and Genetic Models of Hypertension. Elsevier Science Publ., New York, pp 240–255

Yamori Y, Horie R, Nara Y, Kihara M (1983) Pathogenesis, prediction and prevention of stroke in stroke-prone SHR In: Stefanovich V (ed) Stroke: Animal models. pp 99–113. Pergamon Press Oxford, New York, Paris, Kronberg

Zolk O, Flesch M, Schnabel P, Teisman AC, Pinto YM, van Gilst WH, Paul M, Böhm M (1998) Effects of quinapril, losartan and hydralazine on cardiac hypertrophy and β-adrenergic neuroeffector mechanisms in transgenic TGR(mREN2)27 rats. Br J Pharmacol 123:405–412

A.2.0.8 Hypertension Induced by Chronic NO-Synthase Inhibition

PURPOSE AND RATIONALE

Tonic basal release of nitric oxide (NO) by vascular endothelial cells controls blood pressure in the basal state. Chronic blockade of NO synthesis in the rat produces systemic hypertension and glomerular damage (Baylis et al. 1992). This was recommended by Ribeiro et al. (1992) as a model of hypertension. Yang et al. (1996) found an increase of vascular angiotensin II receptor expression after chronic inhibition of NO synthase in spontaneously hypertensive rats. The detrimental sequels of chronic NO synthase inhibition in rats can be inhibited by treatment with ACE inhibitors (Hropot et al. 1994; Küng et al. 1995). Hsieh et al. (2004) reported that NO inhibition by L-NAME accelerates hypertension and induces perivascular inflammation in rats.

Hropot et al. (2003) reported that angiotensin II subtype AT_1 receptor blockade prevents hypertension and renal insufficiency induced by chronic NO synthase inhibition in rats.

PROCEDURE

Male Wistar rats at an age of 7–8 weeks weighing 210 ± 10 g were placed at random in metabolic cages,

divided in four to six groups of six to eight rats each. Group 1 (control) had free access to tap water and food. Groups 2–4 were treated with 0.02% L-NAME water solution for 6 weeks in a daily dose of 25 mg/kg. Groups 3 and 4 received the angiotensin receptor antagonists fonsartan (10 mg/kg) or lorsatan (30 mg/kg) for 6 weeks daily per stomach tube. Groups 5 and 6 received fonsartan and lorasatan alone. At the end of the study, 24-h urine samples were collected and retrobulbar blood samples were taken in short inhalation anesthesia. Plasma values of creatine, PRA and electrolytes were determined. For clearance evaluation rats were anesthetized with 50 mg/kg thiopentone i.p. In order to determine glomerular filtration rate and renal plasma flow, clearances of inulin and *para*-aminohippurate were performed. After the clearance experiments, the rats were sacrificed and hearts and kidneys removed. Hearts were perfused in the isolated working heart preparation via the aorta with modified Krebs-Henseleit buffer containing solvents or drugs. After a preischemic period of 20 min, acute regional myocardial ischemia was produced by clamping the left coronary artery close to its origin for 15 min (ischemic period). Thereafter, the clip was removed and changes during reperfusion were monitored (reperfusion period). The following cardio-dynamic and cardio-metabolic parameters were measured: incidence and duration of ventricular fibrillation, left ventricular pressure, contractility (d*P*/d*t* max), heart rate and coronary flow; in the coronary effluent lactate dehydrogenase, creatine kinase and lactate; in the myocardial tissue lactate, glycogen, ATP and creatine phosphate. Left ventricular pressure was measured with a Statham pressure transducer (P 23 DB) which on differentiation yielded LV d*P*/d*t* max and heart rate. Coronary flow was determined by electromagnetic flow probes in the aortic cannula. For the determination of lactate release, lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate samples were taken from the coronary effluent and analyzed spectrophotometrically.

EVALUATION

Results are presented as arithmetical means ± SEM. A one-way ANOVA was calculated with SYSTAT for Windows (SYSTAT, Evanston, Ill., USA) followed by multiple pairwise comparisons according to Tukey.

MODIFICATIONS OF THE METHOD

Arnal et al. (1993) measured cardiac weight of rats in hypertension induced by NO synthase blockade.

Linz et al. (1999) reviewed the interactions between ACE, kinins and NO.

Sampaio et al. (2002) reported that hypertension plus diabetes mimics the cardiomyopathy induced by NO inhibition in rats.

Rossi et al. (2003) found that chronic inhibition of NO synthase induces hypertension and cardiomyocyte mitochondrial and myocardial remodeling in the absence of hypertrophy.

REFERENCES AND FURTHER READING

Arnal JF, el Amrani AI, Chatellier G, Menard J, Michel JB (1993) Cardiac weight in hypertension induced by nitric oxide synthase blockade. Hypertension 22:380–387

Baylis C, Mitruka B, Deng A (1992) Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. J Clin Invest 90:276–281

Hropot M, Grötsch H, Klaus E, Langer KH, Linz W, Wiemer G, Schölkens BA (1994) Ramipril prevents the detrimental sequels of chronic NO synthase inhibition in rats: hypertension, cardiac hypertrophy and renal insufficiency. Naunyn-Schmiedebergs Arch Pharmacol 350:646–652

Hropot M, Langer KH, Wiemer G, Grötsch H, Linz W (2003) Angiotensin II subtype AT_1 receptor blockade prevents hypertension and renal insufficiency induced by chronic NO-synthase inhibition in rats. Naunyn-Schmiedebergs Arch Pharmacol 367:312–317

Hsieh NK, Wang JY, Liu JC, Wang SD, Chen HI (2004) Nitric oxide inhibition accelerates hypertension and induces perivascular inflammation in rats. Clin Exp Pharmacol Physiol 31:212–218

Küng CF, Moreau P, Takase H, Lüscher TF (1995) L-NAME hypertension alters endothelial and smooth muscle function in rat aorta. Prevention by trandolapril and verapramil. Hypertension 26:744–751

Linz W, Wohlfart P, Schölkens BA, Malinski T, Wiemer G (1999) Interactions between ACE, kinins and NO. Cardiovasc Res 43:549–561

Ribeiro MO, Anuntes E, de Nucci G, Lovisolo SM, Zatz R (1992) Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20:298–303

Rossi MA, Ramos SG, Prado CM (2003) Chronic inhibition of nitric oxide synthase induces hypertension and cardiomyocyte mitochondrial and myocardial remodeling in the absence of hypertrophy. J Hypertens 21:993–1001

Sampaio RC, Tanus-Santos JE, Melo SESFC, Hyslop S, Franchinini KG, Luca IM, Moreno H Jr (2002) Hypertension plus diabetes mimics the cardiomyopathy induced by nitric oxide inhibition in rats. Chest 122:1412–1420

Yang Y, Macdonald GJ, Duggan KA (1996) A study of angiotensin II receptors after chronic inhibition of nitric oxide synthase in the spontaneously hypertensive rat. Clin Exp Pharmacol Physiol 23:441–443

A.2.0.9 Pulmonary Hypertension Induced by Monocrotaline

PURPOSE AND RATIONALE

The pyrrolizidine alkaloid monocrotaline, derived from *Crotalaria spectabilis*, is hepatotoxic and pneumotoxic in the rat. A single injection of monocrotaline

leads to progressive pulmonary hypertension resulting in right ventricular hypertrophy and cardiac failure (Gillespie et al. 1986, 1988; Todorovich-Hunter et al. 1988). Pathologic changes and hemodynamic changes associated with monocrotaline administration include blebbing of the lung, degeneration and fragmentation of endothelial cells, perivascular edema, extravasation of red blood cells, and muscularization of the pulmonary arteries and arterioles (Valdiva et al. 1967; Lalich et al. 1977; Huxtable et al. 1978; Hislop and Reid 1979; Meyrick and Reid 1979; Meyrick et al. 1980; Ghodsi and Will 1981; Hilliker et al. 1982; Sugita et al. 1983; Hilliker and Roth 1985; Stenmark et al. 1985; Altieri et al. 1986; Molteni et al. 1986; Lai et al. 1996). Rats given monocrotaline develop severe right ventricular hypertrophy often accompanied by ascites and pleural effusions (Ceconi et al. 1989).

Amelioration by angiotensin-converting enzyme inhibitors and by penicillamine has been demonstrated (Molteni et al. 1985, 1986).

PROCEDURE

Treatment of male Sprague Dawley rats weighing 200–225 g with the test drug (angiotensin converting enzyme inhibitor or vehicle) is started one week prior to a single subcutaneous injection of 100 mg/kg monocrotaline up to sacrifice 4, 7, or 14 days later by pentobarbital anesthesia and exsanguination. Heart and lungs are excised from thoracic cavity. After removing atria from the heart, the right ventricle is separated from the left ventricle plus septum which are blotted and weighed separately. Left lung is blotted, weighed, minced and reweighed after drying at room temperature for 14 days. Three pulmonary artery segments, main pulmonary artery, right extrapulmonary artery and an intrapulmonary artery from the from the right lower lobe, are isolated for study of vascular responsiveness. Cylindrical segments of each vessel are suspended between stainless steel hooks in 10-ml isolated tissue baths containing modified Krebs-Henseleit buffer aerated with 95% O_2/5% CO_2 at 37°C. At the end of each experiment, vessel segments are blotted and weighed and their dimensions measured. Cross-sectional area of each artery is determined from tissue weight and diameter.

Arteries are equilibrated for 1 h at 1 g of passive applied load and then are made to contract to KCl (6×10^{-2} M). After washout, the procedure is repeated with applied loads increased by 1 g increments. Responses are normalized to the maximum active force development generated by an artery in each experiment and the data are plotted as a function of applied force. Changes in isometric force are monitored through force displacement transducers (Grass FT03) and recorded on a polygraph.

Responsiveness to contractile and relaxant agonists is assessed in pulmonary arteries from saline- and monocrotaline-treated rats both in verum- and placebo-treated groups. Cumulative concentration-response curves to hypertonic KCl, angiotensin II and norepinephrine are generated sequentially in vessels at resting tone. Arteries are then contracted submaximally with norepinephrine and cumulative concentration-response curves to the vasorelaxants isoproterenol and acetylcholine are determined.

EVALUATION

Contractions are expressed as active tension development, force generated per cross-sectional area and relaxations are normalized to precontraction tone. Both contractile and relaxation responses are plotted as a function of the negative logarithm of agonist concentration. Differences in mean responses are compared by a *t*-test for grouped data.

MODIFICATIONS OF THE METHOD

Molteni et al. (1986) treated rats continuously with monocrotaline in the drinking water at a concentration of 2.4 mg/kg/day for a period of 6 weeks. Test rats received an ACE-inhibitor during this time in the drinking water and controls the vehicle only. At the end of the experiment, hearts and lungs were weighed and examined by light and electron microscopy.

Madden et al. (1995) determined L-arginine-related responses to pressure and vasoactive agents in monocrotaline-treated rat pulmonary arteries.

Ono et al. (1995) studied the effects of prostaglandin E_1 (PGE_1) on pulmonary hypertension and lung vascular remodelling in the rat monocrotaline model of human pulmonary hypertension.

Yamauchi et al. (1996) studied the effects of an orally active endothelin antagonist on monocrotaline-induced pulmonary hypertension in rats.

Gout et al. (1999) evaluated the effects of adrenomedullin in isolated vascular rings from rats treated with monocrotaline (60 mg/kg s.c.) causing pulmonary hypertension and ventricular hypertrophy within 3 to 4 weeks.

Kanno et al. (2001) studied the effect of an angiotensin-converting enzyme inhibitor on pulmonary arterial hypertension and endothelial nitric oxide synthase expression in monocrotaline-treated rats. For evaluation of right ventricular hypertrophy as a result of pulmonary arterial hypertension, multislice spin-

echo MRI images were acquired at 8–12 time points in a cardiac cycle with respiratory and ECG gating (Kanno et al. 2000) at 2, 3, 4, and 5 weeks after monocrotaline treatment.

Kang et al. (2003) reported that a phosphodiesterase-5 inhibitor attenuated monocrotaline-induced pulmonary hypertension in rats.

REFERENCES AND FURTHER READING

Altiere RJ, McIntyre MJ, Petrenka J, Olson JW, Gillespie MN (1986) Altered pulmonary vascular smooth muscle responsiveness in monocrotaline-induced pulmonary hypertension. J Pharmacol Exp Ther 236:390–395

Ceconi C, Condorelli ER, Quinzanini M, Rodella A, Ferrari R, Harris P (1989) Noradrenaline, atrial natriuretic peptide, bombesin and neurotensin in myocardium and blood of rats in congestive cardiac failure. Cardiovasc Res 23:674–682

Ghodsi F, Will JA (1981) Changes in pulmonary structure and function induced by monocrotaline intoxication. Am J Physiol 240:H149–H155

Gillespie MN, Olson JW, Reinsel CN, O'Connor WN, Altiere RJ (1986) Vascular hyperresponsiveness in perfused lungs from monocrotaline-treated rats. Am J Physiol 251:H109–H114

Gillespie MN, Goldblum SE, Cohen DA, McClain CJ, (1988) Interleukin-1 bioactivity in the lungs of rats with monocrotaline-induced pulmonary hypertension. Proc Soc Exp Biol Med 187:26–32

Gout B, Quiniou MJ, Khandoudi N, Le Dantec C, Saiag B (1999) Impaired endothelium-dependent relaxation by adrenomedullin in monocrotaline-treated rat arteries. Eur J Pharmacol 380:23–30

Hilliker KS, Roth RA (1985) Increased vascular responsiveness in lungs of rats with pulmonary hypertension induced by monocrotaline pyrrole. Am Rev Resp Dis 131:46–50

Hilliker KS, Bell TG, Roth RA (1982) Pneumotoxicity and thrombocytopenia after single injection of monocrotaline. Am J Physiol 242:H573–H579

Hislop A, Reid L (1979) Arterial changes in Crotalaria spectabilis-induced pulmonary hypertension in rats. Br J Exp Pathol 55:153–163

Huxtable RD, Ciaramitaro D, Eisenstein D (1978) The effect of a pyrrolizidine alkaloid, monocrotaline, and a pyrrole, dehydroretronecine, on the biochemical functions of the pulmonary endothelium. Mol Pharmacol 14:1189–1203

Kang KK, Ahn GJ, Sohn YS, Ahn BO, Kim WB (2003) DA-8159, a potent cGMP phosphodiesterase inhibitor, attenuates monocrotaline-induced pulmonary hypertension in rats. Arch Pharm Res 26:612–619

Kanno S, Lee PC, Zhang Y et al (2000) Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. Circulation. 101:2742–2748

Kanno S, Wu YJ, Lee PC, Billiar TR, Ho C (2001) Angiotensin-converting enzyme inhibitor preserves p21 and endothelial nitric oxide synthase expression in monocrotaline-induced pulmonary arterial hypertension in rats. Circulation 104:945–950

Lai YL, Thacker AA, Diana JN (1996) Hypoxemia and elevated tachykinins in rat monocrotaline pneumotoxicity. Lung 174:195–203

Lalich JJ, Johnson WD, Raczniak JJ, Shumaker RC (1977) Fibrin thrombosis in monocrotaline pyrrole induced cor pulmonale in rats. Arch Path Lab Med 101:69–73

Madden JA, Keller PA, Choy JS, Alvarez TA, Hacker AD (1995) L-arginine-related responses to pressure and vasoactive agents in monocrotaline-treated rat pulmonary arteries. J Appl Physiol 79:589–593

Meyrick B, Reid L (1979) Development of pulmonary arterial changes in rats fed Crotalaria spectabilis. Am J Pathol 94:37–50

Meyrick B, Gamble W, Reid L (1980) Development of Crotonolaria pulmonary hypertension: A hemodynamic and structural study. Am J Physiol 239:H692–H702

Molteni A, Ward WF, Ts'Ao CH, Solliday NH, Dunne M (1985) Monocrotaline-induced pulmonary fibrosis in rats: Amelioration by captopril and penicillamine. Proc Soc Exp Biol Med 180:112–120

Molteni A, Ward WF, Ts'Ao CH, Solliday NH (1986) Monocrotaline-induced cardiopulmonary damage in rats: amelioration by the angiotensin-converting enzyme inhibitor CL242817. Proc Soc Exp Biol Med 182:483–493

Ono S, Tanita T, Hoshikawa Y, Song C, Maeda S, Tabata T, Noda M, Ueda S, Ashino Y, Fujimura S (1995) Effects of prostaglandin E_1 (PGE_1) on pulmonary hypertension and lung vascular remodelling in a rat monocrotaline model of human pulmonary hypertension. Jpn J Thorac Dis 33:862–867

Pelá G, Missale C, Raddino R, Condorelli E, Spano PF, Visioli O (1990) β_1-and β_2-receptors are differentially desensitized in an experimental model of heart failure. J Cardiovasc Pharmacol 16:839–846

Stenmark KR, Morganroth ML, Remigo LK, Voelkel NF, Murphy RC, Henson PM, Mathias MM, Reeves JT (1985) Alveolar inflammation and arachidonate metabolism in monocrotaline-induced pulmonary hypertension. Am J Physiol 248:859–866

Sugita T, Hyers TM, Dauber IM, Wagner WW, McMurtry IF, Reeves JT (1983) Lung vessel leak precedes right ventricular hypertrophy in monocrotaline-treated rats. J Appl Physiol 54:371–374

Todorovich-Hunter L, Johnson DJ, Ranger P, Keeley FW, Rabinovitch M (1988) Altered elastin and collagen synthesis associated with progressive pulmonary hypertension induced by monocrotaline. A biochemical and ultrastructural study. Lab Invest 58:184–195

Valdiva E, Lalich JJ, Hayashi Y, Sonnard J (1967) Alterations in pulmonary alveoli after a single injection of monocrotaline. Arch Pathol 84:64–76

Yamauchi R, Hoshino T, Ban Y, Kikkawa K, Murata S, Nawano M, Toriumi W (1996) Effects of T-0115, a novel orally active endothelin antagonist, on monocrotaline-induced pulmonary hypertension in rats. Jpn J Pharmacol 71 (Suppl 1): 236P

A.2.0.10 Portal Hypertension in Rats

PURPOSE AND RATIONALE

Portal hypertension is associated with hyperdynamic splanchnic circulation and reduced vascular resistance (Vorobioff et al. 1983). Tanoue et al. (1991) developed a method for inducing portal hypertension and esophageal varices in rats – partial ligation of the portal vein after devascularization of the circumference of the left renal vein and complete ligation of the portal vein on the fifth day thereafter. Tsugawa et al. (2000)

used this model to study the role of nitric oxide and endothelin-1 in rat portal hypertension.

PROCEDURE

Male Sprague-Dawley rats were anesthetized with 50 mg/kg Nembutal intraperitoneally. The portal vein was isolated and stenosis created by a single ligature of 3–0 silk placed around the portal vein and a 20-gauge blunt-tipped needle after devascularization of the left renal vein. This devascularization is indispensable in preventing the development of excess collateral vessels, which inhibit the formation of esophageal varices and the portal hypertensive state. The needle was then removed from the ligature. In addition, 3–0 silk was also placed at the area of partial ligation (loose ligation), and both ends were then drawn out through the abdominal wall. Five days after the operation, the ends of the silk that had been placed in the flank were simultaneously pulled to induce complete portal vein ligation. Two weeks later, this portal hypertension model was completed.

Portal venous pressure, blood flow volume in the intra-abdominal viscera, plasma NO and plasma endothelin-1 were measured.

EVALUATION

Results were expressed as mean ± standard deviation. The Student's *t*-test was used to determine significance between portal hypertension rats and sham-operated controls.

MODIFICATIONS OF THE METHOD

Portal hypertension by portal vein ligation without devascularization of the left renal vein was used by Lee et al. (1985), Braillon et al. (1986), Oren et al. (1995), Fernandez et al. (1996), Moreno et al. (1996), Connolly et al. (1999), Hilzenrat et al. (1999), Chagneau et al. (2000), Yu et al. (2000), and Sakurabayashi et al. (2002).

Dieguez et al. (2002) used a surgical technique based on the development of a triple stenosing ligation to worsen the complications inherent to the prehepatic chronic portal hypertension.

Jaffe et al. (1994) and Li et al. (1998) used injection of different sized microspheres into the portal vein of male Wistar rats to induce portal hypertension.

REFERENCES AND FURTHER READING

Braillon A, Lee SS, Girod C, Peignoux-Martinot M, Valla D, Lebrec D (1986) Role of portasystemic shunts in the hyperkinetic circulation of the portal hypertensive rat. J Lab Clin Med 108:543–548

Chagneau C, Tazi KA, Heller J, Sogni P, Poirel O, Moreau R, Lebrec D (2000) The role of nitric oxide in the reduction of protein kinase C-induced contractile response in aortae from rats with portal hypertension. J Hepatol 33:26–32

Connolly C, Cawley T, McCormick PA, Docherty JR (1999) Portal hypertension increases vasoconstrictor responsiveness of rat aorta. Clin Sci 96:41–47

Dieguez B, Aller MA, Nava MP, Palma MD, Arias JL, Lopez L, Arias J (2002) Chronic portal hypertension in the rat by triple-portal stenosis ligation. J Invest Surg 15:329–336

Fernandez M, Garcia-Pagan JC, Casadevall M, Mourelle MI, Pique JM, Bosch J, Rodes J (1996) Acute and chronic cyclooxygenase blockage in portal-hypertensive rats: influence on nitric oxide biosynthesis. Gastroenterology 110:1529–1535

Jaffe V, Alexander B, Mathie RT (1994) Intrahepatic portal occlusion by microspheres: a new model of portal hypertension in the rat. Gut 35:815–819

Hilzenrat N, Arish A, Sikuler E (1999) Acute hemodynamic changes following hemorrhage and volume restitution, using a low viscosity plasma expander, in anesthetized portal hypertensive rats. J Hepatol 31:874–879

Lee SS, Girod C, Valla D, Geoffroy P, Lebrec D (1985) Effect of pentobarbital anesthesia on splanchnic hemodynamics of normal and portal-hypertensive rats. Am J Physiol 249:G528–G532

Li X, Benjamin IS, Alexander B (1998) The relationship between intrahepatic portal systemic shunts and microsphere induced portal hypertension in the rat liver. Gut 42:276–282

Moreno L, Martinez-Cuesta MA, Pique JM, Bosch J, Esplugues JV (1996) Anatomical differences in responsiveness to vasoconstrictors in the mesenteric veins from normal and portal hypertensive rats. Naunyn-Schmiedebergs Arch Pharmacol 354:474–480

Oren R, Hilzenrat N, Maaravi Y, Yaari A, Sikuler R (1995) Hemodynamic effects of hypothyroidism induced by methimidazole in normal and portal hypertensive rats. Dig Dis Sci 40:1941–1945

Sakurabayashi S, Koh KC, Chen L, Groszmann RJ (2002) Octreotide ameliorates the increase in collateral blood flow during postprandial hyperemia in portal hypertensive rats. J Hepatol 36:507–512

Tanoue K, Kitano S, Hshizume M, Wada H, Sugimachi K (1991) A rat model of esophageal varices. Hepatology 13:353–358

Tsugawa K, Hashizume M, Migou S, Kisihara F, Kawanaka H, Tomikawa M, Tanoue K (2000) Role of nitric oxide and endothelin-1 in a portal hypertensive rat model. Scand J Gastroenterol 35:1097–1105

Vorobioff J, Bredfeldt JE, Groszmann RJ (1983) Hyperdynamic circulation in portal-hypertensive rat model: a primary factor of maintenance of chronic portal hypertension. Am J Physiol 244:G52–G57

Yu Q, Shao R, Qian HS, George SE, Rockey DC (2000) Gene transfer of the neuronal NO synthase isoforms to cirrhotic rat liver ameliorates portal hypertension. J Clin Invest 105:741–748

A.3
Coronary (Cardiac) Drugs

A.3.1
Isolated Organs

A.3.1.1
Heart-Lung Preparation

PURPOSE AND RATIONALE

The isolated heart-lung of the dog was introduced by Knowlton and Starling (1912). Since then, the dog model has been used for many physiological and pharmacological studies (Krayer 1931; Krayer and Mendez 1942; Somani and Blum 1966; Takeda et al. 1973; Ishikawa et al. 1978, 1983; Ono et al. 1984a, b; Caffrey et al. 1986; Hausknecht et al. 1986; Fessler et al. 1988; Seifen et al. 1987, 1988; Naka et al. 1989). More recently, the rat model has been preferred (Dietz 1984, 1987; Onwochei et al. 1987, 1988; Kashimoto et al. 1987, 1990, 1994,1995, Fukuse et al. 1995).

PROCEDURE

Wistar rats weighing 300–320 g are anesthetized with 50 mg/kg pentobarbitone i.p. Tracheotomy is performed and intermittent positive pressure ventilation is instituted with air. The chest is opened and flooded with ice-cold saline and the heart arrested. Cannulae are inserted into the aorta and the superior (for measurement of central venous pressure) and inferior venae cavae. The heart-lung preparation is perfused with a solution containing rat blood cells from another rat and Krebs-Ringer bicarbonate buffer, with hematocrit and pH of 25% and 7.4, respectively. Concentration of the buffer constituents (mM): NaCl 127, KCl 5.1, $CaCl_2$ 2.2, KH_2PO_4 1.3, $MgSO_4$ 2.6, $NaHCO_3$ 15, glucose 5.5 and heparin. The perfusate pumped from the aorta passes through a pneumatic resistance and is collected in a reservoir maintained at 37°C and then returned to the inferior vena cava. In this model, no other organs except the heart and lung are perfused. Cardiac output is determined by the inflow as long as the heart does not fail. Mean arterial pressure is regulated by the pneumatic resistance. Heart rate is recorded by a bioelectric amplifier and cardiac output is measured with an electromagnetic blood flow meter. Arterial pressure and right atrial pressure are measured with transducers and amplifiers. The heart is perfused initially with cardiac output of 30 ml/min and mean arterial pressure of 80 mm Hg. Test drugs are administered into the perfusate 5 min after start of the experiment.

EVALUATION

Hemodynamic data within groups are analyzed by two-way analysis of variance (ANOVA) with repeated measures. Recovery time is measured by the Kruskal-Wallis test. The other data are analyzed by one-way ANOVA followed by the Dunnett test for multiple comparisons.

MODIFICATIONS OF THE METHOD

Using the Starling heart-lung preparation in dogs, Wollenberger (1947) studied the energy-rich phosphate supply of the failing heart.

Shigei and Hashimoto (1960) studied the mechanism of the heart failure induced by pentobarbital, quinine, fluoroacetate and dinitrophenol in dog's heart-lung preparation and effects of sympathomimetic amines and ouabain on it.

Imai et al. (1961) used heart-lung preparations of the dog to study the cardiac actions of methoxamine with special reference to its antagonistic action to epinephrine.

Capri and Oliverio (1965), Beaconsfield et al. (1974) used the heart-lung preparation of the **guinea pig**.

Robicsek et al. (1985) studied the metabolism and function of an autoperfused heart-lung preparation of the **dog**.

The **dog heart-lung preparation** was used by Seifen et al. (1988) to study the interaction of a calcium channel agonist with the effects of digoxin, by Somani and Blum (1966) to study blockade of epinephrine- and ouabain-induced cardiac arrhythmias in the dog,
by Riveron et al. (1988) to investigate the energy expenditure of an autoperfusing heart-lung preparation,
by Namakura et al. (1987) to study the role of pulmonary innervation in an *in situ* lung-perfusion preparation as a new model of neurogenic pulmonary edema,
by Hausknecht et al. (1986) to investigate the effects of lung inflation on blood flow during cardiopulmonary resuscitation,
by Caffrey et al. (1986) to evaluate the effect of naloxone on myocardial responses to isoproterenol,
by Ono et al. (1984) to estimate the cardiodepressant potency of various beta-blocking agents,
by Ishikawa et al. (1983) for a graphical analysis of drug effects in the dog heart-lung preparation – with particular reference to the pulmonary circulation and effects of norepinephrine and 5-hydroxytryptamine,
by Iizuka (1983) to study the cardiac effects of acetylcholine and its congeners,

by Fessler et al. (988) to investigate the mechanism of reduced LV afterload by systolic and diastolic positive pleural pressure,
by Takeda et al. (1973) to study the cardiac actions of oxprenolol.

Beaconsfield et al. (1974) used the heart-lung preparation of **guinea pigs** to study the cardiac effect of delta-9-tetrahydrocannabinol.

The **rabbit** autoperfusing heart-lung preparation was used by Muskett et al. (1986, 1988).

The isolated heart-lung preparation in the **cat** was described by Beaufort et al. (1993).

Kontos et al. (1987, 1988) harvested heart-lung blocks from **calves**.

REFERENCES AND FURTHER READING

Beaconsfield P, Oakley C, Carpi A, Rainsbury R, del Basso P (1974a) Cardiac effect of delta-9-tetrahydrocannabinol on a heart-lung preparation and on the intact animal. Eur J Cardiol 2:167–173

Beaconsfield P, Oakley C, Carpi A (1974b) Cardiac effect of delta-9-tetrahydrocannabinol on a heart-lung preparation and on the intact animal. Eur J Cardiol 2:167–173

Beaufort AM, Wierda JMKH, Houwertjes MC, Kleef UW, Meijer DKF (1993) The isolated heart-lung preparation in the cat. An *in situ* model to study the role of the lungs in the disposition of drugs. J Pharmacol Toxicol Meth 29:147–156

Caffrey JL, Wooldridge CB, Gaugl JF (1986) Naloxone enhances myocardial responses to isoproterenol in dog isolated heart-lung. Am J Physiol 250 (Heart Circ Physiol 19): H749–H754

Capri A, Oliverio A (1965) Effect of reserpine on the heart-lung preparation of guinea pig. Arch Int Pharmacodyn Ther 157:470–486

Dietz JR (1984) Release of natriuretic factor from rat heart-lung preparation by atrial distension. Am J Physiol 247 (Regulatory Integrative Comp Physiol 16) R1093–R1096

Dietz JR (1987) Control of atrial natriuretic factor release from a rat heart-lung preparation. Am J Physiol 252 (Regulatory Integrative Comp Physiol 21): R498–R502

Fessler HE, Brower RG, Wise RA, Permutt S (1988) Mechanism of reduced LV afterload by systolic and diastolic positive pleural pressure. J Appl Physiol 65:1244–1250

Fukuse T, Albes JM, Takahashi Y, Brandes H, Hausen B, Schäfers HJ (1995) Influence of red blood cells in an ex vivo rat heart-lung model. J Surg Res 59:399–404

Hausknecht MJ, Wise RA, Brower RG, Hassapoyannes C, Weisfeldt ML, Suzuki J, Permutt S (1986) Effects of lung inflation on blood flow during cardiopulmonary resuscitation in the canine isolated heart-lung preparation. Circ Res 59:676–683

Iizuka H (1983) Cardiac effects of acetylcholine and its congeners as assessed in canine heart-lung preparation. Folia Pharmacol Jpn 81:441–449

Imai S, Shigei T, Hashimoto K (1961) Cardiac actions of methoxamine with special reference to its antagonistic action to epinephrine. Circ Res 9:552–560

Jerusalem E, Starling EH (1910) On the significance of carbon dioxide for the heart beat. J Physiol 40:279–294

Ishikawa N, Taki K, Hojo Y, Hagino Y, Shigei T (1978) Direct recording of cardiac output- and venous return-curves in the dog heart-lung preparation for a graphical analysis of the effects of cardioactive drugs. Jpn Heart J 19:775–782

Ishikawa N, Taki K, Hojo Y, Hagino Y, Shigei T (1983) Graphical analysis of drug effects in the dog heart-lung preparation – with particular reference to the pulmonary circulation and effects of norepinephrine and 5-hydroxytryptamine. Jpn J Pharmacol 33:785–794

Kashimoto S, Tsuji Y, Kumazawa T (1987) Effects of halothane and enflurane on myocardial metabolism during postischaemic reperfusion in the rat. Acta Anaesth Scand 31:44–47

Kashimoto S, Kume M, Kumazawa T (1990) Functional and metabolic effects of bupivacaine and lignocaine in the rat heart-lung preparation. Br J Anaesth 65:521–526

Kashimoto S, Nakamura T, Kume M, Nonaka A, Kumazawa T (1994) Effects and interaction of nicardipine and volatile anesthetics in the rat heart-lung preparation. J Anesth 8:78–83

Kashimoto S, Namakura T, Furuya A, Kume M,. Kumazawa T (1995) Alteration of cardiac function and metabolism in the rat heart-lung preparation by methyl methacrylate and their protection by ulinastatin. Jpn J Anesth 44:1477–1481

Knowlton FP, Starling EH (1912) The influence of variations in temperature and blood pressure on the performance of the isolated mammalian heart. J Physiol 44:206–219

Kontos GJ Jr, Borkon AM, Adachi H, Baumgartner WA, Hutchins GM, Brawn J, Reitz BA (1987) Successful extended cardiopulmonary preservation in the autoperfused working heart-lung preparation. Surgery 102:269–276

Kontos GJ Jr, Borkon AM, Baumgartner WA, Fonger JD, Hutchins GM, Adachi H, Galloway E, Reitz BA (1988) Improved myocardial and pulmonary preservation by metabolic substrate enhancement in the autoperfused working heart-lung preparation. J Heart Transplant 7:140–144

Krayer O (1931) Versuche am isolierten Herzen. Naunyn Schmiedeberg's Arch exper Path Pharmakol 162:1–28

Krayer O, Mendez R (1942) Studies on veratrum alkaloids. I. The action of veratrine upon the isolated mammalian heart. J Pharmacol Exper Ther 74:350–364

Merin RG (1988) The isolated heart preparation. Br J Anesth 60:28S–34S

Muskett AD, Burton NA, Gay WA, Miller M, Rabkin MS (1986) Preservation in the rabbit autoperfusing heart-lung preparation: a potential role of indomethacin. Surgical Forum 37:252–254

Muskett AD, Burton NA, Grossman M, Gay WA Jr (1988) The rabbit autoperfusing heart-lung preparation. J Surg Res 44:104–108

Namakura J, Zhang S, Ishikawa N (1987) Role of pulmonary innervation in canine *in situ* lung-perfusion preparation: a new model of neurogenic pulmonary edema. Clin exp Pharmacol Physiol 14:535–543

Naka Y, Hirose H, Matsuda H, Nakano S, Shirakura R, Kawaguchi A, Miyamoto Y, Miyagawa S, Fukushima N, Kawashima Y (1989) Prevention of pulmonary edema in autoperfusing heart-lung preparations by FUT-175 and leukocyte depletion. Transplant Proc 21:1353–1356

Ono H, Kanazawa Y, O'Hara N, Hashimoto K (1984a) Estimation of cardiodepressant potency of nadolol, alprenololol, propranolol and pindolol, β-blocking agents, in heart-lung preparation and blood-perfused excised papillary muscle preparation of the dog. Japan J Pharmacol 36:507–517

Ono H, O'Hara N (1984b) A study of the cardiodepressant action of a β-blocking agent carteolol in heart-lung preparation of the dog. Jpn Circ J 86:1030–1044

Onwochei MO, Rapp JP (1988) Biochemically stimulated release of atrial natriuretic factor from heart-lung preparation in Dahl rats. Proc Soc Exper Biol Med 188:395–404
Onwochei MO, Snajdar RM, Rapp JP (1987) Release of atrial natriuretic factor from heart-lung preparations of inbred Dahl rats. Am J Physiol 253 (Heart Circ Physiol 22):H1044–1052
Riveron FA, Ross JH, Schwartz KA, Casey G, Sanders ON, Eisiminger R, Magilligan DJ Jr (1988) Energy expenditure of autoperfusing heart-lung preparation. Circulation 78, II Suppl III-103–III-109
Robicsek F, Masters TN, Duncan GD, Denyer MH, Rise HE, Etchison M (1985) An autoperfused heart-lung preparation: metabolism and function. Heart Transplant 4:334–338
Seifen E, Seifen AB, Kennedy RH, Bushman GA, Loss GE, Williams TG (1987) Comparison of cardiac effects of enflurane, isoflurane, and halothane in the dog heart-lung preparation. J Cardiothor Anesth 1:543–553
Seifen E, Kennedy RH, Seifen AB (1988) Interaction of BAY K-8644 with effects of digoxin in the dog heart-lung preparation. Eur J Pharmacol 158:109–117
Shigei T, Hashimoto K (1960) Study on the mechanism of the heart failure induced by pentobarbital, quinine, fluoroacetate and dinitrophenol in dog's heart-lung preparation and effects of sympathomimetic amines and ouabain on it. Jpn J Pharmacol 9:109–122
Somani P, Blum BK (1966) Blockade of epinephrine- and ouabain-induced cardiac arrhythmias in the dog heart-lung preparation. J Pharmacol Exp Ther 152:235–242
Takeda K, Iizuka K, Imai S (1973) Cardiac actions of oxprenolol as studied in dog heart-lung preparations. Arzneim Forsch/Drug Res 23:1446–1450
Wollenberger A (1947) On the energy-rich phosphate supply of the failing heart. Am J Physiol 150:733–745

A.3.1.2 Isolated Heart According to Langendorff

PURPOSE AND RATIONALE

More than 100 years ago Langendorff (1895) described studies on isolated surviving mammalian hearts using mainly cats as donors. Since then, the method has been improved from the technical site and is nowadays used for studies with guinea-pig, rabbit or rat hearts. In principle, the heart is perfused in retrograde direction from the aorta either at constant pressure or at constant flow with oxygenated saline solutions. Retrograde perfusion closes the aortic valves, just as in the *in situ* heart during diastole. The perfusate is displaced through the coronary arteries flowing off the coronary sinus and the opened right atrium. In this original set-up the ventricles do not fill with perfusate and therefore do not perform pressure-volume work. Parameters usually measured are: contractile force, coronary flow and cardiac rhythm.

PROCEDURE

Guinea pigs of either sex weighing 300–500 g are sacrificed by stunning. For studies of biochemical parameters in tissue and perfusate, removal of the heart during barbiturate anesthesia and artificial respiration is recommended. The heart is removed as quickly as possible and placed in a dish containing Ringer's solution at 37°C. Associated pericardial and lung tissue are removed. The aorta is located and cut just below the point of its division. A glass or plastic cannula is introduced into the aorta, tied with two threads and perfusion is started with oxygenated Ringer's solution or Krebs-Henseleit buffer. The heart is transferred to a double walled plexiglass perfusion apparatus which is kept at 37°C by the water from a thermostat. Oxygenated Ringer's solution is perfused at a constant pressure of 40 mm Hg and at a temperature of 37°C from a reservoir. A small steel hook with a string is attached to the apex of the heart. Contractile force is measured isometrically by a force transducer with a preload of 2.5 g and recorded on a polygraph. Coronary flow is measured by a drop counter. Alternatively, flow measurements can be performed using a mechanic-electronic flow meter consisting of a vertical pipe and a magnetic valve (Hugo Sachs Electronic KG, Germany). Heart rate is measured through a chronometer coupled to the polygraph. Drugs are injected into the perfusion medium just above the aortic cannula.

CRITICAL ASSESSMENT OF THE METHOD

A reappraisal of the LANGENDORFF heart preparation was given by Broadley (1979) underlining the usefulness to test coronary vasodilating drugs. The value of the LANGENDORFF method can be best assessed by demonstrating a few of its applications in physiology and pharmacology. Direct effects can be measured as well as the antagonism against various physiological and pharmacological agents.

MODIFICATIONS OF THE METHOD

A survey on various modifications of the LANGENDORFF-technique and the **isolated working heart preparation** has been given by Ross (1972).

Neely et al. (1967) inserted a second cannula into a pulmonary vein or the left atrium. Perfusate from a reservoir flows via this cannula through the mitral valve into the left ventricle. During the systole of the heart, the left ventricle repumps the perfusate through the aorta into the reservoir. The perfusate flowing through the coronary arteries and dripping off from the outside of the heart is collected in a vessel below the heart and recirculated into the reservoir with a roller pump.

Flynn et al. (1978) underlined the difference of this working heart preparation to the original LAN-

GENDORFF method and reported the effects of histamine and noradrenaline on peak left ventricular systolic pressure, contractility, sinus rate, coronary flow, aortic flow, total cardiac output, and external pressure-volume work. Therefore, this method is reported separately.

Ishiu et al. (1996) measured simultaneously Ca^{2+}-dependent indo-1 fluorescence and left ventricular pressure on a beat-to-beat basis in Langendorff guinea pig hearts and investigated the changes in Ca^{+2} transient and left ventricular function during positive inotropic stimulation and myocardial ischemia.

Hukovic and Muscholl (1962) described the preparation of the isolated **rabbit** heart with intact sympathic nervous supply from the right stellate ganglion.

Hendrikx et al. (1994) used the isolated perfused rabbit heart to test the effects of an Na^+/H^+ exchange inhibitor on postischemic function, resynthesis of high-energy phosphate and reduction of Ca^{2+} overload.

Michio et al. (1985) modified the Langendorff method in rabbits to a working heart preparation by cannulating the left atrium. At a pressure of 20 cm H_2O in the left atrium, the heart pumped the solution against a hydrostatic pressure of 100 cm H_2O. Aortic flow, systolic aortic pressure, coronary flow, and heart rate were measured.

The influence of an ACE-inhibitor on heart rate, lactate in the coronary effluate and GTP-level in the myocardium after 60-min hypothermic cardiac arrest was studied in working heart preparation of rabbits by Zegner et al. (1996).

Gottlieb and Magnus (1904) introduced the so called **"balloon method"**. A small balloon fixed to the tip of a catheter is filled with water and inserted into the left ventricle via one of the pulmonary veins, the left atrium and the mitral valve. The balloon size has to fit the volume of the left ventricle and therefore its size depends on the animal species and body weight. The catheter can be fixed by tying the pulmonary vein stems. Via a three-way valve, the balloon can be extended to a given preload. The beating heart now exerts a rhythmic force to the balloon and thus to the membrane of a pressure transducer. The advantages of this method are that force development and preload can be stated reproducibly in pressure units [mm Hg], left ventricular contraction curves can be used for further calculations, and continuous heart rate recordings can be carried out without any problems when using a rate meter.

Sakai et al. (1983) reported a similar method adapted to **mice**.

Bardenheuer and Schrader (1983) described a method whereby the balloon is inserted into the left ventricle as described above. However, isovolumetric pressure in the left ventricle is not measured. Instead, the fluid in the balloon is pumped through the cannula into a closed extra corporal circulation. The fluid is forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume expelled from the balloon corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated **rat** hearts (Linz et al. 1986; Linz et al. 1990):

- LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.
- Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation:

$$MVO_2 = CF \times (P_a = P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37°C (Zander and Euler 1976).

For the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities in the perfusate, samples are taken from the coronary effluent.

After the experiments, hearts are rapidly frozen in liquid nitrogen and stored at –80°C. Of the left ventricle, 500 mg are taken, put into 5 ml ice-cold $HClO_4$ and disrupted with an Ultra-Turrax (Junke and Kunkel, Ika-Werk, Type TP). Glycogen is hydrolyzed with amyloglycosidase (pH 4.8) and determined as glucose. Furthermore, ATP and creatine phosphate are measured.

Avkiran and Curtis (1991) constructed a dual lumen aortic cannula which permits independent perfusion of left and right coronary beds in isolated rat hearts without necessitating the cannulation of individual arteries.

Igic (1996) described a modification of the isolated perfused working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted into the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses coronary vessels and the rest is expelled. Because the inner cannula can be easily retracted into the outer cannula, which is placed in the aorta, the preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

By labeling glucose, lactate, or fatty acids in the perfusate with ^{3}H or ^{14}C, Barr and Lopaschuk (1997) directly measured energy metabolism in the isolated rat heart.

Krzeminski et al. (1991) described a new concept of the isolated heart preparation with on-line computerized data evaluation. Left ventricular pressure was recorded by means of a balloon-catheter, while special suction electrodes obtained the high-amplitude, noise-free electrogram recordings. The coronary effluent partial pressure of oxygen was continuously monitored, which enabled the calculation of myocardial oxygen consumption (MVO_2). The effluent partial pressure of carbon dioxide and pH value were also measured simultaneously. A computerized system of data acquisition, calculation, storage, and end report was described.

Döring and Dehnert (1988) described continuous simultaneous **ultrasonic recording** of two cardiac diameters in an isolated perfused guinea-pig heart. For the measurement of the left ventricular transversal diameter the ultrasonic transmitter was positioned at the epicardium at the largest cardiac diameter. The corresponding ultrasonic receiver was inserted through the right atrium into the right ventricle to approximately the same height as the transmitter. In the right ventricle, which is empty in the isolated perfused LANGENDORFF-heart, it was automatically positioned opposite to the transmitter. Additional transducers were placed both at the heart's base and apex for assessment of the ventricular longitudinal diameter.

Several authors used the **isolated perfused mouse heart**.

Bittner et al. (1996) described a work-performing heart preparation for myocardial performance analysis in murine hearts using a modified Langendorff apparatus.

Sumaray and Yellon (1998a, b) constructed a specially designed Langendorff apparatus that allows perfusion of the isolated **mouse** heart. These authors reported that ischemic preconditioning reduces infarct size following global ischemia in the murine myocardium.

Brooks and Apstein (1996) measured left ventricular systolic and diastolic pressures in the isovolumically contracting (balloon in the left ventricle) mouse hearts.

Sutherland et al. (2003) reviewed characteristics and cautions in the use of the isolated perfused heart of mice.

Wang et al. (2001) studied the relationship between ischemic time and ischemia/reperfusion injury in isolated Langendorff-perfused mouse hearts.

Tejero-Taldo et al. (2002) reported that α-adrenergic receptor stimulation produces late preconditioning through inducible nitric oxide synthase in mouse heart.

Ross et al. (2003) found that the α_{1B}-adrenergic receptor decreases the inotropic response in the mouse Langendorff heart model.

Bratkovsky et al. (2004) measured coronary flow reserve in isolated hearts from mice.

Plumier et al. (1995) generated **transgenic mice** expressing the human heart heat shock protein 70. Upon reperfusion of the hearts after 30 min of ischemia in the Langendorff preparation, transgenic hearts versus non transgenic hearts showed significantly improved recovery of contractile force.

Hannan et al. (2000) compared ENOS knockout and wild-type mouse hearts which were perfused in a Langendorff apparatus with Krebs bicarbonate buffer and subjected to 20 min of global normothermic ischemia followed by 30 min of reperfusion. Myocardial function was measured using a ventricular balloon to determine time to onset of contraction, left ventricular

developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and heart rate-pressure product (RPP).

Sheikh et al. (2001) generated transgenic mice overexpressing fibroblast growth factor (FGF)-2 protein in the heart. An isolated mouse heart model of ischemia-reperfusion injury was used to assess the potential of endogenous FGF-2 for cardioprotection.

APPLICATIONS

Positive Inotropic Effects

While negative inotropic substances can be tested in a heart beating with normal force, the evaluation of a positive inotropic compound usually requires that cardiac force is first reduced. Acute experimental heart failure can be induced by an overdose of barbiturates, such as sodium thiopental, or calcium antagonists. This kind of cardiac failure can be reversed by β-sympathomimetic drugs, cardiac glycosides, or increased Ca^{+2} concentration. In this way, the potential β-sympathomimetic activity of a new drug can be measured using isoproterenol as standard. After thiopental-Na treatment, left ventricular pressure (LVP) and dp/dt_{max} decrease considerably, whereas coronary flow is slightly enhanced. β-Sympathomimetic drugs restore LVP and dp/dt_{max} and keep coronary blood flow elevated.

Cardiac glycosides increase LVP and dp/dt_{max} and leave coronary flow unchanged.

Negative Inotropic Effects

The effects of a β-sympathomimetic drug such as isoproterenol at doses of 0.05 to 0.2 µg increasing contractile force as well as heart frequency are registered. After injection of a β-blocker, the effects of isoproterenol are attenuated. The effects of a potential β-blocking agent can be tested comparing the isoproterenol inhibition versus a standard such as propranolol (0.1 mg).

Coronary Vessel Dilating Effect

The LANGENDORFF heart has been extensively used for assessing the coronary dilating activity of drugs (Broadley 1979). Rothaul and Broadley (1982) demonstrated the release of coronary vasodilator mediators from guinea pig isolated hearts by a technique employing donor and recipient hearts in series.

Calcium-Antagonism

In order to demonstrate the effect of calcium-antagonists, 1 to 5 mg $BaCl_2$ are injected which induce a pronounced spasm of the coronary arteries thereby reducing the coronary flow. Five min later, the test drug is injected. Active compounds have a relaxing effect on coronary arteries indicated by an increase of coronary flow. After this effect has weaned, $BaCl_2$ is injected again and the test drug or a standard drug, e. g. nifedipine, is tested. The increase of coronary flow is expressed as percentage of flow during $BaCl_2$ spasm and compared with the effect of the standard. Using various doses, dose-response curves can be established.

Effect on Potassium Outflow Induced by Cardiac Glycosides

Lindner and Hajdu (1968) described a method using the LANGENDORFF heart in which contractile force, coronary flow, and the potassium content in the coronary outflow was determined by flame photometry. Increase in potassium outflow correlates well with the positive inotropic effect.

Gradual Determination of Hypoxic Damage

Lindner and Grötsch (1973) measured the enzymes creatine phosphokinase (CPK), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (α-HBDH), and glutamicoxalacetic transaminase (GOT) in the effluent of a guinea pig heart preparation under varying degrees of hypoxia. Potassium content and oxygen tension in the inflowing and outflowing solution were determined. The heart rate, the amplitude of contraction and the rate of coronary vessel perfusion were recorded additionally.

Metabolic Studies with Nuclear Magnetic Resonance

Using ^{31}P, studies on metabolism of nucleotides and phosphorylated intermediates of carbohydrates in isolated hearts have been performed (Garlick et al. 1977; Jacobus et al. 1977; Hollis et al. 1978; Matthews and Radda 1984).

Arrhythmogenic, Anti-Arrhythmic and Antifibrillatory Effects

The LANGENDORFF heart preparation is also used to test the influence of compounds on cardiac rhythm. For recording monophasic action potentials, suction electrodes are applied on the heart. Ventricular fibrillation can be induced by simultaneous injection of digitoxin (12.5–25.0 µg) and aconitine (12.5–25.0 µg) into the perfusion fluid (Lindner 1963). Cardiac glycosides shorten the refractory period, decrease the conduction velocity and increase heterotopic stimulus generation. Aconitine increases markedly heterotopic stimulus generation. Both compounds together induce invariably ventricular fibrillation. Anti-arrhythmic com-

pounds can be tested in this way. Fibrillation is inhibited, at least partially, by 20 µg prenylamine, 10–20 µg quinidine or 20 µg ajmaline.

Takeo et al. (1992) described protective effects of anti-arrhythmic agents on oxygen-deficiency-induced contractile dysfunction of isolated perfused hearts. Hypoxia in isolated rabbit hearts was induced by perfusing the heart for 20 min with Krebs-Henseleit buffer saturated with a gas mixture of 95% N_2 and 5% CO_2 containing 11 mM mannitol. After hypoxic perfusion, the heart was reoxygenated for 45 min with oxygenated buffer containing glucose.

Dhein et al. (1989) studied the pathway and time course of the epicardial electrical activation process by means of a computer-assisted epicardial potential mapping, using a matrix of 256 unipolar AgCl electrodes (1 mm spatial and 0.25 ms temporal resolution) in isolated rabbit hearts perfused according to the Langendorff technique. From the activation times of the surrounding electrodes, the direction and velocity of activation for each electrode were calculated, thereby allowing construction of an epicardial vector field. The method was used for the assessment of arrhythmogenic and anti-arrhythmic drug activity.

Electrical Stimulation and Antifibrillatory Effect

Ventricular fibrillation can be induced in the LANGENDORFF preparation by reducing the glucose content of the perfusion medium to 0.25 g/1000 ml and the KCl content to 0.12 g/1000 ml. (Burn et al. 1957, 1960; Lindner 1963). After a perfusion period of 20 min, 10 µg epinephrine are injected into the perfusion cannula. Immediately afterwards, the heart is stimulated with a current of 40 Hz and 5 mA for 2 min. This procedure is repeated every 10 min. Standard conditions are achieved when the fibrillation continues without further electrical stimulation. Hearts treated in this way serve as controls. Other hearts stimulated in the same way are treated with continuous infusion of the test drug or the standard via the perfusion medium. Differences in the incidence of fibrillations are calculated using the χ^2 test.

Electrophysiological Evaluation of Cardiovascular Agents

Balderston et al. (1991) modified the Langendorff technique in rabbit hearts in order to perform electrophysiologic studies. His bundle electrograms were measured with a plunge electrode and allowed atrioventricular nodal physiology to be evaluated directly. Atrial conduction and refractoriness, atrioventricular node conduction and refractoriness, His-Purkinje conduction, and ventricular conduction and refractoriness could be accurately measured. The effects of verapamil and flecainide were described.

EDRF Release from the Coronary Vascular Bed

Lamontagne et al. (1992) isolated platelets from blood of healthy human donors and injected platelets boluses into the perfusion line of the Langendorff preparation of a rabbit heart. In the effluent cyclic GMP was determined as an index for EDRF release.

REFERENCES AND FURTHER READING

Avkiran M, Curtis MJ (1991) Independent dual perfusion of left and right coronary arteries in isolated rat hearts. Am J Physiol 261 (Heart Circ Physiol 30): H2082–H2090

Balderston SM, Johnson KE, Reiter MJ (1991) Electrophysiologic evaluation of cardiovascular agents in the isolated intact rabbit heart. J Pharmacol Meth 25:205–213

Bardenheuer H, Schrader J (1983) Relationship between myocardial oxygen consumption, coronary flow, and adenosine release in an improved isolated working heart preparation of guinea pigs. Circ Res 51:263–271

Barr RL, Lopaschuk GD (1997) Direct measurement of energy metabolism in the isolated rat heart. J Pharmacol Toxicol Meth 38:11–17

Bittner HB, Chen EP, Peterseim DS, Van Trigt P (1996) A work-performing heart preparation for myocardial performance analysis in murine hearts. J Surg Res 64:57–62

Bratkovsky S, Aasum E, Birkeland CH, Riemersma RA, Myrhe ESP, Larsen TS (2004) Measurement of coronary flow reserve in isolated hearts from mice. Acta Physiol Scand 181:167–172

Broadley KJ (1979) The Langendorff heart preparation – Reappraisal of its role as a research and teaching model for coronary vasoactive drugs. J Pharmacol Meth 2:143–156

Brooks WW, Apstein CS (1996) Effect of treppe on isovolumic function in the isolated blood-perfused mouse heart. J Mol Cell Cardiol 28:1817–1822

Brown TG, Lands AM (1964) Cardiovascular activity of sympathomimetic amines. In: Laurence DR, Bacharach AL (eds) Evaluation of Drug Activities: Pharmacometrics. Academic Press, London and New York, pp 353–368

Burn HJ, Hukovic S (1960) Anoxia and ventricular fibrillation: With a summary of evidence on the cause of fibrillation. Br J Pharmacol 15:67–70

Burn HJ, Goodford PJ (1957) Effect of lack of glucose and of lack of oxygen on ventricular fibrillation. J Physiol 137:20P–21P

Chevalier B, Mouas C, Mansier P, Aumont MC, Swynghedauw B (1987) Screening of inotropic drugs on isolated rat and guinea pig hearts. J Pharmacol Meth 17:313–326

Dhein S, Müller A, Klaus W (1989) The potential of epicardial activation mapping in isolated hearts for the assessment of arrhythmogenic and antiarrhythmic drug activity. J Pharmacol Meth 22:197–206

Döring HJ (1990) The isolated perfused warm-blooded heart according to LANGENDORFF. Technique – Function – Application. Physiologie bohemoslovaca 39:481–496

Flynn SB, Gristwood RW, Owen DAA (1978) Characterization of an isolated, working heart guinea-pig heart including effects of histamine and noradrenaline. J Pharmacol Meth 1:183–195

Garlick PP, Radda GK, Seeley PJ, Chance B (1977) Phosphorus NMR studies on perfused heart. Biochem Biophys Res Commun 74:1256–1262

Gottlieb R, Magnus R (1904) Digitalis und Herzarbeit. Nach Versuchen an überlebenden Warmblüterherzen. Naunyn-Schmiedeberg's Arch exper Path Pharmakol 51:30–63

Hannan RL, John MC, Kouretas PC, Hack BD, Matherne GP, Laubach VE (2000) Deletion of endothelial nitric oxidase exacerbates myocardial stunning in an isolated mouse heart model. J Surg Res 93:127–132

Hendrikx M, Mubagawa K, Verdonk F, Overloop K, Van Hecke P, Vanstapel F, Van Lommel A, Verbeken E, Lauweryns J, Flameng W (1994) Na^+-H^+ exchange inhibitor HOE 694 improves postischemic function and high-energy phosphate resynthesis and reduces Ca^{2+} overload in isolated perfused rabbit heart. Circulation 89:2787–2798

Hollis DP, Nunnally RL, Taylor GJ, Weisfeldt ML, Jacobus WE (1978) Phosphorus NMR studies of heart physiology. J Mag Reson 29:319–330

Hukovic S, Muscholl E (1962) Die Noradrenalin-Abgabe aus dem isolierten Kaninchenherzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. Naunyn-Schmiedeberg's Arch exp Path Pharmak 244:81–96

Igic R (1996) The isolated perfused "working" rat heart: a new method. J Pharmacol Toxicol Meth 35:63–67

Ishiu R, Abe Y, Onishi K, Ueda Y, Sekioka K, Nakano T (1996) Changes in calcium transient and left ventricular function during inotropic stimulation and myocardial ischemia in indo-1-loaded beating guinea pig heart. J Pharmacol Toxicol Meth 35:55–61

Jacobus WE, Taylor GJ, Hollins DP, Nunnally RL (1977) Phosphorus nuclear magnetic resonance of perfused working rat hearts. Nature 265:756–758

Krzeminski T, Kurcok A, Kapustecki J, Kowalinski J, Slowinski Z, Brus R (1991) A new concept of the isolated heart preparation with on-line computerized data evaluation. J Pharm Meth 25:95–110

Lamontagne D, König A, Bassenge E, Busse R (1992) Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. J Cardiovasc Pharmacol 20:652.657

Langendorff O (1895) Untersuchungen am lebenden Säugethierherzen. Pflüger's Arch ges Physiol 61:291–332

Lindner E (1963) Untersuchungen über die flimmerwidrige Wirkung des N-(3′-phenylpropyl-(2′))-1,1-diphenylpropyl-(3)-amins (Segontin). Arch Int Pharmacodyn 146:485–500

Lindner E, Grötsch H (1973) Methode zur graduellen Bestimmung hypoxischer Schädigung am isolierten Meerschweinchenherzen nach Langendorff. Arzneim Forsch/Drug Res 23:926–929

Lindner E, Hajdu P (1968) Die fortlaufende Messung des Kaliumverlustes des isolierten Herzens zur Bestimmung der Wirkungsstärke digitalisartiger Körper. Arch Int Pharmacodyn 175:365–372

Linz W, Schölkens BA, Han YF (1986) Beneficial effects of the converting enzyme inhibitor, ramipril, in ischemic rat hearts. J Cardiovasc Pharmacol 8 (Suppl 10):S91–S99

Linz W, Martorana PA, Schölkens BA (1990) Local inhibition of bradykinin degradation in ischemic hearts. J Cardiovasc Pharmacol 15 (Suppl 6): S99–S109

Matthews PM, Radda GK (1984) Applications of nuclear magnetic resonance to the study of myocardial metabolism and pharmacology. In: Schwartz A (ed) Methods in Pharmacology, Vol 5, Myocardial Biology., Plenum Press, New York and London, pp 175–228

Michio F, Hideo I, Tetsuya A (1985) *In vitro* assessment of myocardial function using a working rabbit heart. J Pharmacol Meth 14:49–60

Neely JR, Liebermeister H, Batterbsy EJ, Morgan HE (1967) Effect of pressure development on oxygen consumption by isolated rat heart. Am J Physiol 212:804–814

Plumier JCL, Ross BM, Currie RW, Angelidis CA, Kazlaris H, Kollias G, Pagoulatos GN (1995) Transgenic mice expressing the human heart heat shock protein 70 have improved postischemic myocardial recovery. J Clin Invest 95:1854–1860

Ross BD (1972) Perfusion techniques in biochemistry. A laboratory manual in the use of isolated perfused organs in biochemical experimentation. Clarendon Press, Oxford, Chapter 5: Heart and skeletal muscle. pp 258–320

Ross SA, Rorabaugh BR, Chalothorn D, Yun J, Gonzalez-Cabrera PJ, McCune DF, Piasik MT, Perez DM (2003) The α_{1B}-adrenergic receptor decreases the inotropic response in the mouse Langendorff heart model. Cardiovasc Res 60:598–607

Rothaul AL, Broadley KJ (1982) Measurements of oxygen tension in perfusates from guinea pig isolated hearts and the demonstration of coronary vasodilator material. J Pharmacol Meth 7:91–103

Sakai K, Akima M, Tsuyama K (1983) Evaluation of the isolated perfused heart of mice, with special reference to vasoconstriction caused by intracoronary acetylcholine. J Pharmacol Meth 10:263–270

Sheikh F, Sontag DP, Fandrich RR, Kardami E, Cattini PA (2001) Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts. Am J Physiol, Heart Circ Physiol 280:H1030–H1050

Sumaray MS, Yellon DM (1998a) Characterization and validation of a murine model of global ischaemia-reperfusion injury. Mol Cell Biochem 186:61–68

Sumaray MS, Yellon DM (1998b) Ischemic preconditioning reduces infarct size following global ischemia in the murine myocardium. Bas Res Cardiol 93:384–390

Sutherland FJ, Shattock MJ, Baker KE, Hearse DJ (2003) Mouse isolated perfused heart: characteristics and cautions. Clin Exp Pharmacol Physiol 30 :867–898

Takeo S, Tanonaka K, Liu JX, Ohtsuka Y (1992) Protective effects of antiarrhythmic agents on oxygen-deficiency-induced contractile dysfunction of isolated perfused hearts. In: Yasuda H, Kawaguchi H (eds) New Aspects in the Treatment of Failing Heart. Springer, Tokyo, Berlin, Heidelberg, pp 13–219

Tejero-Taldo MI, Gursoy E, Zhao TC, Kukreja RC (2002) α-Adrenergic receptor stimulation produces late preconditioning through inducible nitric oxide synthase in mouse heart. J Mol Cell Cardiol 34:185–195

Wang QD, Swärdh A, Sjöquist PO (2001) Relationship between ischemic time and ischemia/reperfusion injury in isolated Langendorff-perfused mouse hearts. Act Physiol Scand 171:123–128

Xiang JZ, Linz W, Becker H, Ganten D, Lang RE, Schölkens B, Unger Th (1985) Effects of converting enzyme inhibitors: Ramipril and enalapril on peptide action and neurotransmission in the isolated heart. Eur J Pharmacol 113:215–223

Zander B, Euler H (1976) Concentration measurements of physically dissolved oxygen by the classical van Slyke principle. In: Deng H, Balslen J, Brook R (eds) Measurement of oxygen. Elsevier Scientific Publ. C., Amsterdam, pp 271–276

Zegner M, Podesser B, Koci G, Weisser J, Hallström S, Schima H, Wollenek GH (1996) Evaluation of the in-

fluences of ramiprilat on the reperfusion. – Studied on the isolated working heart model. Acta Chir Austriatica 28:343–346

A.3.1.3 Coronary Artery Ligation in Isolated Working Rat Heart

PURPOSE AND RATIONALE

In working heart preparations of rats, ischemia can be induced by clamping the left coronary artery close to its origin. After removal of the clip, changes in the reperfusion period can be observed. Prevention of these symptoms can be an indicator of the efficacy of coronary drugs.

PROCEDURE

The preparation used is a modification of an isolated working heart preparation originally used for guinea pig hearts (Bardenheuer and Schrader 1983). Wistar rats of either sex weighing 280–300 g are sacrificed by decapitation. The hearts are removed and dissected free from the epicard and surrounding connective tissue. A cannula is introduced into the aorta from where the coronary vessels are perfused with the non-recirculated perfusion medium according to the Langendorff technique. In the left ventricle a balloon closely fitting the ventricular cavity is placed and connected to an artificial systemic circulation. The fluid in the balloon is pumped through a cannula into the closed extra corporal circulation being forced into one direction by 2 recoil valves. The balloon is made of silicone material using a Teflon form (Linz et al. 1986). The dimensions of the form are derived from casts of the left ventricle of K^+-arrested heart by injection of dental cement (Palavit 55, Kulzer and Co, GmbH, Germany). During each heart beat the fluid volume pressed from the balloon, corresponding to the stroke volume of the heart, can be recorded by means of a flow meter probe and an integrator connected in series. Preload and afterload can be adjusted independently from each other. The perfusate flow (retrograde into the aorta and through the coronary arteries) is recorded separately.

The following parameters were measured in isolated rat hearts (Linz et al. 1986):

LVP (left ventricular pressure) with Statham pressure transducer P 23 DB, which on differentiation yielded LV dp/dt_{max} and HR (heart rate). Cardiac output and coronary flow (CF) are determined by electromagnetic flow probes in the outflow system and in the aortic cannula, respectively. Coronary venous pO_2 is measured with a catheter placed in the pulmonary artery by a type E 5046 electrode connected to a PMH 73 pH/blood gas monitor (Radiometer). An epicardial electrocardiogram recording is obtained via two silver electrodes attached to the heart. All parameters are recorded on a Brush 2600 recorder.

Myocardial oxygen consumption (MWO_2) [ml/min/g wet weight] is calculated according to the equation:

$$MVO_2 = CF \times (P_a - P_v) \times (c/760) \times 100$$

where CF is the coronary flow [ml/min/g], P_a is the oxygen partial pressure of arterial perfusate (650 mm Hg), P_v is the oxygen partial pressure of the venous effluent perfusate [mm Hg], and c is the 0.0227 ml O_2/ml perfusate representing the Bunsen solubility coefficient of oxygen dissolved in perfusate at 37°C (Zander and Euler 1976).

Coronary Artery Ligation

For coronary artery occlusion experiment (Scholz et al. 1992, 1993), the isolated working hearts are perfused for a period of 20 min (pre-ischemic period) with modified Krebs-Henseleit buffer at a constant pressure of 65 mm Hg. Thereafter, acute myocardial ischemia is produced by clamping the left coronary artery close to is origin for 15 min (ischemic period). The clip is then reopened, and changes during reperfusion are monitored for 30 min (reperfusion period). After coronary artery ligation and reperfusion the hearts develop ventricular fibrillation.

From the coronary effluent samples are taken for lactate, lactate dehydrogenase (LDH), and creatine kinase (CK) determinations. After the experiment, glycogen, lactate, ATP, and creatine phosphate in myocardial tissue are measured.

The test drugs are given into the perfusion medium either before occlusion or 5 min before reperfusion. For *ex vivo* studies, the rats are treated orally with the test drug 1 h before sacrifice and preparation of the isolated working heart.

EVALUATION

The incidence and duration of ventricular fibrillation after treatment with coronary drugs is compared with controls. Left ventricular pressure, LV dP/dt max, and coronary flow are reduced after coronary constriction by angiotensin II, whereas enzyme activities in the effluent are increased and the myocardial content of glycogen, ATP and creatine phosphate are decreased. Cardiac protective drugs have the opposite effects. The values of each parameter are statistically compared with controls.

MODIFICATIONS OF THE METHOD

Vogel and Lucchesi (1980) described an isolated, blood perfused, **feline** heart preparation for evaluating pharmacological interventions during myocardial ischemia. Ventricular function was measured with a fluid-filled latex balloon within the left ventricle.

Vleeming et al. (1989) ligated the left coronary artery in rats after thoracotomy in ether anesthesia. Forty-eight hours after the operation, the hearts were prepared for retrograde constant pressure perfusion, according to the Langendorff technique.

Igic (1996) presented a new method for the isolated working rat heart. A special double cannula was designed consisting of an outer cannula that is inserted in the aorta and an inner cannula that is advanced into the left ventricle. The perfusion fluid flows through the inner cannula into the left ventricle, and is ejected from there into the aorta. If the outer cannula system is closed, the fluid perfuses the coronary vessels and drips off outside the heart. When the outer cannula is open and certain pressure resistance is applied, a fraction of the ejected fluid perfuses the coronary vessels and the rest is expelled. Because the inner cannula can easily be retracted into the outer cannula, which is placed in the aorta, this preparation provides an opportunity to use the same heart as a "working" or "non-working" model for investigating functions of the heart.

Pepe and McLennan (1993) described a maintained afterload model of ischemia in erythrocyte-perfused isolated working hearts of rats.

Further characterization of the pathophysiological reactions or the isolated working heart was performed by Linz et al. (1999). The external heart power (EHP) [mJ/min/g] was calculated using the formula:

$$\begin{aligned} EHP_{\mathrm{LV}} &= \text{pressure} - \text{volume} + \text{acceleration work} \\ &= [SV(MAP - LAP)] \\ &\quad + [1/2SV \times d \times (SV/\pi r^2 e^2)]HR\, g_{\mathrm{LVwwt}}^{-1} \end{aligned}$$

SV indicates stroke volume; *MAP*, mean aortic pressure; *LAP*, mean left arterial pressure; *d*, specific weight perfusate (1.004 g/cm^3); *r*, inner radius of aortic cannula; *e*, ejection time; *HR*, heart rate; LV, left ventricle; LVwwt, left ventricular wet weight.

The function of the left ventricle was altered by changing the aortic pressure (afterload) at constant left atrial filling load (preload). By adjusting the Starling resistance, the aortic outflow could be switched during 1 min from the fixed baseline afterload to a preset higher afterload producing step-wise rises in mean arterial pressure.

Lee et al. (1988) studied the effects of acute global ischemia on cytosolic calcium transients in perfused isolated **rabbit** hearts with the fluorescent calcium indicator indo 1. Indo 1-loaded hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm from the epicardial surface of the left ventricle. The F_{400}/F_{550} ratio was calculated by an analog circuit, which allowed cancellation of optical motion artifact. The resulting calcium transients were registered simultaneously with the ventricular pressure and demonstrated a rapid upstroke and slow decay similar to those recorded in isolated ventricular myocytes. Global ischemia rapidly suppressed contraction, but it produced a concurrent increase in the systolic and diastolic levels of calcium transients, together with an increase in the duration of the peak.

REFERENCES AND FURTHER READING

Grupp IL, Grupp G (1984) Isolated heart preparations perfused or superfused with balanced salt solutions. In: Schwartz A (ed) Methods in Pharmacology, Vol 5: Myocardial Biology. pp 111–128. Plenum Press, New York and London

Igic R (1996) The isolated perfused "working" rat heart: a new method. J Pharmacol Toxicol Meth 35:63–67

Kannengieser GJ, Lubbe WF, Opie LH (1975) Experimental myocardial infarction with left ventricular failure in the isolated perfused rat heart. Effects of isoproterenol and pacing. J Mol Cell Cardiol 7:135–151

Linz W, Schölkens BA, Han YF (1986) Beneficial effects of the converting enzyme inhibitor, ramipril, in ischemic rat hearts. J Cardiovasc Pharmacol 8 (Suppl 10):S91–S99

Linz W, Schölkens BA, Manwen J, Wilhelm M, Ganten D (1986) The heart as a target for converting enzyme inhibitors: Studies in ischaemic isolated working hearts. J Hypertension 4, Suppl 6:S477–S479

Linz W, Schölkens BA, Kaiser J, Just M, Bei-Yin Q, Albus U, Petry P (1989) Cardiac arrhythmias are ameliorated by local inhibition of angiotensin formation and bradykinin degradation with the converting-enzyme inhibitor ramipril. Cardiovasc Drugs Ther 3:873–882

Linz W, Wohlfart P, Schoelkens BA, Becker RHA, Malinski T, Wiemer G (1999) Late treatment with ramipril increases survival in old spontaneously hypertensive rats. Hypertension 34:291–295

Martorana PA, Linz W, Göbel H, Petry P, Schölkens BA (1987) Effects of nicainoprol on reperfusion arrhythmia in the isolated working rat heart and on ischemia and reperfusion arrhythmia and myocardial infarct size in the anesthetized rat. Eur J Pharmacol 143:391–401

Pepe S, McLennan PL (1993) A maintained afterload model of ischemia in erythrocyte-perfused isolated working hearts. J Pharm Toxicol Meth 29:203–210

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydro-xy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43:313–319

Schölkens BA, Linz W, Lindpaintner K, Ganten D (1987) Angiotensin deteriorates but bradykinin improves cardiac function following ischaemia in isolated rat hearts. J Hypertens 5: Suppl 5:S7–S9

Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Schölkens BA (1992) Effects of Na^+/H^+ exchange inhibitors in cardiac ischaemia. J Mol Cell Cardiol 24:731–740
Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Schölkens BA (1993) Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. Br J Pharmacol 109:562–568
van Gilst WH, de Graeff PA, Wesseling H, de Langen CDJ (1986) Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: A comparison of captopril, enalapril, and HOE 498. J Cardiovasc Pharmacol 8:722–728
Vleeming W, van der Wouw PA, van Rooij HH, Wemer J, Porsius AJ (1989) *In vitro* method for measurement of cardiac performance and responses to inotropic drugs after experimentally induced myocardial infarction in the rat. J Pharmacol Meth 21:95–102
Vogel WM, Lucchesi BR (1980) An isolated, blood perfused, feline heart preparation for evaluating pharmacological interventions during myocardial ischemia. J Pharmacol Meth 4:291–303

A.3.1.4 Isolated Working Heart Model in Infarcted Rat Heart

PURPOSE AND RATIONALE

The model of chronic heart failure in spontaneously hypertensive rats described by Itter et al. (2004a) (see A.6.0.1.2) has been used by the same group to study the isolated working heart in rats after chronic infarction (Itter et al. 2004b).

PROCEDURE

Animals and Methods

WKY/NHsd and SHR/NHsd rats at an age of 4 months were randomized into two groups – sham and myocardial infarction (MI). The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the left ventricle (LV). Chronic heart failure (CHF) was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function and geometric properties were determined by the "working heart" mode and *in vivo* determinations by MRI and heart weight.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg i.p.). The left ventrolateral thorax was shaved and prepared to create a disinfected surgical access area. When stable anesthesia was achieved the animals were placed on a small animal operation table, intubated and ventilated with room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstedten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. Reflexes were evaluated before surgery. The operation took 5 min. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Nordersredt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point the lungs were hyperinflated by increasing the positive end-expiratory pressure, and the chest was closed. The rats were placed on a heating pad and covered with a layer of unbleached tissue paper. The rats were extubated following return of reflexes. They were continuously monitored until they started moving in their cages. To avoid ventricular arrhythmias, lidocaine (2 mg/kg i.m.) was given before surgery. To prevent acute lung edema, the rats received furosemide (Lasix, 2 mg/kg body weight twice daily for 3 days) via the drinking water. To avoid pain and distress the rats received metamizol treatment (Novalgin, 0.1 mg/kg body weight i.m.) once, directly after the recovery period.

Before killing the animals 8 weeks after MI, non-invasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter the animals were anesthetized with pentobarbitone (180 mg/kg i.p. Pentobarbital) and subsequently heparinized (Heparin Natrium 500 I.U./100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE (polyethylene) tube inserted into the trachea and ventilated with room air. A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. The heart was immersed in physiological buffer chilled to 4°C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95% O2/5% CO_2) non-circulating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4$, 1.64; $NaHCO_3$,

24.88; KH_2PO_4, 1.18; glucose, 5.55; and Na pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue, thymus or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous pO_2 measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria.

When a tight seal with no leaks had been established and after a 15-min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters the afterload pressure was enhanced in a cumulative manner from an additional 20 mmHg to 140 mmHg. Thereafter the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5 mmHg to 30 mmHg.

Flow and pressure signals for computation were obtained from the PLUGSYS-measuring system (Hugo Sachs Elektronik, March-Hugstedten, Germany). Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstedten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight, and the left and right ventricular weights were determined. The left ventricle was then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices. The areas of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the left ventricular mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well.

EVALUATION

The data are given as mean ± SEM. Statistics were performed using the SAS system statistics package (SAS Insititute, Cary, N.C., USA) with a sequential rejection *t*-test according to Holm (1979).

REFERENCES AND FURTHER READING

Holm S (1979) A simple sequential rejective multiple test procedure. Scand J Stat 6:65–70

Itter G, Jung W, Juretschke P, Schölkens BA, Linz W (2004a) A model of chronic heart failure in spontaneous hypertensive rats (SHR). Lab Anim 38:138–148

Itter G, Jung W, Schölkens BA, Linz W (2004b) The isolated working heart model in infarcted rat hearts. Lab Anim 39:178–193

A.3.1.5 Relaxation of Bovine Coronary Artery

PURPOSE AND RATIONALE

Eicosanoids can regulate the tonus of coronary arteries. Prostacyclin induces relaxation, whereas thromboxane A_2 causes contraction. Spiral strips from bovine coronary artery can be used for assaying relaxation activity of test compounds (Dusting et al. 1977)

PROCEDURE

Freshly slaughtered beef hearts are immersed in cold oxygenated Krebs solution and immediately transported in a thermos flask to the laboratory. The left descending coronary artery and several of its primary branches are cut into spiral strips (about 20 mm long and 2–3 mm wide). The specimens can be stored up to 48 h at 4°C. The artery strips are suspended in a 4 ml organ bath under an initial tension of 2 g and immersed in a Krebs' bicarbonate solution at 37°C being gassed with oxygen containing 5% CO_2 throughout the experiment. The Krebs solution contains a mixture of antagonists to inhibit any actions from endogenous acetylcholine, 5-hydroxytryptamine, histamine or catecholamines (hyoscine hydrobromide 10^{-7} g/ml, methysergide maleate 2×10^{-7} g/ml, mepyramine maleate 10^{-7} g/ml, propranolol hydrochloride 2×10^{-6} g/ml). The strips are superfused with a solution of the test compounds in concentrations of 0.01, 0.1, 1.0 µg/ml at a rate of 10–20 ml/min with oxygenated Krebs solution containing the mixture of antagonists. Isometric contractions are recorded with Grass force-displacement transducers (type FT 03 C) on a Grass polygraph. The strips are superfused with Krebs' solution 3 h prior to the experiment. Standard compounds are 100 ng/ml PGE_2 inducing contraction and 100 ng/ml PGI_2 inducing pronounced relaxation.

EVALUATION

The relaxation induced by the test compound is expressed as percentage of maximal response to 100 ng/ml PGI_2.

MODIFICATIONS OF THE METHOD

Campell and Paul (1993) measured the effects of diltiazem on isometric force generation, $[Ca^{2+}]_i$, and energy metabolism in the isolated **porcine** coronary artery.

Li et al. (1997) determined the ability of analogues of human α-calcitonin gene-related peptide to relax isolated porcine coronary arteries precontracted with 20 mM KCl.

REFERENCES AND FURTHER READING

Dusting GJ, Moncada S, Vane JR (1977) Prostacyclin (PGX) is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachidonic acid. Prostaglandins 13:3–15

Gilmore N, Vane JR, Wyllie JH (1968) Prostaglandins released by the spleen. Nature 218:1135–1140

Li J, Matsuura JE, Waugh DJJ, Adrian TE, Abel PW, Manning MC, Smith DD (1997) Structure – activity studies on position 14 of human α-calcitonin gene-related peptide. J Med Chem 40:3071–3076

A.3.2 In Vivo Methods

A.3.2.1 Isoproterenol Induced Myocardial Necrosis in Rats

PURPOSE AND RATIONALE

Cardiac necrosis can be produced by injection of natural and synthetic sympathomimetics in high doses. Infarct-like myocardial lesions in the rat by isoproterenol have been described by Rona et al. (1959). These lesions can be totally or partially prevented by several drugs such as sympatholytics or calcium-antagonists.

PROCEDURE

Groups of 10 male Wistar rats weighing 150–200 g are pretreated with the test drug or the standard either s.c. or orally for 1 week. Then, they receive 5.25 and 8.5 mg/kg isoproterenol s.c. on two consecutive days. Symptoms and mortality in each group are recorded and compared with those of rats given isoproterenol alone. Forty-eight hours after the first isoproterenol administration, the rats are sacrificed and autopsied. The hearts are removed and weighed, and frontal sections are embedded for histological examination.

EVALUATION

Microscopic examination allows the following grading:

Grade 0: no change

Grade 1: focal interstitial response

Grade 2: focal lesions in many sections, consisting of mottled staining and fragmentation of muscle fibres

Grade 3: confluent retrogressive lesions with hyaline necrosis and fragmentation of muscle fibres and sequestrating mucoid edema

Grade 4: massive infarct with occasionally acute aneurysm and mural thrombi

For each group the main grade is calculated with the standard deviation to reveal significant differences.

CRITICAL ASSESSMENT OF THE METHOD

The test has been used by many authors for evaluation of coronary active drugs, such as calcium-antagonists and other cardioprotective drugs like nitroglycerin and molsidomine (Vértesy et al. 1991; Classen et al. 1993).

MODIFICATIONS OF THE METHOD

Yang et al. (1996) reported a protective effect of human adrenomedullin$^{13–52}$, a C-terminal fragment of adrenomedullin$^{10–52}$ on the myocardial injury produced by subcutaneous injection of isoproterenol into rats.

REFERENCES AND FURTHER READING

Bhargava AS, Preus M, Khater AR, Günzel P (1990) Effect of iloprost on serum creatine kinase and lactate dehydrogenase isoenzymes after isoprenaline-induced cardiac damage in rats. Arzneim Forsch/Drug Res 40:248–252

Brodowicz GR, Lamb DR (1991) Exercise training, indomethacin, and isoproterenol-induced myocardial necrosis in rats. Bas Res Cardiol 86:40–48

Campell JD, Paul RJ (1993) Effects of diltiazem on force, $[Ca^{2+}]_i$, and energy metabolism in porcine coronary artery. J Cardiovasc Pharmacol 22:408–415

Ciplea AG, Kretschmar R, Heimann W, Kirchengast M, Safer A (1988) Protective effect of the new calcium antagonist Anipamil against isoprenaline-induced cardionecrosis in rats. Arzneim-Forsch/Drug Res 38:215–221

Classen L, Michalsky G, Kammermeier H (1993) Catecholamine-induced cardiac necroses: Protective effect of leucocytopenia, influence of an S2 antagonist, thromboxan-synthetase inhibitor and prostacyclin analogue. Bas Res Cardiol 88:52–59

Ferrans VJ, Hibbs RG, Black WC, Weilbaecher DG (1964) Isoproterenol-induced myocardial necrosis. A histochemical and electron microscopic study. Am Heart J 68:71–90

Genovese A, Chiariello M, de Alfieri W, Latte S, Ferro G, Condorelli M (1982) Quantitative assessment of infarct size in isoproterenol-infarcted rats. Jpn Heart J 23:997–1006

Handforth CP (1962) Isoproterenol-induced myocardial infarction in animals. Arch Path 73:161–165

Joseph X, Bloom S, Pledger G, Balazs T (1983) Determinants of resistance to the cardiotoxicity of isoproterenol in rats. Toxicol Appl Pharmacol 69:199–205

Knufman NMJ, van der Laarse A, Vliegen HW, Brinkman CJJ (1987) Quantification of myocardial necrosis and cardiac hypertrophy in isoproterenol-treated rats. Res Commun Chem Pathol Pharmacol 57:15–32

Meijer AEFH, Hettwer H, Ciplea AG (1988) An enzyme histochemical study of isoproterenol-induced myocardial necroses in rats. Histochem J 20:697–707

Preus M, Bhargava AS, Khater AER, Günzel P (1988) Diagnostic value of serum creatine kinase and lactate dehydrogenase isoenzyme determinations for monitoring early cardiac damage in rats. Toxicol Letters 42:225–253

Rona G (1967) Experimental drug-induced myocardial infarction for animal pharmacologic screening. In: Siegeler PE, Moyer JH (eds) Animal and clinical pharmacologic techniques in drug evaluation. Vol II, pp 464–470, Year Book Medical Publ. Chicago

Rona G, Chappel CI, Balazs T, Gaudry R (1959) An infarct-like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. Arch Path 76:443–455

Rona G, Chappel CI, Kahn DS (1963) The significance of factors modifying the development of isoproterenol-induced myocardial necrosis. Am Heart J 66:389–395

Vértesi C, Knopf E, Gaál, Körmöczy PS (1991) Comparison of the cardioprotective effects of nitroglycerin, molsidomine, and SIN-1 in rats. J Cardiovasc Pharmacol 17 (Suppl 3) S141–S144

Wexler BC (1985) Prolonged protective effects following propranolol withdrawal against isoproterenol-induced myocardial infarction in normotensive and hypertensive rats. Br J exp Path 66:143–154

Yang J, Zhao D, Chang YZ, Tin Q, Zhao YT, Shi XY, Zhang ZK, Tang CS (1996) Protective effect of adrenomedullin$^{10–52}$ on isoproterenol-induced myocardial injury in rats. Chin Pharmacol Bull 12:530–533

A.3.2.2 Myocardial Infarction After Coronary Ligation in Rodents

PURPOSE AND RATIONALE

Ligation of the left coronary artery in rats as described by Selye (1960) induces an acute reduction in pump function and a dilatation of left ventricular chamber. The method has been used to evaluate beneficial effects of drugs after acute (Chiariello et al. 1980; Flaim and Zelis 1981; Bernauer 1985) or chronic (Innes and Weisman 1981; Pfeffer et al. 1985; Linz et al. 1996) treatment.

PROCEDURE

Male Sprague Dawley rats weighing 200–300 g are anesthetized with diethylether. The chest is opened by a left thoracotomy, and a thread is inserted near the middle of the lateral margin of the cutaneous wound and carried through a tunnel of the left pectoral muscle around the cranial half of the incision. The heart is gently exteriorized by pressure on the abdomen. A ligature is placed around the left coronary artery, near its origin, and is tightened. Within seconds, the heart is repositioned in the thoracic cavity, and the ends of the musculocutaneous thread are tightened to close the chest wall and enable the animal to breathe spontaneously. The speed of the procedure renders mechanical respiration unnecessary.

To evaluate drug effects, the rats are treated 5 min after and 24 h after occlusion by subcutaneous injection (standard 5 mg/kg propranolol).

Two days after surgery, the rats are anesthetized with 60 mg/kg i.p. pentobarbital and the right carotid artery is cannulated with a polyethylene catheter connected to a pressure transducer. The fluid-filled catheter is then advanced into the left ventricle through the aortic valve for measurement of left ventricular systolic and end-diastolic pressure.

After hemodynamic measurements, the heart is arrested by injecting 2 ml of 2.5 M potassium chloride. The chest is opened, and the hearts are isolated and rinsed with 300 mM KCl to maintain a complete diastole. A double-lumen catheter is advanced into the left ventricle through the ascending aorta, the right and left atria are tied off with a ligature, and the right ventricle is opened. The left ventricular chamber is filled with a cryostatic freeze medium through the smaller of the two catheter lumens and connected to a hydrostatic pressure reservoir maintained at a level corresponding to the end-diastolic pressure measured *in vivo*. The outlet (larger lumen) is then raised to the same level as the inlet to allow fluid in the two lumens to equilibrate. The heart is rapidly frozen with hexane and dry-ice.

The hearts are serially cut with a cryostat into 40-µm-thick transverse sections perpendicularly to the longitudinal axis from apex to base. At a fixed distance, eight sections are obtained from each heart and collected on gelatin-coated glass slides. Sections are air-dried and incubated at 25°C for 30 min with 490 µM nitroblue tetrazolium and 50 mM succinic acid in 0.2 M phosphate buffer (pH 7.6), rinsed in cold distilled water, dehydrated in 95% ethyl alcohol, cleared in xylene, and mounted with a synthetic resin medium. Viable tissue appears dark blue, contrasting with the unstained necrotic tissue.

EVALUATION

The infarct size can be determined by planimetry and expressed as percentage of left ventricular area, and thickness can be expressed as percentage of non-infarcted ventricular wall thickness (MacLean et al. 1978; Chiariello 1980; Roberts et al. 1983). An automatic method for morphometric analysis with image acquisition and computer processing was described by Porzio et al. (1995).

CRITICAL ASSESSMENT OF THE METHOD

Myocardial infarction following coronary artery ligation in Sprague-Dawley rats is a widely used rat model of heart failure. If the left coronary artery is not completely ligated, heart failure may occur as a consequence of chronic myocardial ischemia (Kajstura et al. 1994).

MODIFICATIONS OF THE METHOD

Johns and Olson (1954) described the coronary artery patterns for mouse, rat, hamster and guinea pig.

Kaufman et al. (1959), Fishbein et al. (1978, 1980) used various histochemical methods for identification and quantification of border zones during the evolution of myocardial infarction.

Sakai et al. (1981) described an **experimental model of angina pectoris in the intact anesthetized rat**. In anesthetized rats the tip of a special carotid cannula was placed closely to the right and left coronary ostium. Single intra-aortic injections of methacholine or acetylcholine (in the presence of physostigmine) developed a reproducible elevation of the ST segment and the T wave of the electrocardiogram. Coronary drugs were tested to prevent these changes.

Ytrehus et al. (1994) analyzed the effects of anesthesia, perfusate, risk zone, and method of infarct sizing in rat and rabbit heart infarction.

Leprán et al. (1981) placed a loose ligature of atraumatic silk around the left anterior descending coronary artery under ether anesthesia in **rats**. Ten days later, acute myocardial infarction was produced by tightening the ligature.

Kouchi et al. (2000) found an increase in $G_{i\alpha}$ protein accompanying progression of post-infarction remodelling in hypertensive cardiomyopathy in **rats.** G protein α subunits were studied with immunoblotting techniques (Böhm et al. 1990). The polyclonal antiserum MB1 was raised in rabbits against the carboxyl-terminal decapeptide of retinal transduction (KENLKDCGLF) coupled to keyhole limpet hemocyanine. The MB1 recognized $G_{i\alpha 1}$ and $G_{i\alpha 2}$ but not $G_{0\alpha}$ and $G_{i\alpha 3}$ (Böhm et al. 1994). The membrane fractions were electrophoresed in SDS-polyacrylamide gels and were transferred to nitrocellulose filters. The filters were incubated with the first antibodies for $G_{i\alpha}$ (MB1) or $G_{s\alpha}$ (RM/1) and then with the second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Amersham). Immunoreactive signals were detected by means of the ECL kit (Amersham).

Liu et al. (1997) found that ligation of the left descending coronary artery in Lewis inbred **rats** produces an uniformly large infarct with low mortality. The model may be superior to the usual model in Sprague-Dawley rats with a marked variability in infarct size and cardiac dysfunction.

Coronary artery ligation induces left ventricular remodeling with cardiomyocyte apoptosis, myocardial fibrosis indicated by morphological studies and by collagen accumulation, which can be prevented by drug treatment (Yang et al. 1992; Belichard et al. 1994; Nguyen et al. 1998; Sia et al. 2002; Bäcklund et al. 2004).

The naturally occurring peptide ***N*-acety-seryl-aspartyl-lysyl-proline (Ac-SDKP)** is a inhibitor of pluripotent hematopoietic stem cell proliferation and is normally present in human plasma and circulating mononuclear cells. It is cleaved to an inactive form by the NH_2-terminal catalytic domain of ACE (Azizi et al. 1996). Acute angiotensin converting enzyme inhibition increases the plasma level of *N*-acetyl-seryl-aspartyl-lysyl-proline (Azizi et al. 1996). By morphological studies and collagen determinations, Rasoul et al. (2004) found an antifibrotic effect of Ac-SDKP and angiotensin converting enzyme inhibition in hypertension in rats. Similarly, Yang et al. (2004) found that Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction.

Moreover, Azizi et al. (1997) and Le Meur et al. (1998) discussed whether the plasma Ac-SDKP level is a reliable marker of chronic angiotensin converting enzyme inhibition in hypertensive patients. An AcSDKP EIA Kit is available from Cayman, Ann Arbor, Mich., USA.

Chen et al. (2004) found inhibition and reversal of myocardial -infarction-induced hypertrophy and heart failure by NHE-1 inhibition.

Johns and Olson (1954) described a method of experimental **myocardial infarction by coronary occlusion in small animals**, such as **mouse**, **hamster**, **rat** and **guinea pig**.

Scholz et al. (1995) described a dose-dependent reduction of myocardial infarct size in **rabbits** by a selective sodium-hydrogen exchange subtype 1 inhibitor.

Gomoll and Lekich (1990) tested the **ferret** for a myocardial ischemia/salvage model. Varying combinations of duration of left anterior descending coronary occlusion and reperfusion were evaluated.

Coronary artery ligation in mice

Michael et al. (1995, 1999), and Gould et al. (2001) described the surgical procedure to induce myocardial ischemia in **mice** by ligation of the left anterior descending branch of the left coronary artery.

Infarct and Reperfusion Model

Male C57BL/6 mice 12–16 weeks of age (22.5–30.5 g body weight) were used. Anesthesia was produced by an intraperitoneal injection of pentobarbital sodium (4 mg/ml; 10 µl/g body weight). Mice were placed in a supine position with paws taped to the operating table. With direct visualization of the trachea, an endotracheal tube was inserted and connected to a Harvard rodent volume-cycled ventilator cycling at 100/min with volume sufficient to adequately expand the lungs but not overexpand. The inflow valve was supplied with 100% oxygen.

For studies of the myocardial response to permanent occlusion, ligation of the anterior descending branch of the left coronary artery was achieved by tying an 8–0 silk suture around the artery. The suture was passed under the artery at a position ~ 1 mm from the tip of the normally positioned left auricle.

For studies of the effect of reperfusion after coronary artery occlusion, the ligature was tied at the same location on the coronary artery used for the permanent occlusion. However, to allow subsequent reestablishment of blood flow, occlusion was produced by placing a 1-mm length of polyethylene (PE) tubing (OD = 0.61 mm) on the artery and fixing it in place with the ligature. The artery was then compressed by tightening the ligature, producing myocardial blanching and electrocardiographic (ECG) S-T segment elevation as observed in permanent ligations. After occlusion for the desired time, blood flow was restored by removing the ligature and PE tubing. The chest wall was then closed by a 6–0 Ticron suture with one layer through the chest wall and muscle and a second layer through the skin and subcutaneous layer.

After surgical closing of the chest, the endotracheal tube was removed, warmth was provided by a heat lamp, and 100% oxygen was provided via a nasal cone. The animal was given 0.1 mg/kg butorphanol tartrate as an analgesic, and it became sternally recumbent within 1 h. After surviving the experimental infarct the mice recovered, and this allowed postoperative physiological measurement. Sham-operated mice underwent an identical procedure with placement of the ligature but did not undergo coronary artery occlusion.

MODIFICATIONS OF THE METHOD

Guo et al. (1998) demonstrated the effects of an early and a late phase of ischemic preconditioning in mice. The results demonstrated that, in the mouse, a robust infarct-sparing effect occurred during both the early and the late phases of ischemic preconditioning, although the early phase was more powerful.

Guo et al. (2005) found that late preconditioning induced by NO donors, adenosine A_1 receptor agonists, and δ_1-opioid receptor agonists is mediated by inducible NO synthase.

Lutgens et al. (1999) reported cardiac structural and functional changes after chronic myocardial infarction in the mouse.

Scherrer-Crosbie et al. (1999) described echocardiographic determination of risk area in a murine model of myocardial ischemia. Myocardial contrast echocardiography was performed before and after coronary artery ligation in anesthetized mice by intravenous injection of contrast microbubbles and transthoracic echo imaging. Time-video intensity curves were obtained for the anterior, lateral, and septal myocardial walls. After myocardial ischemia, myocardial contrast echocardiography defects were compared with the area of no perfusion measured by Evans blue staining.

Jones and Lefer (2001) described cardioprotective actions of acute HMG-CoA reductase inhibition in the setting of myocardial infarction.

Janssens et al. (2004) reported that cardiomyocyte-specific overexpression of NO synthase 3 (NOS3) improves left ventricular (LV) performance and reduces compensatory hypertrophy after myocardial infarction. The effect of cardiomyocyte-restricted overexpression of one NO synthase isoform, NOS3, on LV remodeling after myocardial infarction in mice was tested. LV structure and function before and after permanent left anterior descending (LAD) coronary artery ligation were compared in transgenic mice with cardiomyocyte-restricted NOS3 overexpression (NOS3-TG) and their wild-type littermates (WT). Before myocardial infarction, systemic hemodynamic measurements, echocardiographic assessment of LV fractional shortening (FS), heart weight, and myocyte width (as assessed histologically) did not differ in NOS3-TG and WT mice. The inotropic response to graded doses of isoproterenol was significantly reduced in NOS3-TG mice. One week after LAD ligation, the infarcted fraction of the LV did not differ in WT and NOS3-TG mice. Four weeks after myocardial infarction, however, end-systolic LV internal diameter (LVID) was greater, and FS and maximum and minimum rates of LV pressure development were less in WT than in NOS3-TG mice. LV weight/body weight ratio was greater in WT than in NOS3-TG mice.

LaPointe et al. (2004) found that inhibition of cyclooxygenase-2 (COX-2) improves cardiac function after myocardial infarction in the mouse. Myocardial infarction was produced by ligation of the LAD coro-

nary artery in mice. Two days later, mice were treated with a selective COX-2 inhibitor, or vehicle in drinking water for 2 weeks. After the treatment period, mice were subjected to two-dimensional M-mode echocardiography to determine cardiac function. Hearts were then analyzed for determination of infarct size, interstitial collagen content, brain natriuretic peptide (BNP) mRNA, myocyte cross-sectional area, and immunohistochemical staining for transforming growth factor (TGF)β and COX-2.

Shibuya et al. (2005) reported that *N*-acetyl-seryl-aspartyl-lysine-proline prevents renal insufficiency and matrix expansion in diabetic d*b*/d*b* **mice**.

Weinberg et al. (2005) found in coronary ligation experiments in mice, that rosuvastatin reduces experimental left ventricular infarct size after ischemia-reperfusion injury but not total coronary occlusion.

Yang et al. (2005) found that the infarct-sparing effect of A_{2A}-adenosine receptor activation is due primarily to its action on lymphocytes. Chimeric mice were created by bone marrow transplantation from A_{2A}AR-knockout or green fluorescent protein (GFP) donor mice to irradiated congenic C57BL/6 (B6) recipients. In the GFP chimeras, we were unable to detect GFP-producing cells in the vascular endothelium, indicating that bone-marrow-derived cells were not recruited to endothelium at appreciable levels after bone marrow transplantation and/or acute myocardial infarction. Injection of 5 or 10 µg/kg of a potent and selective agonist of A_{2A} adenosine receptor had no effect on hemodynamic parameters but reduced infarct size in B6 mice after 45 min of LAD artery occlusion followed by 24 h of reperfusion.

Kanno et al. (2003) found **connexin**43 to be a determinant of myocardial infarct size following coronary occlusion in mice.

Regulation of myocardial connexins during hypertrophic remodeling was reviewed by Teunissen et al. (2004).

Kuhlmann et al. (2006) reported that granulocyte colony stimulating factor (G-CSF), alone or in combination with stem cell factor (SCF), can improve hemodynamic cardiac function after myocardial infarction in mice and reduces inducible arrhythmias in the infarcted heart potentially via increased connexin43 expression and arteriogenesis.

REFERENCES AND FURTHER READING

Azizi M, Rousseau A, Ezan E, Guyene TT, Michelet S, Grognet JM, Lefant M, Corvol P, Ménard J (1996) Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator *N*-acetyl-seryl-aspartyl-lysyl-proline. J Clin Invest 97:839–844

Azizi M, Rousseau A, Ezan E, Guyene TT, Michelet S, Grognet JM, Lenfant M, Corvol P, Menard J (1997) Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator *N*-acetyl-seryl-aspartyl-lysyl-proline. J Clin Invest 97.839–844

Azizi M, Ezan E, Nicolet L, Grognet JM, Ménard J (1997) High plasma level of *N*-acetyl-seryl-aspartyl-lysyl-proline. A new marker of chronic angiotensin-converting enzyme inhibition. Hypertension 30:1015–1019

Bäcklund T, Palojoki E, Saraste A, Eriksson A, Finkenberg P, Kytö V, Lakkisto P, Mervaala E, Voipio-Pulkki LM, Laine M, Tikkanen L (2004) Sustained cardiomyocyte apoptosis and left ventricular remodelling after myocardial infarction in experimental diabetes. Diabetologia 47:325–330

Belichard P, Savard P, Cardinal R, Naddeau R, Gosselin H, Paradis P, Rouleau JL (1994) Markedly different effects on ventricular remodelling result in a decrease in inducibility of ventricular arrhythmias. J Am Coll Cardiol 23:505–513

Bernauer W (1985) The effect of β-adrenoreceptor blocking agents on evolving myocardial necrosis in coronary ligated rats with and without reperfusion. Naunyn-Schmiedeberg's Arch Pharmacol 328:288–294

Böhm M, Gierschik P, Jakobs KH, Pieske B, Schnabel P, Ungerer M, Erdmann E (1990) Increase of $G_{i\alpha}$ in human hearts with dilated but not ischemic cardiomyopathy. Circulation 82:1249–1265

Böhm M, Eschenhagen T, Gierschik P, Larisch K, Lensche H, Mende U, Schmitz W, Schnabel P, Scholz H, Steinfath M, Erdmann H (1994) Radioimmunochemical quantification of $G_{i\alpha}$ in right and left ventricles from patients with ischemic and dilated cardiomyopathy and predominant left ventricular failure. J Mol Cell Cardiol 26:133–149

Chen L, Chen CX, Gan XT, Beier N, Scholz W, Karmazyn M (2004) Inhibition and reversal of myocardial infarction-induced hypertrophy and heart failure by NHE-1 inhibition. Am J Physiol 286:H381–H387

Chiariello M, Brevetti G, DeRosa G, Acunzo F, Petillo F, Rengo F, Condorelli M (1980) Protective effects of simultaneous alpha and beta adrenergic receptor blockade on myocardial cell necrosis after coronary arterial occlusion in rats. Am J Cardiol 46:249–254

Colatsky Th (1989) Models of myocardial ischemia and reperfusion injury: Role of inflammatory mediators in determining infarct size. Pharmacological Methods in the Control of Inflammation, pp 283–320, Alan R. Liss, Inc.

Fishbein MC, MacLean D, Maroko PR (1978) Experimental myocardial infarction in the rat. Qualitative and quantitative changes during pathologic evolution. Am J Pathol 90:57–70

Fishbein MC, Hare AC, Gissen SA, Spadaro J, MacLean D, Maroko PR (1980) Identification and quantification of histochemical border zones during the evolution of myocardial infarction in the rat. Cardiovasc Res 14:41–49

Flaim SF, Zelis R (1981) Diltiazem pretreatment reduces experimental myocardial infarct size in rat. Pharmacology 23:281–286

Gomoll AW, Lekich RF (1990) Use of the ferret for a myocardial ischemia/salvage model. J Pharmacol Meth 23:213–223

Gould KE, Taffet GE, Michael LH, Christie RM, Konkol DL, Pocius JS, Zachariah JP, Chaupin DF, Daniel SL, Sandusky GE, Jr, Hartley CJ, Entman ML (2001) Heart failure and greater infarct expansion in middle-aged mice: a relevant model for postinfarction failure. Am J Physiol 282:H615–H621

Guo Y, Wu WJ, Qiu Y, Tang XL, Yang Z, Bolli R (1998) Demonstration of an early and a late phase of ischemic preconditioning in mice. Am J Physiol 275:H1375–H1387

Guo Y, Stein AB, Wu WJ, Zhu X, Tan W, Li Q, Bolli R (2005) Late preconditioning induced by NO donors, adenosine A_1 receptor agonists, and δ_1-opioid receptor agonists is mediated by iNOS. Am J Physiol 289:H2251–H2257

Innes IR, Weisman H (1981) Reduction in the severity of myocardial infarction by sulfinpyrazone. Am Heart J 102:153–157

Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szelid Z, Gillijns H, Van de Werf F, Collen D, Bloch KD (2004) Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. Circ Res 94:1256–1262

Johns TNP, Olson BJ (1954) Experimental myocardial infarction. I. A method of by coronary occlusion in small animals. Ann Surg 140:675–682

Jones SP, Lefer DJ (2001) Cardioprotective actions of acute HMG-CoA reductase inhibition in the setting of myocardial infarction. Acta Physiol Scand 173:139–143

Kajstura J, Zhang X, Reiss K, Szoke E, Li P, Lagastra C, Cheng W, Darzynkiewicz Z, Olivetti G, Anversa P (1994) Myocyte cellular hyperplasia and myocyte cellular hypertrophy contribute to chronic ventricular remodeling in coronary artery narrowing-induced cardiomyopathy in rats. Circ Res 74:382–400

Kanno S, Kovacs A, Yamada KA, Saffitz JE (2003) Connexin43 as a determinant of myocardial infarct size following coronary occlusion in mice. J Am Coll Cardiol 41:681–686

Kaufman N, Gavan TL, Hill RW (1959) Experimental myocardial infarction in the rat. Arch Pathol 57:482–488

Kouchi I, Zolk O, Jockenhövel F, Itter G, Linz W, Cremers B, Böhm M (2000) Increase in $G_{i\alpha}$ protein accompanies progression of post-infarction remodelling in hypertensive cardiomyopathy. Hypertension 36:42–47

Kuhlmann MT, Kirchhof P, Klocke R, Hasib L, Stypmann J, Fabritz L, Stelljes M, Tian W, Zwiener M, Mueller M, Kienast J, Breithardt G, Nikol S (2006) G-CSF/SCF reduces inducible arrhythmias in the infracted heart potentially via increased connexin43 expression and arteriogenesis. J ExpMed 203:87–97

LaPointe MC, Mendez M, Leung A, Tao Z, Yang XP (2004) Inhibition of cyclooxygenase-2 improves cardiac function after myocardial infarction in the mouse. Am J Physiol 286:H1416–H1424

Le Meur Y, Aldigier JC, Praloran V (1998) Is plasma Ac-SDKP level is a reliable marker of chronic angiotensin-converting enzyme inhibition in hypertensive patients? Hypertension 31:1201–1202

Leprán I, Koltai M, Szekeres L (1981) Effect of non-steroid anti-inflammatory drugs in experimental myocardial infarction in rats. Eur J Pharmacol 69:235–238

Linz W, Wiemer G, Schmidts HL, Ulmer W, Ruppert D, Schölkens BA (1996) ACE inhibition decreases postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction. Clin Exper Hypertension 18:691–712

Liu YH, Yang XP, Nass O, Sabbah HN, Peterson E, Carretero OA (1997) Chronic heart failure induced by coronary artery ligation in Lewis inbred rats. Am J Physiol 272 (2 Pt2):H722–H727

Lutgens E, Daemen MJ, de Muinck ED, Debets J, Leenders P, Smits JF (1999) Chronic myocardial infarction in the mouse: cardiac structural and functional changes. Cardiovasc Res 41:586–593

MacLean D, Fishbein MC, Braunwald E, Maroko PR (1978) Long-term preservation of ischemic myocardium after experimental coronary artery occlusion. J Clin Invest 61:541–551

Michael LH, Entman ML, Hartley CJ, Youker KA, Zhu J, Hall SR, Hawkins HK, Berens K Balantyne CM (1995) Myocardial ischemia and reperfusion: a murine model. Am J Physiol 269 (Heart Circ Physiol 38): H2147–H2154

Michael LH, Ballantyne CM, Zachariah JP, Gould KE, Pocius JS, Taffet GE, Hartley CJ, Pham TT, Daniel SL, Funk E, Entman ML (1999) Myocardial infarction and remodeling in mice: effect of reperfusion. Am J Physiol 277:H660–H668

Nguyen T, Salibi EE, Rouleau JL (1998) Postinfarction survival and inducibility of ventricular arrhythmias in the spontaneously hypertensive rat. Effects of ramipril and hydralazine. Circulation 98:2074–2080

Pfeffer JM, Pfeffer MA, Braunwald E (1985) Influence of chronic captopril therapy on the infarcted left ventricle of the rat. Circ Res 57:84–95

Porzio S, Masseroli M, Messori A, Forloni G, Olivetti G, Jeremic G, Riva E, Luvara G, Latini R (1995) A simple, automatic method for morphometric analysis of the left ventricle in rats with myocardial infarction. J Pharmacol Toxicol Meth 33:221–229

Rasoul S, Carretero OA, Peng H, Cavasin MA, Zhuo J, Sanchez-Mendoza A, Brigstock DR, Rhaleb NE (2004) Antifibrotic effect of Ac-SDKP and angiotensin converting enzyme inhibition in hypertension. J Hypertens 22:593–603

Roberts CS, MacLean D, Braunwald E, Maroko PR, Kloner RA (1983) Topographic changes in the left ventricle after experimentally induced myocardial infarction in the rat. Am J Cardiol 51:873–876

Sakai K, Akima M, Aono J (1981) Evaluation of drug effects in a new experimental model of angina pectoris in the intact anesthetized rat. J Pharmacol Meth 5:325–336

Scherrer-Crosbie M, Steudel W, Ullrich R, Hunziker PR, Liel-Cohen N, Newell J, Zaroff J, Zapol WM, Picard MH (1999) Echocardiographic determination of risk area in a murine model of myocardial ischemia. Am J Physiol 277:H986–H992

Scholz W, Albus U, Counillon L, Gögelein H, Lang HJ, Linz W, Weichert A, Schölkens BA (1995) Protective effects of HOE 642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischemia and reperfusion. Cardiovasc Res 29:260–268

Selye H, Bajusz E, Grasso S, Mendell P (1960) Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11:398–407

Shibuya K, Kanasaki K, Isono M, Sato H, Omata M, Sugimoto T, Araki SI, Isshiki K, Kashiwagi A, Haneda M, Koya D (2005) *N*-Acetyl-seryl-aspartyl-lysine-proline prevents renal insufficiency and matrix expansion in diabetic d*b*/d*b* mice. Diabetes 54:838–835

Sia YT, Lapointe N, Parker TG, Tsoporis JN, Deschepper CP, Calderose A, Pourdjabbar A, Jasmin JF, Sarrazin JF, Liu P, Asam A, Butany J, Rouleau JL (2002) Beneficial effects of long-term use of the antioxidant probucol in heart failure in the rat. Circulation 105:2549–2555

Teunissen BEJ, Jongsma HJ, Bierhuizen MFA (2004) Regulation of myocardial connexins during hypertrophic remodelling. Eur Heart J 25:1979–1989

Walker MJA, MacLeod BA, Curtis MJ (1991) Myocardial ischemia and infarction. Comp Pathol Bull 23:3–4

Weinberg EO, Scherrer-Crosbie M, Picard MH, Nasseri BA, MacGillivray C, Gannon J, Lian Q, Bloch KD, Lee RT (2005) Rosuvastatin reduces experimental left ventricular infarct size after ischemia-reperfusion injury but not total coronary occlusion. Am J Physiol 288:H1802–H1809

Yang XP, Sabbah HN, Liu YH, Sharov VG, Mascha EJ, Alwan I, Carretero OA (1992) Ventriculographic evaluation in three rat models of cardiac dysfunction, Am J Physiol 265(6 Pt 2):H1946–1952

Yang F, Yang XP, Liu YH, Xu J, Cingolani O, Rhaleb NE, Carretero OA (2004) Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction. Hypertension 43:229–236

Yang Z, Day YJ, Toufektsian MC, Ramos SI, Marshall M, Wang XQ, French BA, Linden J (2005) Infarct-sparing effect of A_{2A}-adenosine receptor activation is due primarily to its action on lymphocytes. Circulation 111:2190–2197

Ytrehus K, Liu Y, Tsuchida A, Miura T, Liu GS, Yang XM, Herbert D, Cohen MV, Downey JM (1994) Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing. Am J Physiol 267 (Heart Circ Physiol 36):H2383–H2390

A.3.2.3 Occlusion of Coronary Artery in Anesthetized Dogs and Pigs

PURPOSE AND RATIONALE

The size of infarcts is studied after proximal occlusion of the left anterior descending coronary artery in open-chest dogs. Compounds potentially reducing infarct-size are tested. To delineate the post-mortem area at risk, coronary arteriograms are made after injection of a $BaSO_4$-gelatin mass into the left coronary ostium. The infarct's area is visualized with nitro-blue tetrazolium chloride in myocardial sections.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are used. The animals are anesthetized by intravenous injection of pentobarbital sodium (bolus of 35 mg/kg followed by continuous infusion of 4 mg/kg/h). The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator. Arterial blood gases are checked, and the ventilation rate and/or oxygen flow rate are adjusted to achieve physiological blood gas values (P_{O2}: 100–140 mm Hg, P_{CO2}: 32–40 mm Hg, and pH 7.47). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously from lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral vein is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured from a high-sensitivity scale. From the pressure curve, d*p*/d*t* max is differentiated and heart rate is counted.

Experimental Procedure

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is exposed. After reaching steady state conditions for the hemodynamic parameters (approx. 45 min), the LAD is ligated just below the first diagonal branch for 360 min. No attempt is made to suppress arrhythmic activity after the ligation.

The test substance or the vehicle (controls) is administered by intravenous bolus injection and/or continuous infusion. The schedule of administration may vary. Hemodynamic parameters are registered continuously during the whole experiment. At the end of the experiment, the animals are sacrificed with an overdose of pentobarbital sodium and the heart is dissected.

Preparation to Determine Area at Risk

Coronary arteriograms are made according to Schaper et al. (1979) to delineate the anatomic post-mortem area at risk. A purse-string suture is placed around the left coronary ostium in the sinus of Valsalva; a cannula is then placed in the ostium and the purse-string suture is tightened. Micronized $BaSO_4$ suspended in 12% gelatin solution (37°C) is injected under increasing pressure (2 min at 100 mm Hg, 2 min at 150 mm Hg and 2 min at 200 mm Hg). The heart is placed in crushed ice to gel the injectate. The right ventricle is removed and the left ventricle plus septum is cut into transverse sections (approx. 1 cm thick) from the apex to the level of the occlusion (near the base). From each slice angiograms are made with a X-ray tube at 40 kV to assess the post-mortem area at risk (by defect opacity: reduction of $BaSO_4$-filled vessels in infarct tissue).

Preparation to Determine Infarct Size

The slices are then incubated in p-nitro-blue tetrazolium solution (0.25 g/L in Sörensen phosphate buffer, pH 7.4, containing 100 mM D,L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of the infarct area.

Left ventricle and infarct area, and area at risk are measured by planimetry from projections of all slices with the exclusion of the apex and of the slice containing the ligature.

EVALUATION

Mortality and the different hemodynamic parameters are determined. Changes of parameters in drug-treated animals are compared to vehicle controls. The different characteristics are evaluated separately. Mean values ± SEM of infarct area and of area at risk are calculated. Statistical analyses consist of regression and correlation analyses and of the Student's *t*-test. Results are considered significant at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Nachlas and Shnitka (1963) described the macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity in dogs by staining the cardiac tissue with Nitro-BT [2,2′-di-p-nitrophenyl-5,5′ diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride] yielding a dark blue formazan in viable muscle but not in necrotic muscle fibers.

Chiariello et al. (1976) compared the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction in dogs.

Black et al. (1995) studied the cardioprotective effects of heparin or *N*-acetylheparin in an *in vivo* dog model of myocardial and ischemic reperfusion injury. The hearts were removed after 90 min of coronary occlusion and a 6 h-reperfusion period. Area at risk was determined by the absence of Evans blue dye after perfusion of the aorta in a retrograde fashion and infarct zone by the absence of formazan pigment within the area at risk after perfusion of the circumflex coronary artery with triphenyltetrazolium chloride.

Reimer et al. (1985) tested the effect of drugs to protect ischemic myocardium in unconscious and conscious dogs. In the conscious model, dogs of either sex weighing 10–25 kg were anesthetized with thiamyal sodium (30–40 mg/kg i.v.) and underwent thoracotomy through the 4th intercostal space. Heparin-filled polyvinyl chloride catheters were positioned in the aortic root, the left atrium via the left atrial appendage, and a systemic vein. A mechanical adjustable snare type occluder was placed around the proximal left circumflex coronary artery above or below the first marginal branch, so that temporary occlusion resulted in cyanosis of at least 75% of the inferior wall. The catheters and snare were either exteriorized or positioned in a subcutaneous pocket at the back of the neck. Penicillin, 1000,000 units, and streptomycin, 1.0 g, were given i.m. for the first 4 postoperative days, and at least 7 days were allowed for recovery from surgery.

Dogs were fasted overnight prior to the study. After exteriorization and flushing of the catheters, 30–40 min were allowed for the animals to adjust to laboratory conditions. Morphine sulfate, 0.25 mg/kg, i.m., was given 30 min before occlusion, and an additional 0.25 mg/kg, i.v., was given 20 min later. Heart rate and aortic and left atrial pressures were monitored continuously. Permanent coronary occlusion was produced by a sudden one-stage tightening of the snare occluder. Drugs were administered by continuous i.v. infusion over 6 h. Hemodynamic measurements were taken 5 min before occlusion and 10, 25, 105, 180, and 360 min after occlusion.

Raberger et al. (1986) described a model of **transient myocardial dysfunction in conscious dogs**. Mongrel dogs, trained to run on a treadmill, were chronically instrumented with a miniature pressure transducer in the left ventricle and a hydraulic occluder placed around the circumflex branch of the left coronary artery. Two pairs of piecoelectrical crystals for sonomicrometry were implanted subendocardially to measure regional myocardial functions. Comparable episodes of regional dysfunction of the left coronary artery area during treadmill runs were found after partial left coronary artery stenosis induced by external filling of the occluder.

Hartman and Warltier (1990) described a model of **multivessel coronary artery disease** using conscious, chronically instrumented dogs. A hydraulic occluder was implanted around the left anterior descending coronary artery (LAD) and an Ameroid constrictor around the left circumflex coronary artery (LCCA). Pairs of piecoelectric crystals were implanted within the subendocardium of the LAD and LCCA perfusion territories to measure regional contractile function. A catheter was placed in the left atrial appendage for injection of radioactive microspheres to measure regional myocardial perfusion. Bolus injections of adenosine were administered daily via the left atrium to evaluate LAD and LCCA coronary reserve. After stenosis by the Ameroid constrictor, radioactive microspheres were administered to compare regional perfusion within normal myocardium to flow in myocardium supplied by the occluded or stenotic coronary arteries.

Holmborn et al. (1993) compared triphenyltetrazolium chloride staining versus detection of fi-

bronectin in experimental myocardial infarction in **pigs**.

Klein et al. (1995) used intact **pigs** and found myocardial protection by Na^+/H^+ exchange inhibition in ischemic reperfused hearts.

Klein et al. (1997) measured the time delay of cell death by Na^+/H^+ exchange inhibition in regionally ischemic, reperfused **porcine** hearts.

Garcia-Dorado et al. (1997) determined the effect of Na^+/H^+ exchange blockade in ischemic rigor contracture and reperfusion-induced hypercontracture in *pigs* submitted to 55 min of coronary occlusion and 5 h reperfusion. Myocardial segment length analysis with ultrasonic microcristals was used to detect ischemic rigor (reduction in passive segment length change) and hypercontracture (reduction in end-diastolic length).

Symons et al. (1998) tested the attenuation of regional dysfunction in response to 25 cycles of ischemia (2 min) and reperfusion (8 min) of the left circumflex coronary artery in **conscious swine** after administration of a Na^+/H^+ exchange inhibitor. The animals were instrumented to measure arterial blood pressure, regional myocardial blood flow (colored microspheres), systolic wall thickening in the normally perfused left anterior descending and left circumflex coronary artery regions (sonomicrometry), left circumflex coronary artery blood flow velocity (Doppler) and reversibility to occlude the left circumflex coronary artery (hydraulic occluder).

Etoh et al. (2001) studied myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in **pigs**.

REFERENCES AND FURTHER READING

Abendroth RR, Meesmann W, Stephan K, Schley G, Hübner H (1977) Effects of the β-blocking agent Atenolol on arrhythmias especially ventricular fibrillation and fibrillation threshold after acute experimental coronary artery occlusion. Z Kardiol 66:341–350

Black SC, Gralinski MR, Friedrichs GS, Kilgore KS, Drsicoll EM, Lucchesi BR (1995) Cardioprotective effects of heparin or *N*-acetylheparin in an *in vivo* model of myocardial and ischemic reperfusion injury. Cardiovasc Res 29:629–636

Chiariello M, Gold HL, Leinbach RC, Davis MA, Maroko PR (1976) Comparison between the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction. Circulation 54:766–773

Etoh T, Joffs C, Deschamps AM, Davis J, Dowdy K, Hendrick J, Baicu S, Mukherjee R, Manhaini M, Spinale FG (2001) Myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in pigs. Am J Physiol 281:H987–994

Garcia-Dorado D, Ganzález MA, Barrabés JA, Ruiz-Meana M, Solares J, Lidon RM, Blanco J, Puigfel Y, Piper HM, Soler-Soler J (1997) Prevention of ischemic rigor contracture during coronary occlusion by inhibition of Na^+-H^+ exchange. Cardiovasc Res 35:80–89

Hartman JC, Warltier DC (1990) A model of multivessel coronary artery disease using conscious, chronically instrumented dogs. J Pharmacol Meth 24:297–310

Holmborn B, Näslund U, Eriksson A, Virtanen I, Thornell LE (1993) Comparison of triphenyltetrazolium chloride (TTC) staining versus detection of fibronectin in experimental myocardial infarction. Histochemistry 99:265–275

Klein HH, Pich S, Bohle RM, Wollenweber J, Nebendahl K (1995) Myocardial protection by Na^+/H^+ exchange inhibition in ischemic, reperfused porcine hearts. Circulation 92:912–917

Klein HH, Bohle RM, Pich S, Lindert-Heimberg S, Wollenweber J, Nebendahl K (1997) Time delay of cell death by Na^+/H^+ exchange inhibition in regionally ischemic, reperfused porcine hearts. J Cardiovasc Pharmacol 30:235–240

Martorana PA, Göbel H, Kettenbach B, Nitz RE (1982) Comparison of various methods for assessing infarct-size in the dog. Basic Res Cardiol 77:301–308

Martorana PA, Kettenbach B, Breipohl G, Linz W (1990) Reduction of infarct size by local angiotensin-converting enzyme inhibition is abolished by a bradykinin antagonist. Eur J Pharmacol 182:395–396

Nachlas MN, Shnitka TK (1963) Macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity. Am J Pathol 42:379–396

Raberger G, Krumpl G, Mayer N (1986) A model of transient myocardial dysfunction in conscious dogs. J Pharmacol Meth 16:23–37

Reimer KA, Jennings RB, Cobb FR, Murdock RH, Greenfield JC, Becker LC, Bulkley BH, Hutchins GM, Schwartz RP, Bailey KR, Passamani ER (1985) Animal models for protecting ischemic myocardium: Results of the NHLBI cooperative study. Comparison of unconscious and conscious dog models. Circ Res 56:651–665

Schaper W, Frenzel H, Hort W (1979) Experimental coronary artery occlusion. I. Measurement of infarct size. Basic Res Cardiol. 74:46–53

Scherlag BJ, El-Sherif N, Hope R, Lazzara R (1974) Characterization and localization of ventricular arrhythmias resulting from myocardial ischemia and infarction. Circ Res 35:372–383

Symons JD, Correa SD, Schaefer S (1998) Na-H exchange inhibition with cariporide limits functional impairment due to repetitive ischemia. J Cardiovasc Pharmacol 32:

A.3.2.4 Acute Ischemia by Injection of Microspheres in Dogs

PURPOSE AND RATIONALE

Severe left ventricular failure is induced by repeated injections of 50 µm plastic microspheres into the left main coronary artery of anesthetized dogs. Hemodynamic measurements are performed under theses conditions testing drugs which potentially improve cardiac performance. The test can be used to evaluate the influence of drugs on myocardial performance during acute ischemic left ventricular failure in dogs.

PROCEDURE

Dogs of either sex weighing approximately 30 kg are anesthetized by an intravenous bolus injection of 35–40 mg/kg pentobarbital sodium continued by an infusion of 4 mg/kg/h. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator and controlled by measuring end-expiratory CO_2 concentration as well as blood gases. Two peripheral veins are cannulated for the administration of narcotic (brachial vein) and test compounds (saphenous vein). The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of the right femoral vein is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (Gould PC 350) is inserted via the left carotid artery. Left ventricular enddiastolic pressure (LVEDP) is measured on a high-sensitivity scale. From the pressure curve, dp/dt max is differentiated and heart rate (HR) is counted. To measure right ventricular pressure, a Millar microtip catheter is inserted via the right femoral vein. Systolic, diastolic and mean pulmonary artery pressure (PAP), mean pulmonary capillary pressure, and cardiac output are measured by a thermodilution technique using a Cardiac Index Computer (Gould SP 1435) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. To measure coronary blood flow, an electromagnetic flow probe (Hellige Recomed) is placed on the proximal part of the LCX.

Polystyrol microspheres (3M Company, St. Paul, Minnesota, USA) with a diameter of $52.5 \pm 2.24\,\mu m$ are diluted with dextran 70, 60 mg/ml and saline at a concentration of 1 mg microspheres/ml (1 mg = approx. 12,000 beads). For administration of microspheres, an angiogram catheter (Judkins-Schmidt Femoral-Torque, William Cook, Europe Aps. BP 7) is inserted into the left ostium via the left femoral artery.

Induction of Failure

The microspheres are injected through the angiogram catheter into the left ostium initially as 10 ml and later as 5 ml boluses about 5 min apart. The microsphere injections produce stepwise elevations of LVEDP. Embolization is terminated when LVEDP has increased to 16–18 mm Hg and/or PAPm has increased to 20 mm Hg and/or heart rate has reached 200 beats/min. The embolization is completed in about 70 min and by injection of an average dose of 3–5 mg/kg microspheres. Hemodynamic variables are allowed to stabilize after coronary embolization for at least 30 min.

Experimental Course

The test substance or the vehicle (controls) is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application.

Recordings are obtained

- before embolization
- after embolization
- before administration of test compound
- 5, 30, 45, 60, 90, 120 and, eventually, 150 and 180 min following administration of test drug. At the end of the experiment, the animal is sacrificed by an overdose of pentobarbital sodium.

EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formula:

stroke volume [ml/s],

$$SV = \frac{\text{cardiac output}}{\text{heart rate}}$$

tension index [mmHgs],

$$IT = \frac{BPs \times \text{heart rate}}{1000}$$

Coronary vascular resistance [mm Hgmin/ml],

$$CVR = \frac{BPm \times RAPm}{CBF}$$

total peripheral resistance [dyns/cm^5],

$$TPR = \frac{BPm \times RAPm}{\text{cardiac output}} \times 79.9$$

Pulmonary artery resistance [dyn s/cm^5],

$$PAR = \frac{PAPm - PCPm}{\text{cardiac output}} \times 79.9$$

right ventricle work [kgm/min],

$$RVW = (PAPm - RAPm) \times \text{cardiac output} \times 0.0136$$

left ventricle work [kgm/min],

$$LVW = (BPm - LVEDP) \times \text{cardiac output} \times 0.0136$$

left ventricular myocardial oxygen consumption [ml O_2/min/100 g],

$$MVO_2 = K_1(BPs \times HR) + K_2 \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} + 1.43$$

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25 \times 10^{-4}$
BPs = systolic blood pressure [mm Hg]
BPd = diastolic blood pressure [mm Hg]
BPm = mean blood pressure [mm Hg]
CBF = coronary blood flow in left circumflex coronary artery [ml/min]
RAPm = mean right atrial pressure [mm Hg]
PAPm = mean blood pressure A. pulmonalis [mm Hg]
PCPm = mean pulmonary capillary pressure
HR = heart rate [beats/min]
SV = stroke volume [ml]
BW = body weight [kg]

Changes of parameters in drug-treated animals are compared to vehicle controls; statistical significance of the differences is calculated with the Student's *t*-test.

Mean embolization times, doses of microspheres and number of microsphere applications are evaluated.

MODIFICATIONS OF THE METHOD

Gorodetskaya et al. (1990) described a simple method to produce acute heart failure by coronary vessel embolization with microspheres in rats.

REFERENCES AND FURTHER READING

Gorodetskaya EA, Dugin SF, Medvedev OS, Allabergenova AE (1990) A simple method to produce acute heart failure by coronary vessel embolization in closed chest rats with microspheres. J Pharmacol Meth 24:43–51

Rooke GA, Feigl EO (1982) Work as a correlate of canine left ventricular oxygen consumption, and the problem of catecholamine oxygen wasting. Circ Res 50:273–286

Schölkens BA, Martorana PA, Göbel H, Gehring D (1986) Cardiovascular effects of the converting enzyme inhibitor ramipril (Hoe 498) in anesthetized dogs with acute ischemic left ventricular failure. Clin and Exp Theory and Practice A8(6):1033–1048

Smiseth OA (1983) Effects of the β-adrenergic receptor agonist pirbuterol on cardiac performance during acute ischaemic left ventricular failure in dogs. Eur J Pharmacol 87:379–386

Smiseth OA, Mjøs OD (1982) A reproducible and stable model of acute ischemic left ventricular failure in dogs. Clin Physiol 2:225–239

A.3.2.5 Influence on Myocardial Preconditioning

PURPOSE AND RATIONALE

Damage to the mammalian heart produced by prolonged ischemia and reperfusion can be reduced by "preconditioning" the myocardium via a brief cycle of ischemia and reperfusion prior to the protracted ischemic event. Ischemic preconditioning has been shown to decrease infarct size and increase recovery of post-ischemic ventricular function (Murray et al. 1986), and to reduce leakage of cellular marker proteins indicative for cardiac myocyte death (Volovsek et al. 1992). In addition, preliminary preconditioning also attenuates cardiac arrhythmia associated with subsequent occlusion and reperfusion (Vegh et al. 1990).

The mechanistic basis of this phenomenon is under discussion (Parratt 1994; Parratt and Vegh 1994). Adenosine receptor involvement in myocardial protection after ischemic preconditioning in rabbits has been shown by Baxter et al. (1994). Adenosine (A_1 receptor) antagonists have been demonstrated to block the protection produced by preconditioning (Liu et al. 1991), and short term administration of adenosine was shown to simulate the protective effects of ischemic preconditioning (Toombs et al. 1993). These observations together suggest that adenosine is generated by the short preconditioning ischemia. Other recent pharmacological studies (Gross and Auchampach 1992; Yao and Gross 1994) indicate the involvement of the ATP-sensitive potassium channel. Recent investigations indicate that an increase of NO production after ACE inhibitors may be a part of the protective mechanism (Linz et al. 1992, 1994). Moreover, the involvement of prostanoids and bradykinin in the preconditioning process has been discussed (Wiemer et al. 1991). Gho et al. (1994) found a limitation of myocardial infarct size in the rat by transient renal ischemia, supporting the hypothesis that the mechanism leading to cardiac protection by ischemic preconditioning may not only reside in the heart itself.

PROCEDURE

New Zealand rabbits of either sex weighing 2.5–3.5 kg are initially anesthetized with an intramuscular injection of ketamine (50 mg/ml) /xylazine (10 mg/ml) solution at a dose of 0.6 ml per kg body weight. A tracheotomy is performed to facilitate artificial respiration. The left external jugular vein is cannulated to permit a constant infusion (0.15–0.25 ml/min) of xylazine (2 mg/ml in heparinized saline) to assist in

maintaining anesthesia and fluid volume. Anesthesia is also maintained by i.m. injections (0.4–0.6 ml) of ketamine (80 mg/ml) and xylazine (5 mg/ml) solution. After the xylazine infusion is started, animals are respired with room air at a tidal volume of 10 ml/kg and a frequency of 30 inflations per min (Harvard Apparatus, USA). Thereafter, ventilation is adjusted or inspiratory room air is supplemented (5% CO_2/95% O_2) to maintain arterial blood chemistry within the following ranges: pH 7.35–7.45, P_{CO2} 25–45 mm Hg, P_{O2} 90–135 mm Hg. The right femoral artery and vein are isolated and catheterized for measurement of arterial pressure and administration of drugs, respectively.

A thoracotomy is performed in the fourth intercostal space, and the lungs are retracted to expose the heart. The pericardium is cut to expose the left ventricle, and a solid-state pressure transducer catheter (e. g., MicroTip 3F, Millar Instruments, Houston, USA) is inserted through an apical incision and secured to enable measurement of pulsatile left ventricular pressure. The maximal rate of increase in left ventricular pressure (LVdP/dt max) is determined by electronic differentiation of the left ventricular pressure wave form. A segment of 4–0 prolene suture is looped loosely around a marginal branch of the left main coronary artery to facilitate coronary occlusion during the experiment. Needle electrodes are inserted subcutaneously in a lead II configuration to enable recording of an ECG in order to determine heart rate and help confirm the occurrence of ischemia (ST segment elevation) and reperfusion of the myocardium distal to the coronary occlusion. Continuous recording of pulsatile pressure, ECG, heart rate, and LVdP/dt are simultaneously displayed on a polygraph (e. g. Gould chart recorder, Gould Inc., Valley View, USA) and digitized in real time by a personal computer. Hemodynamic data are condensed for summary and later statistical analysis.

Ischemic preconditioning is induced by tightening the prolene loop around the coronary artery for 5 min and then loosening to reperfuse the affected myocardium for 10 min prior to a subsequent 30 min occlusion. After surgical preparation, and prior to 30 min of occlusion, rabbits are randomly selected to receive ischemic preconditioning, no preconditioning, or ischemic preconditioning plus treatment with test drugs. After 30 min of occlusion, the ligature is released and followed by 120 min of reperfusion. Occlusion is verified by epicardial cyanosis distal to the suture, which is usually accompanied by alterations in hemodynamics and ECG. Reperfusion is validated by return of original color. Systemic hemodynamics are summarized for each experimental period. The experiment is terminated after 120 min of reperfusion, and the heart is excised for determinations of infarct size and area at risk.

Immediately before the animal is sacrificed, the marginal branch of the left coronary artery is reoccluded and India ink is rapidly injected by syringe with a 18-g needle into the left ventricular chamber to demarcate blackened normal myocardium from unstained area at risk. After the rabbits are sacrificed, the heart is removed and sectioned in a breadloaf fashion from apex to base perpendicular to the long axis. The right ventricle is removed from each slice leaving only the left ventricle and septum. After each slice is weighed, the portions are washed and incubated in a phosphate buffered saline solution of triphenyl tetrazolium chloride (1 g/ml, Sigma) for 10–15 min. Salvaged myocardium in the area at risk stains brick red, whereas infarcted tissue remains unaltered in color. Slices are then placed between sheets of Plexiglas and the areas (normal, risk, infarct) of each slice are traced on a sheet of clear acetate. Traces are then digitized and analyzed using computerized planimetry to compare the relative composition of each slice with respect to normal tissue, area at risk, and infarcted myocardium. Planimetry is performed with a computerized analysis system, e. g., Quantimet 570C image analysis system (Leica, Deerfield, USA).

Surface areas of normal tissue, area at risk, and infarcted myocardium on both sides of each slide are averaged for the individual slide. The contribution of each slide to total infarcted and area at risk (%) and area at risk as a percentage of total left ventricular mass for the entire left ventricle is prorated by the weight of each slice (Garcia-Dorado et al. 1987). By adding the adjusted contributions from each slice to infarcted tissue, area at risk, and left ventricular mass, a three-dimensional mathematical representation of total myocardial infarct size and risk zone can be calculated for each rabbit, and a mean tabulated for each treatment group for statistical comparison.

EVALUATION

All data are presented as mean ± SD. Systemic hemodynamic data are analyzed by ANOVA using Statistica/W software. Means are considered significantly different at $p < 0.05$.

MODIFICATIONS OF THE METHOD

Li et al. (1990) found in dog experiments that preconditioning with one brief ischemic interval is as effective as preconditioning with multiple ischemic periods.

In contrast, Vegh et al. (1990) found in other dog experiments that two brief preconditioning periods of coronary occlusion, with an adequate period of reperfusion between, reduce the severity of arrhythmias.

Yang et al. (1996) found a second window of protection after ischemic preconditioning in conscious rabbits which minimizes both infarction and arrhythmias.

Late preconditioning against myocardial stunning in conscious pigs together with an increase of heat stress protein (HSP) 70 was described Sun et al. (1995).

Szilvassy et al. (1994) described the anti-ischemic effect induced by ventricular overdrive pacing as a conscious rabbit model of preconditioning. Rabbits were equipped with right ventricular electrode catheters for pacing and intracavital recording and polyethylene cannulae in the left ventricle and right carotid artery to measure intraventricular pressure and blood pressure. One week after surgery in conscious animals, ventricular overdrive pacing at 500 beats/min over 2, 5, or 10 min resulted in an intracavital S-T segment elevation, shortening of ventricular effective refractory period, decrease in maximum rate of pressure development and blood pressure, and increase in left ventricular end-diastolic pressure proportional to the duration of stimulus. A 5-min preconditioning ventricular overdrive pacing applied 5 or 30 min before a 10-min ventricular overdrive pacing markedly attenuated ischemic changes, whereas a 2-min ventricular overdrive pacing had no effect.

The ventricular overdrive pacing induced preconditioning effect was lost in atherosclerotic rabbits (Szilvassy et al. 1995), however, delayed cardiac protection could be induced in these animals (Szekeres et al. 1997).

REFERENCES AND FURTHER READING

Baxter GF, Marber MS, Patel VC, Yellon DM (1994) Adenosine receptor involvement in a delayed phase of myocardial protection 24 h after ischemic preconditioning. Circulation 90:2993–3000

Garcia-Dorado D, Théroux P, Elizaga J, Galiñanes M, Solares J, Riesgo M, Gomez MJ, Garcia-Dorado A, Aviles FF (1987) Myocardial reperfusion in the pig heart model: infarct size and duration of coronary occlusion. Cardiovasc Res 21:537–544

Gho BC, Schoemaker RG, van der Lee C, Sharma HS, Verdouw PC (1994) Myocardial infarct size limitation in rat by transient renal ischemia. Circulation 90: I-476/2557

Gross JG, Auchampach JA (1992) Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. Circ Res 70:223–233

Hoff PT, Tamura Y, Lucchesi BR (1990) Cardioprotective effects of amlodipine on ischemia and reperfusion in two experimental models. Am J Cardiol 66:10H–16H

Li GC, Vasquez JA, Gallagher KP, Lucchesi BR (1990) Myocardial protection with preconditioning. Circulat 82:609–619

Linz W, Wiemer G, Schölkens BA (1992) ACE-inhibition induces NO-formation in cultured bovine endothelial cells and protects isolated ischemic rat hearts. J Mol Cell Cardiol 24:909–919

Linz W, Wiemer G, Gohlke P, Unger T, Schölkens BA (1994) Kardioprotektive Effekte durch Ramipril nach Ischämie und Reperfusion in tierexperimentellen Studien. Z Kardiol 83: Suppl 4:53–56

Liu GS, Thornton J, Van Winkle DM, Stanley WH, Olsson RA, Downey JM (1991) Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. Circulation 84:350–356

Mickelson JK, Simpson PJ, Lucchesi BR (1989) Streptokinase improves reperfusion blood flow after coronary artery occlusion. Intern J Cardiol 23:373–384

Murry CE, Jennings RB, Reimer KA (1986) Preconditioning with ischaemia: a delay of lethal cell injury in ischaemic myocardium. Circulation 74:1124–1136

Parratt JR (1994) Protection of the heart by ischemic preconditioning: mechanisms and possibilities for pharmacological exploitation. TIPS 15:19–25

Parratt J, Vegh A (1994) Pronounced antiarrhythmic effects of ischemic preconditioning. Cardioscience 5:9–18

Simpson PJ, Fantone JC, Mickelson JK, Gallagher KP, Lucchesi BR (1988) Identification of a time window for therapy to reduce experimental canine myocardial injury: suppression of neutrophil activation during 72 h of reperfusion. Circ Res 63:1070–1079

Sun JZ, Tang XL, Knowton AA, Park SW, Qiu Y, Bolli R (1995) Late preconditioning against myocardial stunning. An endogenous protective mechanism that confers resistance to post-ischemic dysfunction 24 h after brief ischemia in conscious pigs. J Clin Invest 95:388–403

Szekeres L, Szilvássy Z, Fernandy P, Nagy I, Karcsu S, Scáti S (1997) Delayed cardiac protection against harmful consequences of stress can be induced in experimental atherosclerosis in rabbits. J Mol Cell Cardiol 29:1977–1983

Szilvássy Z, Fernandy P, Bor P, Jakab I, Lonovics J, Koltai M (1994) Ventricular overdrive pacing-induced anti-ischemic effect: a conscious rabbit model of preconditioning. Am J Physiol 266 (Heart Circ Physiol 35): H2033–H2041

Szilvássy Z, Fernandy P, Szilvássy J, Nagy I, Karcsu S, Lonovics J, Dux L, Koltai M (1995) The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. J Mol Cell Cardiol 27:2559–2569

Tamura Y, Chi L, Driscoll EM, Hoff PT, Freeman BA, Gallagher KP, Lucchesi BR (1988) Superoxide dismutase conjugated to polyethylene glycol provides sustained protection against myocardial ischemia/reperfusion injury in canine heart. Circ Res 63:944–959

Toombs CF, McGee DS, Johnston WE, Vinten-Johansen J (1993) Protection from ischaemic-reperfusion injury with adenosine pretreatment is reversed by inhibition of ATP sensitive potassium channels. Cardiovasc Res 27:623–629

van Gilst WH, de Graeff PA, Wesseling H, de Langen CDJ (1986) Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: A comparison of captopril, enalapril, and HOE 498. J Cardiovasc Pharmacol 8:722–728

Vegh A, Szekeres L, Parrat JR (1990) Protective effects of preconditioning of the ischaemic myocardium involve cyclooxygenase products. Cardiovasc Res 24:1020–1023

Volovsek A, Subramanian R, Reboussin D (1992) Effects of duration of ischaemia during preconditioning on mechanical function, enzyme release and energy production in the isolated working rat heart. J Mol Cell Cardiol 24:1011–1019

Wiemer G, Schölkens BA, Becker RHA, Busse R (1991) Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. Hypertension 18:585–563

Yang XM, Baxter GF, Heads RJ, Yellon DM, Downey JM, Cohen MV (1996) Infarct limitation of the second window of protection in a conscious rabbit model. Cardiovasc Res 31:777–783

Yao Z, Gross GJ (1994) A comparison of adenosine-induced cardioprotection and ischemic preconditioning in dogs. Efficacy, time course, and role of K_{ATP} channels. Circulation 89:1229–1236

A.3.2.6 MRI Studies of Cardiac Function

PURPOSE AND RATIONALE

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm-thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water and, because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as grey matter or lesions, have higher T_1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected (0.1–0.3 mmol kg^{-1}) and, in areas of increased blood–brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1-weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

MRI has emerged as a highly accurate and quantitative tool for the evaluation of cardiac function (Peshock et al. 1996).

Al-Shafei et al. (2000a, 2002b) performed MRI analysis of cardiac cycle events in diabetic rats and tested the effect of angiotensin-converting enzyme inhibition.

PROCEDURE

Diabetes was induced in Wistar rats at an age of 7, 10, and 13 weeks. The rats were anesthetized using 1%–2% halothane in oxygen, their blood glucose levels were checked. They were then given a single intraperitoneal injection of streptozotocin 65 mg/kg body weight. The control rats received sham injections of the citrated buffer when they were 7 weeks old. One diabetic group was treated with 2 g/l captopril in the drinking water

For **MR imaging**, rats were anesthetized using 1%–2% halothane in oxygen, weighed and their systolic blood pressures measured non-invasively using a rat tail blood pressure monitor both before and after imaging sessions to confirm physiological stability. Electrocardiographic (ECG) monitoring used shielded subcutaneous electrodes and a Tektronix 2225 oscilloscope. The cine imaging protocols were performed with the anesthetized animal placed in a specially designed home-built half-sine-spaced birdcage radiofrequency (RF) probe unit contained within a cylindrical plastic holder fitted within a gradient set of internal diameter 11 cm. The RF probe unit was made up of a half-sine spaced birdcage RF probe of internal diameter of 4.5 cm with open ends, an RF shield consisting of a cylinder of copper gauze surrounding and sliding over the birdcage, a tuning capacitor and a coaxial cable to carry the RF (Ballon et al. 1990). The assembly included ECG leads, attachment plugs for the ECG leads and a unit to anchor anesthetic delivery tubes near the nose of the animal. All experiments used a 2 T Oxford Instruments (UK) superconducting magnet with a horizontal internal bore of 31 cm. A gated cine protocol synchronized line acquisition to set times following alternate electrocardiographic R waves. This acquisition was then repeated at the same slice position at 12 equally incremented times through the cardiac cycle. This sequence in turn was repeated for each of the 128 lines to generate each 128×128 image, which itself was acquired twice for signal averaging. The preceding procedure was in turn repeated 12 times to obtain signal-averaged images for every one of the 12 contiguous transverse slices examined. Each imaging session therefore required ($128 \times 12 \times 2 \times 2$) times the

cardiac cycle duration. The effective repeat time (TR) was approximately 13 ms. The short echo time (TE) of 4.3 ms reduced motion artifacts and ensured good contrast between blood and myocardium.

EVALUATION

The image data were transferred from the MRI console using in-house hardware and software to remote UNIX workstations for quantitative analysis using in-house software based on CaMReS libraries (CaMReS, Dr N. J. Herrod, Herchel Smith Laboratory for Medicinal Chemistry, University of Cambridge).

MODIFICATIONS OF THE METHOD

Itter et al. (2004) used non-invasive MRI techniques in a model of chronic heart failure in spontaneously hypertensive rats.

Bryant et al. (1998) and Franco et al. (1999) described MRI and invasive evaluation of development of heart failure in transgenic **mice** with myocardial expression of tumor necrosis factor-α.

Wiesmann et al. (2002) reported analysis of right ventricular function in healthy mice and a murine model of heart failure by*in vivo* MRI.

Kraitchman et al. (2003) described quantitative ischemia detection during cardiac magnetic resonance stress testing by use of fast harmonic phase MTI (FastHARP) in dogs.

Reddy et al. (2004) discussed the feasibility of a porcine model of healed myocardial infarction by integration of cardiac MRI with three-dimensional electroanatomic mapping to guide left ventricular catheter manipulation.

Pelzer et al. (2005) reported that the estrogen receptor-α agonist 16α-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. Improved left ventricular function upon 16α-LE2 treatment was also observed in cardiac MRI studies.

REFERENCES AND FURTHER READING

Al-Shafei AIM, Wise RG, Gresham GA, Bronns G, Carpenter TA, Hall LD, Huang CHL (2002a) Non-invasive magnetic resonance assessment of myocardial changes and the effects of angiotensin-converting enzyme inhibition in diabetic rats. J Physiol (Lond) 538:541–553

Al-Shafei AIM, Wise RG, Gresham GA, Carpenter TA, Hall LD, Huang CHL (2002b) Magnetic resonance imaging analysis of cardiac cycle events in diabetic rats: the effect of angiotensin-converting enzyme inhibition. J Physiol (Lond) 538:555–572

Ballon D, Graham MC, Midownik S, Koutcher JA (1990) A 64 MHz half-birdcage resonator for clinical imaging. Journal of Magnetic Resonance 90:131–140

Bryant D, Becker L, Richardson J, Shelton J, Franco F, Peshock R, Thompson M, Giroir B (1998) Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-α. Circulation 97:1375–1381

Franco F, Thomas GD, Giroir B, Bryant D, Bullock MC, Chwialkowski MC, Victor RG, Peshock RM (1999) Magnetic resonance imaging and invasive evaluation of development of heart failure in transgenic mice with myocardial expression of tumor necrosis factor-α. Circulation 99:448–454

Itter G, Jung W, Juretschke P, Schölkens BA, Linz W (2004) A model of chronic heart failure in spontaneous hypertensive rats (SHR) Lab Anim 38:138–146

Kraitchman DL, Sampath S, Castillo E, Derbyshire JA, Boston RC, Bluemke DA, Gerber BL, Pronce JL, Osman NF (2003) Quantitative ischemia detection during cardiac magnetic resonance stress testing by use of FastHARP. Circulation 107:2025–2030

Pelzer T, Jazbutyte V, Hu K, Segerer S, Nahrendorf M, Nordbeck P, Bonz AW, Muck J, Fritzemeier KH, Hegele-Hartung C, Ertl G, Neyses L (2005) The estrogen receptor-α agonist 16α-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. Cardiovasc Res 67:604–612

Peshock RM, Willet DL, Sayad DE, Hundley WG, Chwialkowski MC, Clarke GD, Parkey RW (1996) Quantitative MR imaging of the heart. Magn Reson Imaging Clin N Am 4:267–305

Reddy VY, Malchano ZJ, Holmvang G, Schmidt EJ, d'Avila A, Houghtaling C, Chan RC (2004) Integration of cardiac magnetic resonance imaging with three-dimensional electroanatomic mapping to guide left ventricular catheter manipulation. Feasibility of a porcine model of healed myocardial infarction. J Am Coll Cardiol 44:2202–2213

Wiesmann F, Frydrychowicz A, Rautenberg J, Illinger R, Rommel E, Haase A, Neubauer S (2002) Analysis of right ventricular function in healthy mice and a murine model of heart failure by in vivo MRI. Am J Physiol 283:H1065–H1071

A.3.2.7 MRI Studies After Heart and Lung Transplantation

PURPOSE AND RATIONALE

Acute cardiac allograft rejection continues to be the cause of graft loss and contributes to the morbidity and mortality after cardiac transplantation. Endomyocardial biopsy is used routinely for cardiac transplant rejection surveillance. A sensitive and non-invasive method for detecting rejection is desirable. Kanno et al. (2001) developed a rat model of heterotopic heart and lung transplantation for MRI experiments. Allotransplantations were performed with syngeneic transplantations serving as controls. MR images were obtained with a gradient echo method.

PROCEDURE

Animals

All rats used in the experiments were male, 2–3 months of age, and weighed 220–250 g each. Ani-

mals were housed individually and provided with food and water ad libitum. Inbred Brown Norway (BN; $RT1^n$) and DA ($RT1^a$) rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind).

Heart and Lung Transplantation

Under anesthesia with injection of 35 mg/kg body weight of sodium pentobarbital IP, 500 U/kg body weight of heparin was injected. In the syngeneic group, an en bloc donor heart and lung were taken from a BN rat and transplanted to another BN rat. In the allogeneic group, a graft from a DA rat was transplanted to a BN rat. This group was divided into two groups: one group was treated with 3 mg/kg per day ciclosporin (CsA), and the other group was not given CsA. Graft survival was monitored every day by palpating contraction of the transplanted heart.

Operative procedures have been described by Kanno et al. (2000). In brief, after the chest wall of the donor rat was opened, the left lung was ligated and excised. The azygos vein with the left superior and right superior venae cavae was ligated and divided. The descending thoracic aorta was transected, and 10 ml of cold University of Wisconsin solution (UW solution, Dupont Pharma) was infused into the inferior vena cava until the fluid draining from the aorta was clear, followed by ligation and division of the inferior vena cava. The ascending aorta was dissected and transected at the portion between the left common carotid artery and the left subclavian artery, followed by ligation and division of the right brachiocephalic artery and the left common carotid artery. After removal of the heart and lung from the donor, the right lung was washed 3 times through the bronchus with UW solution containing penicillin G. The grafts were then placed into cold UW solution for ≈5 min until transplantation. Next, the left inguinal portion of the recipient rat was opened and dissected to make enough space for the transplanted organs. The left lower part of the abdominal wall was opened in a transverse fashion from the left femoral vessels to the midline. The abdominal organs were retracted to the right, and both the aorta and the inferior vena cava just beyond the bifurcation were dissected. The vessels were clamped, and an appropriate opening of the aorta was made to receive the aorta of the graft in an end-to-side fashion. Rhythmic heartbeats commenced spontaneously as the heart and the lung regained circulation after removal of the clamp. After hemostasis of the surgical field, the abdominal wall was sutured, with care taken not to kink or obstruct the aorta of the graft.

MRI EXPERIMENTS

MRI measurements were carried out on a 4.7-T/40-cm Bruker AVANCE DRX MR instrument equipped with 15-cm, 10-gauss/cm shielded gradients. *In vivo* MR images of transplanted heart-lung were obtained over a period of 24 h after infusion of dextran-coated ultrasmall superparamagnetic iron oxide (USPIO) particles. The imaging sequence consisted of a gradient echo sequence, triggered to ECG and ventilator (60 strokes/min, 10 ml/kg), with TR/TE 500/10 ms, flip angle equal to Ernst angle, slice thickness 1 mm, field of view 6.0 cm, data matrix size 256 × 130 (zero-filled to 256 × 256), and scan time 5 min. ECG leads were placed on both of the hind limbs of the rat with the transplant to pick up the heartbeat from the transplanted heart more effectively. The change of MRI signal intensity was measured in whole ventricular wall in each transplanted heart. The MR signal intensity of the heart was normalized to that of the leg muscle, because USPIO particles are not readily taken up by muscular tissue, according to Gellissen et al. (1999)

Dextran-coated USPIO particles were synthesized according to the method of Palmacci and Josephson (1993) with slight modifications (Dodd et al. 1999) The MR relaxivities R_1 (spin-lattice relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) and R_2 (spin-spin relaxation rate constant, $1/T_2$, per mol of Fe in USPIO) measured at 4.7 T were 3.8×10^4 and 9.1×10^4 (mol/l)/s, respectively. For *in vivo* studies, dextran-coated USPIO particles were dialyzed against PBS solution and diluted to a concentration of 18 μmol Fe/ml, and 0.8 ml of the suspension (i. e., ≈3 mg Fe/kg body weight) was injected intravenously for each study.

At 6 days after transplantation, dextran-coated USPIO particles were injected intravenously as mentioned above, and the animals were subjected to MRI. Then 24 h later, these animals were again placed inside the magnet and scanned. The regions of interest were defined manually with Bruker software. MR signal intensity in the entire ventricular wall in the plane was measured. After injection of USPIO particles at postoperative day (POD) 6, animals with allotransplants were given CsA for 4 (POD 7 to 10) or 7 (POD 7 to 13) days and reinjected with USPIO particles on POD 14.

Pathological Analysis and Immunohistochemistry

After an MR experiment was completed, the transplanted hearts were extirpated, fixed in 3.7% formaldehyde, and embedded in paraffin for 5-μm sections. Hematoxylin-eosin staining and Perl's Prussian blue staining were performed in the Transplantation Pathol-

ogy Laboratory of the University of Pittsburgh Medical Center. Histological analysis for pathological grading of heart rejection, which is based on the criteria established by the International Society for Heart and Lung Transplantation, was also performed by this laboratory in a blinded manner. Monoclonal anti-rat macrophage antibody (ED1, Serotec) was used as a primary antibody for macrophages. Immunohistochemistry was carried out with the ABC staining system (Santa Cruz Biotechnology) according to the manufacturer's protocol.

EVALUATION

The results are presented as mean ± SD. The results were analyzed by ANOVA with StatView software (SAS Institute). A value of $P < 0.05$ was considered to be statistically significant.

REFERENCES AND FURTHER READING

Dodd SJ, Williams M, Suhan JP et al (1999) Detection of single mammalian cells by high-resolution magnetic resonance imaging. Biophys J 76:103–109

Gellissen J, Axmann C, Prescher A et al (1999) Extra- and intracellular accumulation of ultrasmall superparamagnetic iron oxides (USPIO) in experimentally induced abscesses of the peripheral soft tissues and their effects on magnetic resonance imaging. Magn Reson Imaging 17:557–567

Kanno S, Lee PC, Dodd SJ et al (2000) A novel approach using magnetic resonance imaging for the detection of lung allograft rejection. J Thorac Cardiovasc Surg 120:923–934

Kanno S, Wu YJ, Lee PC, Dodd SJ, Williams M, Griffith HP, Ho C (2001) Macrophage accumulation associated with rat cardiac allograft rejection detected by magnetic resonance imaging with ultrasmall superparamagnetic iron oxide particles. Circulation 104:934–938

Palmacci S, Josephson L (1993) Synthesis of polysaccharide covered superparamagnetic oxide colloids. US Patent 5262176 Example 1. November 16, 1993

A.3.3 Ex Vivo Methods

A.3.3.1 Plastic Casts from Coronary Vasculature Bed

PURPOSE AND RATIONALE

Prolonged administration of coronary drugs has been shown to increase the number and size of interarterial collaterals of dogs and pigs after coronary occlusion (Vineberg et al. 1962; Meesmann and Bachmann 1966). An increased rate of development of collateral arteries was observed after physical exercise in dogs (Schaper et al. 1965), as well as after chronic administration of coronary dilating drugs (Lumb and Hardy 1963). An even more effective stimulus for collateral development is an acute or gradual occlusion of one or several major coronary branches. Filling the arterial coronary bed with a plastic provides the possibility to make the collaterals visible and to quantify them (Schmidt and Schmier 1966; Kadatz 1969).

PROCEDURE

Dogs weighing 10–15 kg are anesthetized with pentobarbital sodium 30 mg/kg i.v. They are respirated artificially and the thorax is opened. After opening of the pericard, Ameroid cuffs are placed around major coronary branches. Gradual swelling of the plastic material occludes the lumen within 3–4 weeks. The dogs are treated daily with the test drug or placebo. After 1 week recovery period they are submitted to exercise on a treadmill ergometer. After 6 weeks treatment, the animals are sacrificed, the heart removed and the coronary bed flushed with saline. The liquid plastic Araldite is used to fill the whole coronary tree from the bulbus aortae. The aortic valves are glued together in order to prevent filling of the left ventricle. Red colored Araldite is used to fill the arterial tree. The venous part of the coronary vasculature can be filled with blue colored Araldite from the venous sinus. The uniformity of the filling pressure, the filling time, and the viscosity of the material are important. Polymerization is complete after several hours. Then, the tissue is digested with 35% potassium hydroxide. The method gives stable preparations which can be preserved for a long time.

EVALUATION

Plastic casts from drug treated animals are compared with casts from dogs submitted to the same procedure without drug treatment.

CRITICAL ASSESSMENT OF THE METHOD

The procedure allows impressive demonstration of the formation of arterial collaterals. The results of post mortem Araldit impletion agree with the functional results of experimental coronary occlusion.

MODIFICATIONS OF THE METHOD

Boor and Reynolds (1977) described a simple planimetric method for determination of left ventricular mass and necrotic myocardial mass in postmortem hearts.

REFERENCES AND FURTHER READING

Boor PJ, Reynolds ES (1977) A simple planimetric method for determination of left ventricular mass and necrotic myocardial mass in postmortem hearts. Am J Clin Pathol 68:387–392

Kadatz R (1969) Sauerstoffdruck und Durchblutung im gesunden und koronarinsuffizienten Myocard des Hundes und ihre Beeinflussung durch koronarerweiternde Pharmaka. Arch Kreislaufforsch 58:263–293

Kadatz R (1971) Agents acting on coronary blood vessels. In Turner RA, Hebborn P (eds) Screening methods in pharmacology, Vol II. Academic Press, New York London, pp 41–60

Meesman W (1982) Early arrhythmias and primary ventricular fibrillation after acute myocardial ischemia in relation to pre-existing collaterals. In: Parratt JR (ed) Early Arrhythmias Resulting from Myocardial Ischemia. Mechanisms and Prevention by Drugs. McMillan London, pp 93–112

Meesmann W, Bachmann GW (1966) Pharmakodynamisch induzierte Entwicklung von Koronar-Kollateralen in Abhängigkeit von der Dosis. Arzneim Forsch 16:501–509

Meesmann W, Schulz FW, Schley G, Adolphsen P (1970) Überlebensquote nach akutem experimentellem Coronarverschluß in Abhängigkeit von Spontankollateralen des Herzens. Z ges exp Med 153:246–264

Schaper W, Xhonneux R, Jageneau AHM (1965) Stimulation of the coronary collateral circulation by Lidoflazine (R 7904) Naunyn-Schmiedeberg's Arch exp Path Pharmak 252:1–8

Schmidt HD, Schmier J (1966) Eine Methode zur Herstellung anatomischer Korrosionspräparate – dargestellt am Koronargefäßsystem des Hundes. Zschr Kreislaufforsch 55:297–305

Vineberg AM, Chari RS, Pifarré R, Mercier C (1962) The effect of Persantin on intercoronary collateral circulation and survival during gradual experimental coronary occlusion. Can Med Ass J 87:336–345

A.4 Calcium Uptake Inhibiting Activity

A.4.0.1 General Considerations

Cellular calcium flux is regulated by receptor-operated and voltage-dependent channels, which are sensitive to inhibition by calcium entry blockers. The term calcium antagonist was introduced by Fleckenstein (1964, 1967) when two drugs, prenylamine and verapamil, originally found as coronary dilators in the LANGENDORFF-experiment, were shown to mimic the cardiac effects of simple Ca^{2+}-withdrawal, diminishing Ca^{2+}-dependent high energy phosphate utilization, contractile force, and oxygen requirement of the beating heart without impairing the Na^+-dependent action potential parameters. These effects were clearly distinguishable from β-receptor blockade and could promptly be neutralized by elevated Ca^{2+}, β-adrenergic catecholamines, or cardiac glycosides, measures that restore the Ca^{2+} supply to the contractile system. In the following years many Ca^{2+}-antagonists were introduced to therapy. Specific Ca^{2+}-antagonists interfere with the uptake of Ca^{2+} into the myocardium and prevent myocardial necrotization arising from deleterious intracellular Ca^{2+} overload. They act basically as specific inhibitors of the slow transsarcolemnal Ca^{2+} influx but do not or only slightly affect the fast Na^+ current that initiates normal myocardial excitation.

Calcium channels and the sites of action of drugs modifying channel function have been classified (Bean 1989; Porzig 1990; Tsien and Tsien 1990; Spedding and Paoletti 1992).

Nomenclature and structure–function relationships of voltage-gated calcium channels were reviewed by Catterall et al. (2005).

Kochegarow (2003) reviewed the therapeutic application of modulators of voltage-gated calcium channels.

Four main types of voltage dependent calcium channels are described:

1. L type (for long lasting),
2. T type (for transient),
3. N type (for neuronal), and
4. P type (for Purkinje cells).

They differ not only by their function (Dolphin 1991) and localization in tissues and cells but also by their sensitivity to pharmacological agents (Ferrante and Triggle 1990; Dascal 1990; Kitamura et al. 1997) and by their specificity to radioligands.

The widely distributed **L-type channels** exist in isoforms (L1, 2, 3, 4) and consist of several subunits, known as α_1, α_2, β, γ, δ. They are sensitive to dihydropyridines, phenylalkylamines or benzothiazepines, but insensitive to ω-conotoxin and ω-agatoxin. The segments required for antagonist binding have been analyzed (Peterson et al. (1996, Schuster et al. 1996; Mitterdorfer et al. 1996; Hockerman et al. 1997; Striessnig et al. 1998; Catterall 1998).

Berjukow et al. (2000) discussed the molecular mechanism of calcium channel block by israpidine.

Striessnig et al. (2004) described the role of L-type Ca^{2+} channels in Ca^{2+} channelopathies.

The **T-type channels** are located mainly in the cardiac sinoatrial node and have different electrophysiological characteristics from L-type channels (Massie 1997; Perez-Reyes et al. 1998).

Reviews of molecular physiology of low-voltage-activated T-type calcium channels were given by Perez-Reyes (2003, 2006).

N- and P-type calcium channels blockers occur in neuronal cells and are involved in neurotransmitter release (Olivera et al. 1987; Bertolino and Llinás 1992; Mintz et al. 1992; Woppmann et al. 1994; Diversé-Pierluissi et al. 1995; Miljanich and Ramachandran 1995; Fisher and Bourque 1996; Ikeda 1996; Ertel et al. 1997; Sinnegger et al. 1997).

REFERENCES AND FURTHER READING

Barhanin J, Borsotto M, Coppola T, Fosset M, Hosey MM, Mourre C, Pauron D, Qar J, Romey G, Schmid A, Vandaele S, Van Renterghem C, Lazdunski M (1989) Biochemistry, molecular pharmacology, and functional control of Ca^{2+}-channels. In: Wray DW, Norman RI, Hess P (eds) Calcium Channels: Structure and Function. Ann NY Acad Sci 560:15–26

Bean BP (1989) Classes of calcium channels in vertebrate cells. Annu Rev Physiol 51:367–384

Berjukow S, Marksteiner R, Gapp F, Sinnegger MJ, Hering S (2000) Molecular mechanism of calcium channel block by israpidine. J Biol Chem 275:22114–22120

Bertolino M, Llinás RR (1992) The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. Annu Rev Pharmacol Toxicol 32:399–421

Catterall WA (1998) Receptor sites for blockers of L-type calcium channels. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 2, R 582

Catterall WA, Saegar MJ, Takahashi M, Nunoki K (1989) Molecular properties of dihydropyridine-sensitive calcium channels. In: Wray DW, Norman RI, Hess P (eds) Calcium Channels: Structure and Function. Ann NY Acad Sci 560:1–14

Catterall WA, Perez-Reyes E, Snutch TP, Striennig J (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57:411–425

Dascal N (1990) Analysis and functional characteristics of dihydropyridine-sensitive and -insensitive calcium channel proteins. Biochem Pharmacol 40:1171–1178

Diversé-Pierluissi M, Goldsmith PK, Dunlap K (1995) Transmitter-mediated inhibition of N-type calcium channels in sensory neurons involves multiple GTP-binding proteins and subunits. Neuron 14:191–200

Dolphin AC (1991) Regulation of calcium channel activity by GTP binding proteins and second messengers. Biochim Biophys Acta 1091:68–80

Ertel SI, Ertel EA, Clozel JP (1997) T-Type calcium channels and pharmacological blockade: Potential pathophysiological relevance. Cardiovasc Drugs Ther 11:723–739

Ferrante J, Triggle DJ (1990) Drug- and disease-induced regulation of voltage-dependent calcium channels. Pharmacol Rev 42:29–44

Fisher TE, Bourque CW (1996) Calcium-channel subtypes in the somata and axon terminals of magnocellular neurosecretory cells. Trends Neurosci 19:440–444

Fleckenstein A (1964) Die Bedeutung der energiereichen Phosphate für Kontraktilität und Tonus des Myocards. Verh Dtsch Ges Inn Med 70:81–99

Fleckenstein A (1983) History of calcium antagonists. Circ Res 52 (Suppl I):3–16

Fleckenstein A, Kammermeier H, Döring HJ, Freund HJ (1967) Zum Wirkungsmechanismus neuartiger Koronardilatatoren mit gleichzeitig Sauerstoff einsparenden Myocardeffekten, Prenylamin, Irpoveratril. Z Kreislaufforsch 56:716–744,839–853

Fleckenstein A, Frey M, Fleckenstein-Grün G (1983) Consequences of uncontrolled calcium entry and its prevention with calcium antagonists. Eur Heart J 4 (Suppl H):43–50

Fleckenstein A, Frey M, Fleckenstein-Grün G (1986) Antihypertensive and arterial anticalcinotic effects of calcium antagonists. Am J Cardiol 57:1D–10D

Galizzi JP, Quar J, Fosset M, Van Renterghem C, Lazdunski M (1987) Regulation of calcium channels in aortic muscle cells by protein kinase C activators (diacylglycerol and phorbol esters) and by peptides (vasopressin and bombesin) that stimulate phosphoinositide breakdown. J Biol Chem 262:6947–6950

Hockerman GH, Peterson BZ, Johnson BD, Catterall WA (1997) Molecular determinants of drug binding and action on L-type calcium channels. Annu Rev Pharmacol Toxicol 37:361–396

Hosey MM, Chang FC, O'Callahan CM, Ptasienski J (1989) L-type channels in cardiac and skeletal muscle: purification and phosphorylation. In: Wray DW, Norman RI, Hess P (eds) Calcium Channels: Structure and Function. Ann NY Acad Sci 560:27–38

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$-subunits. Nature 380:255–258

Kitamura N, Ohta T, Ito S, Nakazato Y (1997) Calcium channel subtypes in porcine adrenal chromaffin cells. Pflügers Arch – Eur J Physiol 434:179–187

Kochegarow AA (2003) Pharmacological modulators of voltage-gated calcium channels and their therapeutic application. Cell Calcium 33:145–162

Maggi CA, Tramontana M, Cecconi R, Santicioli P (1990) Neurochemical evidence of N-type calcium channels in transmitter secretion from peripheral nerve endings of sensory nerves in guinea pigs. Neurosci Lett 114:203–206

Massie BM (1997) Mibefradil: A selective T-type calcium antagonist. Am J Cardiol 80A:231–321

Miljanich GP, Ramachandran J (1995) Antagonists of neuronal calcium channels: structure, function and therapeutic implications. Annu Rev Pharmacol Toxicol 35:707–734

Mintz IM, Adams ME, Bean BP (1992) P-Type calcium channels in rat central and peripheral neurons. Neuron 9:85–95

Mitterdorfer J, Wang Z, Sinnegger MJ, Hering S, Striessnig J, Grabner M, Glossmann H (1996) Two amino acid residues in the IIIS5 segment of L-type calcium channels differentially contribute to 1,4-dihydropyridine sensitivity. J Biol Chem 271:30330–30335

Moresco RM, Govoni S, Battaini F, Trivulzio S, Trabucchi M (1990) Omegaconotoxin binding decreases in aged rat brain. Neurobiol Aging 11:433–436

Nakao SI, Ebata H, Hamamoto T, Kagawa Y, Hirata H (1988) Solubilization and reconstitution of voltage-dependent calcium channel from bovine cardiac muscle. Ca^{2+} influx assay using the fluorescent dye Quin2. Biochim Biophys Acta 944:337–343

Olivera BM, Cruz LJ, de Santos V, LeCheminant GW, Griffin D, Zeikus R, McIntosh JM, Galyean R, Varga J, Gray WR, Rivier J (1987) Neuronal calcium channel antagonists. Discrimination between calcium channel subtypes using ω-conotoxin from *Conus magnus* venom. Biochemistry 26:2086–2090

Perez-Reyes E, Cribbs L, Daud A, Jung-Ha Lee (1998) Molecular characterization of T-type calcium channels. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 2:R583

Perez-Reyes E (2003) Molecular physiology of low-voltage-activated T-type calcium channels. Physiol Rev 83:117–161

Perez-Reyes E (2006) Molecular characterization of T-type calcium channels. Cell Calcium 40:89–96

Peterson BZ, Tanada TN, Catterall WA (1996) Molecular determinants of high affinity dihydropyridine binding in L-type calcium channels. J Biol Chem 271:5293–5296

Porzig (1990) Pharmacological modulation of voltage-dependent calcium channels in intact cells. Rev Physiol Biochem Pharmacol 114:209–262

Rampe D, Triggle DJ (1993) New synthetic ligands for L-type voltage-gated calcium channels. Progr Drug Res 40:191–238
Reuter H, Porzig H, Kokubun S, Prod'Hom B (1988) Calcium channels in the heart. Properties and modulation by dihydropyridine enantiomers. Ann NY Acad Sci 522:16–24
Rosenberg RL, Isaacson JS, Tsien RW (1989) Solubilization, partial purification, and properties of ω-conotoxin receptors associated with voltage-dependent calcium channels. In: Wray DW, Norman RI, Hess P (eds) Calcium Channels: Structure and Function. Ann NY Acad Sci 560:39–52
Schuster A, Lacinová L, Klugbauer N, Ito H, Birnbaumer L, Hofmann F (1996) The IVS6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenylalkylamines. EMBO J 15:2365–2370
Sinnegger MJ, Wang Z, Grabner M, Hering S, Striessnig J, Glossmann H, Mitterdorfer J (1997) Nine L-type amino acid residues confer full 1,4-dihydropyridine sensitivity to the neuronal calcium channel α_{1A} subunit. J Bill Chem 272:27686–27693
Spedding M, Paoletti R (1992) Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacol Rev 44:363–376
Striessnig J, Grabner M, Mitterdorfer J, Hering S, Sinneger MJ, Glossmann H (1998) Structural basis of drug binding to L Ca^{2+} channels. Trends Pharmacol Sci 19:108–115
Striessnig J, Hoda JC, Koschak A, Zaghetto F, Müllner C, Sinnegger-Brauns MJ, Wild C, Watschinger K, Trockenbacher A, Pelster G (2004) L-type Ca^{2+} channels in Ca^{2+} channelopathies. Biochem Biophys Res Commun 322:1341–1346
Tsien RW, Tsien RY (1990) Calcium channels, stores and oscillations. Annu Rev Cell Biol 6:715–760
Woppmann A, Ramachandran J, Miljanich GP (1994) Calcium channel subtypes in rat brain: Biochemical characterization of the high-affinity receptors for ω-conopeptides SNX-230 (synthetic MVIIC), SNX-183 (SVIB), and SNX-111 (MVIIA). Mol Cell Neurosci 5:350–357

A.4.1
In Vitro Methods

A.4.1.1
^{3}H-Nitrendipine Binding in Vitro

PURPOSE AND RATIONALE

Radiolabeled dihydropyridine calcium channel antagonists such as ^{3}H-nitrendipine (Salter and Grover 1987; Campiani et al. 1996) are selective ligands for a drug receptor site associated with the voltage-dependent calcium channel. A constant concentration of the radioligand ^{3}H-nitrendipine (0.3–0.4 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of plasma membranes from bovine cerebral cortices. If the test drug exhibits any affinity to calcium channels, it is able to compete with the radioligand for channel binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent is the test drug.

PROCEDURE

Materials and solutions:

Preparation buffer	Tris-HCl pH 7.4	50 mM
Incubation buffer	Tris-HCl Genapol pH 7.4	50 mM 0.001%
Radioligand	^{3}H-nitrendipine Specific activity 2.59–3.22 TBq/mmol (70–87 Ci/mmol) (NEN)	
For inhibition of ^{3}H-ni-trendipine binding in non-specific binding Experiments	Nifedipine (Sigma)	

Two freshly slaughtered bovine brains are obtained from the slaughterhouse and placed in ice-cold preparation buffer. In the laboratory, approx. 5 g wet weight of the two frontal cerebral cortices are separated from the brains.

Membrane Preparation

The tissue is homogenized (glass Teflon potter) in ice-cold preparation buffer, corresponding to 1 g cerebral wet weight/50 ml buffer, and centrifuged at 48,000 *g* (4°C) for 10 min. The resulting pellets are resuspended in approx. 270 ml preparation buffer, and the homogenate is centrifuged as before. The final pellets are dissolved in preparation buffer, corresponding to 1 g cerebral cortex wet weight/30 ml buffer. The membrane suspension is immediately stored in aliquots of 5–10 ml at –77°C. Protein content of the membrane suspension is determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

At the day of the experiment, the required volume of the membrane suspension is slowly thawed and centrifuged at 48,000 *g* (4°C) for 10 min. The resulting pellets are resuspended in a volume of ice-cold incubation buffer, yielding a membrane suspension with a protein content of 0.6–0.8 mg/ml. After homogenization (glass Teflon potter), the membrane suspension is stirred under cooling for 20–30 min until the start of the experiment.

Experimental Course

As 1,4-dihydropyridines tend to bind to plastic material, all dilution steps are done in glass tubes.

For each concentration samples are prepared in triplicate. The total volume of each incubation sample is 200 µl (microtiter plates).

Saturation Experiments

total binding:

- 50 µl ^{3}H-nitrendipine (12 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl incubation buffer

non-specific binding:

- 50 µl ^{3}H-nitrendipine (4 concentrations, 5×10^{-11}–4×10^{-9} M)
- 50 µl nifedipine (5×10^{-7} M)

Competition Experiments

- 50 µl ^{3}H-nitrendipine (1 constant concentration, 3–4×10^{-10} M)
- 50 µl incubation buffer without or with non-labeled test drug (15 concentrations, 10^{-10}–10^{-3} M)

The binding reaction is started by adding 100 µl membrane suspension per incubation sample (0.6–0.8 mg protein/ml). The samples are incubated for 60 min in a bath shaker at 25°C. The reaction is stopped by subjecting the total incubation volume to rapid vacuum filtration over glass fibre filters. Thereby the membrane-bound is separated from the free radioactivity. Filters are washed immediately with approx. 20 ml ice-cold rinse buffer per sample. The retained membrane-bound radioactivity on the filter is measured after addition of 2 ml liquid scintillation cocktail per sample in a Packard liquid scintillation counter.

EVALUATION

The following parameters are calculated:

- total binding
- non-specific binding
- specific binding = total binding - non-specific binding

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{3}H-nitrendipine versus non-labeled drug by a computer-supported analysis of the binding data.

$$\frac{K_D\ ^3\mathrm{H} \times \mathrm{IC}_{50}}{K_D\ ^3\mathrm{H} + [^3\mathrm{H}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of specifically bound ^{3}H-nitrendipine in the competition experiment

[^{3}H] = concentration of ^{3}H-nitrendipine in the competition experiment.

K_D ^{3}H = dissociation constant of ^{3}H-nitrendipine, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the receptors are occupied by the test drug.

The affinity constant K_i [mol/l] is recorded and serves as a parameter to assess the efficacy of the test drug.

Standard data:

- nifedipine $K_i = 2$–4×10^{-9} mol/l

MODIFICATIONS OF THE METHOD

[^{3}H](+)-PN200–100 (israpidine) has been used by many authors as the labeled ligand for binding experiments (Grassegger et al. 1989; Nokin et al. 1990; Striessnig et al. 1991; Yaney et al. 1991; Miwa et al. 1992; Ichida et al. 1993; Kalasz et al. 1993; Ikeda et al. 1994; Rutledge and Triggle 1995; Shimasue et al. 1996; He et al. 1997; Natale et al. 1999; Matthes et al. 2000; Peri et al. 2000).

Yaney et al. (1991) performed binding experiments with [^{3}H](+)-PN200–110 to membranes of RINm5F cells.

The RINm5F pancreatic β-cells were grown on plastic culture flasks in medium RPMI 1640 supplemented with 10% fetal bovine serum, 100 µg/ml of streptomycin and 100 U/ml penicillin. The cells were kept in humidified incubators at 37°C in 5% CO_2-95% air. Maintenance flasks were subcultured every 4–5 days at ~80% confluency. For binding experiments of [^{3}H](+)-PN200–110 to membranes, cells were removed from the flasks by incubation for 15–20 min in 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] and 1 mM EGTA (pH 7.4) at 4°C. The osmotically ruptured cells were homogenized with a Brinkmann Polytron followed by centrifugation at 20,000 *g* for 15 min. The pellet was resuspended and washed twice. The final pellet was resuspended in 1 mM $CaCl_2$ and 20 mM MOPS (pH 7.4) at a protein concentration of 0.2–0.4 mg/ml. Membranes were incubated at room temperature with (+)-[^{3}H]PN200–110 in a final volume of 500 µl (Weiland and Oswald 1985). Bound and free radioligand were separated by vacuum filtration through a glass fiber filter (no. 32, Schleicher and Schüll) followed by three washes of the filter with 3 ml incubation buffer. Non-specific binding was determined in the presence of 1 µM nitrendipine. Radioactivity retained by the filters was determined by liquid scintillation counting. The concentration of free radioligand was calculated by scintillation counting of aliquots of incubation mixtures under nonspecific conditions before and after centrifugation.

Several other calcium entry blockers, such as nimodipine, diltiazem, verapamil and desmethoxyverapamil, have been labeled and used for binding studies in order to elucidate the calcium channel recognition sites and may be used for further classification of calcium antagonists (Ferry and Glossmann 1982; Glossmann et al. 1983; Goll et al. 1984; Lee et al. 1984; Glossmann et al. 1985; Schoemaker and Langer 1985; Ruth et al. 1985; Reynolds et al. 1986).

Vaghy et al. (1987) identified of a 1,4-dihydropyridine- and phenylalkylamine-binding polypeptide in calcium channel preparations.

Naito et al. (1989) described photoaffinity labeling of the purified skeletal muscle calcium antagonist receptor by [^{3}H]azidobutyryl diltiazem.

Watanabe et al. (1993) reported that azidobutyryl clentiazem labels the benzothiazepine binding sites in the α_1 subunit of the skeletal muscle calcium channel. Tissue heterogeneity of calcium channel antagonist binding sites has been demonstrated by Gould et al. (1983).

Photoaffinity labeling of the cardiac calcium channel with 1,4-dihydropyridine(–)-[3H]azidopine was described by Ferry at al (1987).

Knaus et al. (1992) described a unique fluorescent phenylalkylamine probe for L-type Ca^{2+} channels.

Binding sites for ω-conotoxin appear to be primarily associated with the N-type of voltage-dependent calcium channels (Feigenbaum et al. 1988; Wagner et al. 1988).

Cohen et al. (1992) recommended the peptide ω-agatoxin IIIA as a valuable pharmacological tool being the only known ligand that blocks L-type calcium channels with high affinity at all voltages and causes, unlike the 1,4-dihydropyridines, no block of T-type calcium channels.

REFERENCES AND FURTHER READING

Balwierczak JL, Grupp IL, Grupp G, Schwartz A (1986) Effects of bepridil and diltiazem on [^{3}H] nitrendipine binding to canine cardiac sarcolemma. Potentiation of pharmacological effects of nitrendipine by bepridil. J Pharmacol Exp Ther 237:40–48

Bellemann P, Ferry D, Lübbecke F, Glossmann H (1981) [^{3}H]-Nitrendipine, a potent calcium antagonist, binds with high affinity to cardiac membranes. Arzneim Forsch/Drug Res 31:2064–2067

Boles RG, Yamamura HI, Schoemaker H, Roeske WR (1984) Temperature-dependent modulation of [^{3}H]nitrendipine binding by the calcium channel antagonists verapamil and diltiazem in rat brain synaptosomes. J Pharmacol Exp Ther 229:333–339

Bolger GT, Skolnick P (1986) Novel interactions of cations with dihydropyridine calcium antagonist binding sites in brain. Br J Pharmacol 88:857–866

Bolger GT, Genko P, Klockowski R, Luchowski E, Siegel H, Janis RA, Triggle AM, Triggle DJ (1983) Characterization of binding of the Ca^{2+} channel antagonist, [^{3}H]nitrendipine, to guinea pig ileal smooth muscle. J Pharmacol Exp Ther 225:291–309

Campiani G, Fiorini I, De Filippis MP, Ciani SM, Garofalo A, Nacci V, Giorgi G, Sega A, Botta M, Chiarini A, Budriesi R, Bruni G, Romeo MR, Manzoni C, Mennini T (1996) Cardiovascular characterization of pyrrolo[2,1-*d*] [1,5]benzothiazepine derivatives binding selective to the peripheral-type benzodiazepine receptor (PBR): from dual PBR affinity and calcium antagonist activity to novel and selective calcium entry blockers. J Med Chem 39:2922–2938

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Cohen CJ, Ertel EA, Smith MM, Venam VJ, Adams ME, Leibowitz MD (1992) High affinity block of myocardial L-type calcium channels by the spider toxin ω-agatoxin IIIA: advantages over 1,4-dihydropyridines. Mol Pharmacol 42:947–951

Ehlert FJ, Itoga E, Roeske WR, Yamamura HI (1982) The interaction of [^{3}H]nitrendipine with receptors for calcium antagonists in the cerebral cortex and heart of rats. Biochem Biophys Res Commun 104:937–943

Ehlert FJ, Roeske WR, Itoga E, Yamamura HI (1982) The binding of [^{3}H]nitrendipine to receptors for calcium channel antagonists in the heart, cerebral cortex, and ileum of rats. Life Sci 30:2191–2202

Feigenbaum P, Garcia ML, Kaczorowski GJ (1988) Evidence for distinct sites coupled with high affinity ω-conotoxin receptors in rat brain synaptic plasma membrane vesicles. Biochem Biophys Res Commun 154:298–305

Ferry DR, Glossmann H (1982) Identification of putative calcium channels in skeletal muscle microsomes. FEBS Lett 148:331–337

Ferry DR, Goll A, Gadow C, Glossmann H (1984) (–)-^{3}H-desmethoxyverapamil labelling of putative calcium channels in brain: autoradiographic distribution and allosteric coupling to 1,4-dihydropyridine and diltiazem binding sites. Naunyn Schmiedeberg's Arch Pharmacol 327:183–187

Ferry DR, Goll A, Glossmann H (1987) Photoaffinity labelling of the cardiac calcium channel. Biochem J 243:127–135

Fleckenstein A (1977) Specific pharmacology of calcium in myocardium, cardiac pacemakers and vascular smooth muscle. Ann Rev Pharmacol Toxicol 17:149–177

Glossmann H, Ferry DR (1985) Assay for calcium channels. Meth Enzymol 109:513–550

Glossmann H, Linn T, Rombusch M, Ferry DR (1983) Temperature-dependent regulation of d-*cis*-[^{3}H]diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. FEBS Letters 160:226–232

Glossmann H, Ferry DR, Goll A, Striessnig J, Schober M (1985a) Calcium channels: Basic properties as revealed by radioligand binding studies. J Cardiovasc Pharmacol 7 (Suppl 6):S20–S30

Glossmann H, Ferry DR, Goll A, Striessnig J, Zernig G (1985b) Calcium channels and calcium channel drugs: Recent biochemical and biophysical findings. Arzneim Forsch./Drug Res 35:1917–1935

Glossmann H, Ferry DR, Striessnig J, Goll A, Moosburger K (1987) Resolving the structure of the Ca^{2+} channel by photoaffinity labelling. Trends Pharmacol Sci 8:95–100

Goll A, Ferry DR, Striessnig J, Schober M, Glossmann H (1984) (–)-[^{3}H]Desmethoxyverapamil, a novel Ca^{2+} channel probe. FEBS Lett 176:371–377

Gould RJ, Murphy KMM, Snyder SH (1982) [^{3}H]Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc Nat Acad Sci, USA 79:3656–3660

Gould RJ, Murphy KMM, Snyder SH (1983) Tissue heterogeneity of calcium channel antagonist binding sites labeled by [^{3}H]nitrendipine. Mol Pharmacol 25:235–241

Grassegger A, Striessnig J, Weiler M, Knaus HG, Glossmann H (1989) [^{3}H]HOE 166 defines a novel calcium antagonist drug receptor – distinct from the 1,4-dihydropyridine binding domain. Naunyn Schmiedebergs Arch Pharmacol 340:752–759

He M, Bodi I, Mikala G, Schwartz A (1997) Motif III S5 of L-type channels is involved in the dihydropyridine binding site. J Biol Chem 272:2629–2633

Ichida S, Wada T, Nakazaki S, Matsuda N, Kishino H, Akimoto T (1993) Specific bindings of [^{3}H](+)PN200–110 and [^{125}]omega-conotoxin to crude membranes from differentiated NG 108–15 cells. Neurochem Res 18:633–638

Ikeda S, Amano Y, Adachi-Akahane S, Nagao T (1994) Binding of [^{3}H](+)PN200–110 to aortic membranes from normotensive and spontaneously hypertensive rats. Eur J Pharmacol 264:223–226

Janis RA, Sarmianto JG, Maurer SC, Bolger GT, Triggle DJ (1984) Characteristics of the binding of [^{3}H]nitrendipine to rabbit ventricular membranes: Modification by other Ca^{2+} channel antagonists and by the Ca^{2+} channel agonist Bay K 8644. J Pharmacol Exp Ther 231:8–15

Kalasz H, Watanabe T, Yabana H, Itagaki K, Naito K, Nakayama H, Schwartz A, Vaghy PL (1993) Identification of 1,4-dihydropyridine binding domains within the primary structure of the α_1 subunit of the skeletal muscle L-type calcium channel. FEBS Lett 331:177–181

Knaus HG, Moshammer T, Kang HC, Haugland RP, Glossmann H (1992) A unique fluorescent phenylalkylamine probe for L-type Ca^{2+} channels. J Biol Chem 267:2179–2189

Lee HR, Roeske WR, Yamamura HI (1984) High affinity specific [^{3}H](+)PN 200–110 binding to dihydropyridine receptors associated with calcium channels in rat cerebral cortex and heart. Life Sci 35:721–732

Matthes J, Huber I, Haaf O, Antepohl W, Striessnig J, Herzig S (2000) Pharmacodynamic interaction between mibefradil and other calcium channel blockers. Naunyn-Schmiedebergs Arch Pharmacol 361:578–583

Miwa K, Miyagi Y, Araie E, Sasayama S (1992) Effects of diltiazem and verapamil on (+)-PN 200–110 binding kinetics in dog cardiac membranes. Eur J Pharmacol 213:127–132

Marangos PJ, Patel J, Miller Ch, Martino AM (1982) Specific calcium antagonist binding sites in brain. Life Sci 31:1575–1585

Naito K, McKenna E, Schwartz A, Vaghy PI (1989) Photoaffinity labeling of the purified skeletal muscle calcium antagonist receptor by a novel benzodiazepine, [^{3}H]azidobutyryl diltiazem. J Biol Chem 264:21211–21214

Natale NR, Rogers ME, Staples R, Triggle DJ, Rutledge A (1999) Lipophilic 4-isoxazolyl-1,4-dihydropyridines: synthesis and structure-activity relationships. J Med Chem 42:3087–3093

Nokin P, Clinet M, Beaufort P, Meysman L, Laruel R, Chatelain P (1990) SR 33557, a novel calcium entry blocker. II Interactions with 1,4-dihydropyridine, phenylalkylamine, and benzodiazepine binding sites in rat heart sarcolemmal membranes. J Pharmacol Exp Ther 255:600–607

Peri R, Padmanabhan S, Rutledge A, Singh S, Triggle DJ (2000) Permanently charged chiral 1,4-dihydropyridines: molecular probes of L-type calcium channels. Synthesis and pharmacological characterization of methyl-(ω-trimethylalkylammonium 1,4-dihydro-2,6-dimethyl-4-(3-nitro-phenyl)-3,5-pyridinecarboxylate iodide, calcium channel antagonists. J Med Chem 43:2906–2914

Reynolds IJ, Snowman AM, Snyder SH (1986) (–)-[^{3}H]Desmethoxyverapamil labels multiple calcium channel modulator receptors in brain and skeletal muscle membranes: Differentiation by temperature and dihydropyridines. J Pharmacol Exp Ther 237:731–738

Ruth P, Flockerzi V, von Nettelblatt V, Oeken J, Hoffmann F (1985) Characterization of binding sites for nimodipine and (–)-desmethoxyverapamil in bovine sarcolemma. Eur J Biochem 150:313–322

Rutledge A, Triggle DJ (1995) The binding interactions of Ro 40–5967 at the L-type Ca^{2+} channel in cardiac tissue. Eur J Pharmacol 280:155–158

Salter F, Grover AK (1987) Characterization and solubilization of the nitrendipin binding protein from canine small intestinal circular smooth muscle. Cell Calcium 8:145–166

Schoemaker H, Langer SZ (1985) [^{3}H]diltiazem binding to calcium channel antagonists recognition sites in rat cerebral cortex. Eur J Pharmacol 111:273–277

Shimasue K, Urushidani T, Hagiwara M, Nagao T (1996) Effects of anadamide and arachidonic acid on specific binding of (+)-PN200–110 and (?)-desmethoxyverapamil to L-type Ca^{2+} channel. Eur J Pharmacol 296:347–350

Striessnig J, Murphy BJ, Catterall WA (1991) Dihydropyridine receptor of L-type Ca^{2+} channels: identification of binding domains for [^{3}H](+)-PN200–110 and [^{3}H]azidopine within the α1 subunit. Proc Natl Acad Sci USA 88:10769–10773

Vaghy PI, Striessnig J, Miwa K, Knaus HG, Itagati K, McKenna E, Glossmann H, Schwartz A (1987) Identification of a novel 1,4-dihydropyridine- and phenylalkylamine-binding polypeptide in calcium channel preparations. J Biol Chem 262:14337–14342

Wagner JA, Snowman AM,. Biswas A, Olivera BM, Snyder SH (1988) ω-Conotoxin GVIA binding to a high-affinity receptor in brain: Characterization, calcium sensitivity, and solubilization. J Neurosci 8:3354–3359

Watanabe T, Kalasz H, Yabana H, Kuniyasu A, Mershon J, Itagaki K, Vaghy PL, Naito K, Nakayama H, Schwartz A (1993) Azidobutyryl clentiazem, a new photoactivable diltiazem analog, labels benzothiazepine binding sites in the α_1 subunit of the skeletal muscle calcium channel. FEBS Lett 334:261–264

Weiland GA, Oswald RE (1985) The mechanism of binding of dihydropyridine calcium channel blockers to rat brain membranes. J Biol Chem 260:8456–8464

Yaney GC, Stafford A, Henstenberg JD, Sharp GWG, Weiland GA (1991) Binding of the dihydropyridine calcium channel blocker (+)-[^{3}H]isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxy-carbonyl-2,6-dimethyl-3- pyridinecarboxylate (PN200–110) to RINm5F membranes and cells. Characterization and functional significance. J Pharmacol Exp Ther 258:652–662

A.4.2
Calcium Antagonism in Isolated Organs

A.4.2.1
Calcium Antagonism on Action Potential of Isolated Guinea Pig Papillary Muscle

PURPOSE AND RATIONALE

Intracellular action potential in the guinea pig papillary muscle is recorded. Partial depolarization is achieved by potassium enriched Ringer solution and by addition of isoproterenol. Resting potential is increased to 40 mV resulting in inactivation of the fast sodium channel. Under these conditions, upstroke velocity is an indicator for calcium flux through the membrane, which is decreased by calcium blockers.

PROCEDURE

Guinea pigs of either sex (Pirbright White strain) weighing 300–400 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Initially, normal Ringer solution oxygenated with carbogen (95% O_2/5% CO_2) at a temperature of 36°C is used. A standard micro electrode technique is applied to measure the action potential via a glass micro electrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at intervals of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold. The intracellular action potential is amplified, differentiated for registration of upstroke velocity (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

After an incubation period of 30 min the Ringer solution is changed to the following composition containing 5 times more potassium and 10% less sodium.

- NaCl 8,1 g/L
- KCl 1,0 g/L
- $CaCl_2$ 0,2 g/L
- $NaHCO_3$ 0,1 g/L
- glucose 5,0 g/L

For further depolarization, isoproterenol (1.0 mg per 100 ml) is added. By this measure, resting potential is increased to about 40 mV, resulting in inactivation of the fast inward sodium channel. The resulting slow rising action potential is sensitive to calcium antagonistic drugs (Kohlhardt and Fleckenstein 1977).

The test compound is added at a concentration of 1 µg/ml. Effective compounds are tested at lower concentrations and compared with the standard (nifedipin at concentrations of 0.01 and 0.1 µg/ml)

EVALUATION

The decrease of upstroke velocity is tested at various concentrations of the test compound and compared with the standard.

REFERENCES AND FURTHER READING

Grupp IL, Grupp G (1984) Isolated heart preparations perfused or superfused with balanced salt solutions. In: Schwartz A (ed) Methods in Pharmacology, Vol 5: Myocardial Biology. Plenum Press, New York and London, pp 111–128

Kohlhardt M, Fleckenstein A (1977) Inhibition of the slow inward current by nifedipine in mammalian ventricular myocardium. Naunyn Schmiedeberg's Arch Pharmacol 298:267–272

Linz W, Schölkens BA, Kaiser J, Just M, Bei-Yin Q, Albus U, Petry P (1989) Cardiac arrhythmias are ameliorated by local inhibition of angiotensin formation and bradykinin degradation with the converting-enzyme inhibitor ramipril. Cardiovasc Drugs Ther 3:873–882

Striessnig J, Meusburger E, Grabner M, Knaus HG, Glossmann H, Kaiser J, Schölkens B, Becker R, Linz W, Henning R (1988) Evidence for a distinct Ca^{2+} antagonist receptor for the novel benzothiazinone compound Hoe 166. Naunyn-Schmiedeberg's Arch Pharmacol 337:331–340

A.4.2.2
Calcium Antagonism in the Isolated Guinea Pig Atrium

PURPOSE AND RATIONALE

k-Strophanthine (and other cardiac glycosides) inhibit the membrane-bound Na^+/K^+-activated ATP-ase which leads to an increase in intracellular Ca^{2+}-concentration. Ca^{2+} ions activate the contractile apparatus, causing a distinctive enhancement of contractions. The procedure can be used to evaluate a compound's calcium channel blocking activity by measuring its ability to decrease atrial contractions induced by k-strophanthine.

PROCEDURE

Apparatus

HSE-stimulator 1 (Hugo Sachs Elektronik, D-79232 March-Hugstetten, Germany)

Stimulation data:

- frequency 1.5 Hz
- duration 3 ms
- voltage 3–8 V

Experiment

Guinea pigs of either sex weighing 200–500 g are sacrificed with a blow to the nape of the neck and exsanguinated. The left atrium is removed, placed in an organ bath and attached to an isotonic strain gauge, its base being wired to an electrode of the stimulator. The Ringer-solution is aerated with carbogen and kept at 36°C. The atrium is continuously stimulated via stimulator 1, the voltage being slowly increased up to the threshold level. Contractions are recorded on a polygraph. Prior to drug administration, two prevalues are obtained by adding 2 µg/ml k-strophanthine-α (Cymarin) to the organ bath and measuring the increase in contractile force. Following a 15 min washout and recovery period, the test drug is added to the bath followed by administration of k-strophanthine-α 10 min later. The change in contractile force is always measured 10 min after the addition of k-strophanthine-α.

Standard compounds:

- Verapamil hydrochloride
- Nifedipine

EVALUATION

The percent inhibition of k-strophanthine-α induced contraction is determined.

MODIFICATIONS OF THE METHOD

Calcium antagonists can also be evaluated in the LANGENDORFF heart preparation (Lindner and Ruppert 1982).

Leboeuf et al. (1992) reported the protective effect of bepridil and flunarizine against veratrine-induced contracture in rat atria concluding from the results in this model that these agents may be more effective as L-type calcium ion-channel blockers in protecting against calcium overload during ischaemia and reperfusion injury.

REFERENCES AND FURTHER READING

Church J, Zsotér TT (1980) Calcium antagonistic drugs. Mechanism of action. Can J Physiol Pharmacol 58:254–264

Grupp IL, Grupp G (1984) Isolated heart preparations perfused or superfused with balanced salt solutions. In: Schwartz A (ed) Methods in Pharmacology, Vol 5: Myocardial Biology. pp 111–128. Plenum Press, New York and London

Leboeuf J, Baissat J, Massingham R (1992) Protective effect of bepridil and against veratrine-induced contracture in rat atria. Eur J Pharmacol 216:183–189

Lindner E, Ruppert D (1982) Effects of calcium antagonists on coronary spasm and pulmonary artery contraction in comparison to their antagonistic action against K-strophanthin in isolated guinea-pig atria. Pharmacology 24:294–302

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydroxy-labd-14-en-11-one. Arzneim Forsch/ Drug Res 43(I) 313–319

Salako LA, Vaugham Williams EM, Wittig JH (1976) Investigations to characterize a new anti-arrhythmic drug, ORG 6001, including a simple test for calcium antagonism. Br J Pharmacol 57:251–262

A.4.2.3 Calcium Antagonism in the Isolated Rabbit Aorta

PURPOSE AND RATIONALE

Contraction of aorta rings is induced by adding potassium chloride or norepinephrine to the organ bath containing slightly modified Krebs bicarbonate buffer. Test drugs with calcium channel blocking activity have a relaxing effect.

PROCEDURE

Rabbits of either sex weighing 3–4 kg are sacrificed with an overdose of pentobarbital sodium. The chest cavity is opened and the descending thoracic aorta (from the level of the aortic arch to the level of the diaphragm) is rapidly removed and placed in a beaker of oxygenated Krebs bicarbonate buffer at 37°C.

The content of magnesium and calcium is slightly diminished in the Krebs bicarbonate buffer resulting in the following composition:

- NaCl 118.4 mMol
- KCl 4.7 mMol
- KH_2PO_4 1.2 mMol
- $MgSO_4 \cdot 2H_2O$ 1.2 mMol
- $CaCl_2 \cdot 2H_2O$ 1.9 mMol
- $NaHCO_3$ 25.0 mMol
- dextrose 10.0 mMol
- EDTA 0.013 mMol

The tissue is then transferred to a dish containing fresh oxygenated, warmed Krebs solution. Fat and loose connective tissue are carefully removed while keeping the tissue moist with the solution. Eight rings of 4–5 mm width are obtained and each is mounted in a 20 ml tissue bath which contains the oxygenated warmed Krebs solution. Initial tension is set at 1.0 g. The tissue is allowed to incubate over a period of 2 h, during which time the Krebs solution is changed every 15 min. Also during this time, tension is maintained at 1.0 g. Just prior to the end of the 2 h equilibration period, the Krebs solution is changed again and the tissue

is allowed to stabilize at 1.0 g tension. A sustained contraction is then generated by addition of either 40 mM KCl or 2.9×10^{-3} mM norepinephrine.

Twenty min after addition of the agonist, the test drug is added so that the final concentration in the bath is 1×10^{-5} M. The percent relaxation reading is taken 30 min after addition of the test drug. If at least 30% relaxation occurs, an accumulative concentration-relaxation curve is established. There is a 30 min period of time between the addition of each concentration of test compound.

EVALUATION

Active tension is calculated for the tissue at the time point just prior to the addition of the test compound and also at the point 30 min after the addition of each concentration of test compound. Active tension is defined as the difference between the generated tension and the baseline tension. The percent relaxation from the predrug, precontracted level is calculated for each concentration of test compound. A number of 5 experiments constitutes a dose range. An ID_{50} is calculated by linear regression analysis.

MODIFICATIONS OF THE METHOD

Hof and Vuorela (1983) compared three methods for assessing calcium antagonism on rabbit aorta smooth muscle.

Matsuo et al. (1989) reported a simple and specific screening method for Ca-entry blockers. In the presence of various Ca-channel blockers, 1×10^{-4} M Ca^{2+} causes relaxation of rat uterine smooth muscle that has been tonically contracted with oxytocin in calcium-free medium after prolonged preincubation with 3 mM EGTA.

Micheli et al. (1990) used spirally cut preparations of rat aorta and rings of rabbit ear artery to test calcium entry blocker activity.

Rüegg et al. (1985) described a smooth muscle cell line originating from fetal rat aorta to be suitable for the study of voltage sensitive calcium channels. Calcium channel antagonists inhibited both the basal and the potassium chloride stimulated $^{45}Ca^{2+}$ uptake.

REFERENCES AND FURTHER READING

Hof RP, Vuorela HJ (1983) Assessing calcium antagonism on vascular smooth muscle: comparison of three methods. J Pharmacol Meth 9:41–52

Matsuo K, Morita S, Uchida MK, Sakai K (1989) Simple and specific assessment of Ca-entry-blocking activities of drugs by measurement of Ca reversal. J Pharmacol Meth 22:265–275

Micheli D, Collodel A, Semerano C, Gaviraghi G, Carpi C (1990) Lacidipine: A calcium antagonist with potent and long-lasting antihypertensive effects in animal studies. J Cardiovasc Pharmacol 15:666–675

Rüegg UT, Doyle VM, Zuber JF, Hof RP (1985) A smooth muscle cell line suitable for the study of voltage sensitive calcium channels. Biochem Biophys Res Commun 130:447–453

Robinson CP, Sastry BVR (1976) The influence of mecamylamine on contraction induced by different agonists and the role of calcium ions in the isolated rabbit aorta. J Pharmacol Exp Ther 197:57–65

Striessnig J, Meusburger E, Grabner M, Knaus HG, Glossmann H, Kaiser J, Schölkens B, Becker R, Linz W, Henning R (1988) Evidence for a distinct Ca^{2+} antagonist receptor for the novel benzothiazinone compound Hoe 166. Naunyn-Schmiedeberg's Arch Pharmacol 337:331–340

Towart R (1982) Effects of nitrendipine (Bay e 5009), nifedipine, verapamil, phentolamine, papaverine, and minoxidil on contractions of isolated rabbit aortic smooth muscle. J Cardiovasc Pharmacol 4:895–902

Turner RA (1965) Cardiotonic agents; The aortic strip of the rabbit. In: Turner RA (ed) Screening methods in pharmacology. pp 203–209. Academic Press, New York and London

A.4.2.4 Calcium Antagonism in the Isolated Guinea Pig Pulmonary Artery

PURPOSE AND RATIONALE

Contraction of the pulmonary artery is induced by changing the normal Tyrode solution in the organ bath against a potassium enriched solution. This contraction can be inhibited by calcium blockers.

PROCEDURE

The following solutions are used:

	Normal Tyrode solution [mMol]	Potassium Enriched Tyrode solution [mMol]
NaCl	135.0	89.0
KCl	3.7	50.0
$MgSO_4$	0.81	0.81
NaH_2PO_4	0.41	0.41
$NaHCO_3$	11.0	11.0
$CaCl_2$	2.25	2.25
Glucose	5.6	5.6

Guinea pigs (Pirbright White strain) of either sex weighing 400–500 g are sacrificed by stunning. The pulmonary artery is removed and cut spirally at an angle of 45°. The resulting strip is cut to lengths of 2 cm and one piece is suspended in oxygenated normal Tyrode solution in an organ bath at 37°C with a preload of 1 g. Contractions are registered with an isotonic strain transducer and recorded on a polygraph.

After 1 h equilibrium time, normal solution is exchanged with potassium enriched Tyrode solution. The artery strip reacts with a contraction which achieves after 10 min 90–95% of its maximum. After an additional 10 min, exchange to normal Tyrode solution is performed. Ten min later again a contraction is induced by potassium enriched solution. When the height of the contraction has reached a constant level, the test substance is added and again potassium induced contraction recorded. The height of the contraction is expressed as percent of initial potassium induced contraction.

After lavage, the procedure is repeated with a higher dose or the standard.

EVALUATION

For calculation of a regression line, the decrease of contraction versus control after various doses is measured in mm. The percentage of inhibition after various doses is taken for calculation of an ED_{50}.

REFERENCES AND FURTHER READING

Green AF, Boura ALA (1964) Sympathetic nerve blockade. In: Laurence DR, Bacharach AL (eds) Evaluation of drug activities: Pharmacometrics. Academic Press, London and New York, pp 370–430

Lindner E, Ruppert D (1982) Effects of Ca^{2+} antagonists on coronary spasm and pulmonary artery contraction in comparison to their antagonistic action against k-strophanthin in isolated guinea-pig atria. Pharmacology 24:294–302

Striessnig J, Meusburger E, Grabner M, Knaus HG, Glossmann H, Kaiser J, Schölkens B, Becker R, Linz W, Henning R (1988) Evidence for a distinct Ca^{2+} antagonist receptor for the novel benzothiazinone compound Hoe 166. Naunyn-Schmiedeberg's Arch Pharmacol 337:331–340

A.4.3 In Vivo Methods

A.4.3.1 Evaluation of Calcium Blockers in the Pithed Rat

PURPOSE AND RATIONALE

Using the cardioaccelerator response in pithed rats, calcium entry blockers can be distinguished from other agents which have modes of action not involving direct blockade of calcium entry (Clapham 1988).

PROCEDURE

Male Sprague-Dawley rats (250–350 g) are anaesthetized with methohexitone sodium (50 mg/kg i.p.). Following cannulation of the trachea, the rats are pithed through one orbit with a stainless steel rod and immediately artificially respired with room air (78 strokes/min, 1 ml/100 g body weight) via a Palmer small animal respiration pump. A jugular vein is cannulated for administration of drugs. Arterial blood pressure is recorded from a carotid artery using a pressure transducer. Heart rate is derived from the phasic arterial pressure signal with a phase lock loop rate-meter (BRL Instrument Services). Both parameters are displayed on a recorder. The animals are kept warm by an incandescent lamp positioned about 25 cm above them. The pithing rod is withdrawn so that the tip lays in the thoracic portion of the spinal cord. All rats then receive (+)tubocurarine (1.5 mg/kg i.v.) and are bilaterally vagotomized.

The cardioaccelerator response is obtained by continuous electrical stimulation of the thoracic spinal cord with square wave pulses of 0.5 ms duration, at supramaximal voltage at a frequency of 0.5 Hz using the pithing rod as a stimulating electrode. An indifferent electrode is inserted subcutaneously in the femoral region. Only rats with a resulting tachycardia of more than 100 beats/min are included into the experiments.

When the cardioaccelerator response has stabilized for about 3–5 min, cumulative intravenous doses of drug or corresponding vehicle are administered. Succeeding doses are given when the response to the previous dose has stabilized.

Calcium antagonists and β-blockers inhibit dose-dependent the tachycardia elicited by electrical stimulation of the spinal cord, whereas lignocaine and nicorandil are not effective.

Doses of β-blockers or calcium-antagonists, which reduce the tachycardia to 50% are tested again. Three min after administration of the drug, calcium gluconate (1 mg/min) or water (0.1 ml/min) are infused using a Harvard apparatus compact infusion pump. The effects of calcium entry blockers, but not of β-adrenoreceptor blockers, are antagonized.

EVALUATION

The level of tachycardia immediately prior to drug administration is taken as 100% and responses to drugs are expressed as a percentage of this predose tachycardia. If an inhibitory effect > 50% is seen, then an ID_{50} (with 95% confidence limits) is interpolated from linear regression analysis. Significance of differences between the groups receiving calcium gluconate and their parallel vehicle controls is calculated by Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

Differentiation between the effects of β-blockers and calcium-antagonists can be achieved in a relatively simple *in vivo* model.

REFERENCES AND FURTHER READING

Clapham JC (1988) A method for *in vivo* assessment of calcium slow channel blocking drugs. J Cardiovasc Pharmacol 11:56–60

A.5 Anti-Arrhythmic Activity

A.5.0.1 General Considerations

Guidelines for the study of arrhythmias in man and animals regarding the experimental design as well as the classification, quantification, and analysis were given as the Lambeth Conventions by Walker et al. (1988).

Classification

Anti-arrhythmic drugs have been classified into various groups and subgroups (Vaughan Williams 1970, 1975, 1984, 1988, 1991, 1992; Borchard et al. 1989; Frumin et al. 1989; Harumi et al. 1989; Colatsky and Follmer 1990; Podrid 1990; Coromilas 1991; Nattel 1991; Rosen and Schwartz 1991; Scholz 1991; Woosley 1991; Ravens 1992; Sanguinetti 1992; Grant 1992; Nattel 1993; Scholz 1994). This classification is based on electrophysiological effects (e. g., action potential) and on interaction with membrane receptors and ion channels. The heterogeneity of classification criteria resulted in vivid discussions (The Sicilian Gambit 1991; Vaughan Williams 1991, 1992). In particular, a clinical study (CAST investigators 1990) challenged the therapeutic value of some anti-arrhythmic drugs.

Weirich and Antoni (1990, 1991) proposed a subdivision of class-1-anti-arrhythmic drugs according to the saturation behavior of frequency-dependent block and its onset-kinetics.

Class I anti-arrhythmic drugs directly alter membrane conductance of cations, particularly those of Na^+ and K^+. They reduce upstroke velocity, V_{max}, of the cardiac action potential by blockade of the fast Na^+ channel. This leads to a depression of conduction velocity, a prolongation of the voltage- and time-dependent refractory period and an increase in the threshold of excitability in cardiac muscle. Class I anti-arrhythmic drugs are subclassified according their effect on the action potential duration.

Class I A anti-arrhythmic drugs (Quinidine-like substances, e. g., disopyramide, procainamide, ajmaline) lengthen the action potential duration which is reflected in the ECG as lengthening of the QT-interval. This effect is added to that on fast sodium channel resulting in delayed recovery from inactivation.

Class I B anti-arrhythmic drugs (lidoacaine-like drugs, e. g., mexiletine, phenytoin, tocainide), in contrast, shorten the action potential duration.

Class I C anti-arrhythmic drugs (e. g., encainide, flecainide, propafenone, indecainide) produce quinidine- and lidocaine-like effects and exert differential actions on the duration of action potential in Purkinje fibres (shortening) and ventricular muscle.

Class II anti-arrhythmic drugs are β-adrenergic antagonists. They exert their anti-arrhythmic effects by antagonizing the electrophysiological effects of catecholamines which are mainly mediated by an increase in slow calcium inward current.

Class III anti-arrhythmic drugs (e. g., amiodarone, bretylium, sotalol) prolong the action potential and lead to a corresponding increase in the effective refractory period. The action is mainly due to a block of outward repolarizing currents. However, activation of sodium and calcium inward currents that prolong the plateau of the action potential may also be involved.

Class IV anti-arrhythmic drugs (e. g., verapamil, diltiazem) are slow calcium channel blockers suppressing the slow calcium inward current and calcium-dependent slow action potentials.

Experimentally Induced Arrhythmias

Winslow (1984) reviewed the methods for the detection and assessment of antiarrhythmic activity.

Szekeres (1979) suggested a rational screening program for the selection of effective antiarrhythmic drugs.

Arrhythmia models in the rat were reviewed by Cheung et al. (1993).

Arrhythmogenic stimuli can be divided into three groups: chemical, electrical and mechanical (Szekeres and Papp 1975; Wilson 1984).

Chemically Induced Arrhythmias

A large number of chemical agents alone or in combination are capable of inducing arrhythmias. Administration of anesthetics like chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus, such as intravenous adrenaline, or cardiac glycosides (usually ouabain), aconitine, and veratrum alkaloids cause arrhythmias. The sensitivity to these arrhythmogenic substances differs among various species.

Electrically Induced Arrhythmias

The possibilities to produce arrhythmias by electrical stimulation of the heart and the difficulties for evaluation of anti-arrhythmic drugs by this approach have

been discussed by Szekeres (1971). Serial electrical stimulation result in flutter and fibrillation and it is possible to reproduce some of the main types of arrhythmias of clinical importance. The flutter threshold or the ventricular multiple response threshold may be determined in anesthetized dogs before or after the administration of the test drug.

Mechanically Induced Arrhythmias

Arrhythmias can be induced directly by ischemia or by reperfusion. After ischemia either by infarction or by coronary ligation several phases of arrhythmias are found. The two stage coronary artery ligation technique described by Harris (1950) focuses on late arrhythmias.

Curtis and Walker (1988) examined seven scores in an attempt to validate the use of arrhythmia scores in an *in vivo* model of conscious rats.

The influence on reperfusion arrhythmias can be tested in various species, e g., rat, pig, dog and cat (Bergey et al. 1982; Winslow 1984; Curtis et al. 1987; Brooks et al. 1989).

REFERENCES AND FURTHER READING

Bergey JL, Nocella K, McCallum JD (1982) Acute coronary artery occlusion-reperfusion-induced arrhyhythmias in rats, dogs, and pigs: antiarrhythmic evaluation of quinidine, procainamide and lidocaine. Eur J Pharmacol 81:205–216

Borchard U, Berger F, Hafner D (1989) Classification and action of antiarrhythmic drugs. Eur Heart J 10 (Suppl E):31–40

Brooks RR, Miller KE, Carpenter JF, Jones SM (1989) Broad sensitivity of rodent arrhythmia models to class I, II, III, and IV antiarrhythmic agents. Proc Soc Exp Biol Med 191:201–209

Cheung PH, Pugsley MK, Walker MJA (1993) Arrhythmia models in the rat. J Pharmacol Toxicol Meth 29:179–184

Colatsky TJ, Follmer CH (1990) Potassium channels as targets for antiarrhythmic drug action. Drug Dev Res 19:129–140

Coromilas J (1991) Classification of antiarrhythmic agents: electropharmacologic basis and clinical relevance. Cardiovasc Clin 22:97–116

Curtis MJ, Walker MJA (1988) Quantification of arrhythmias using scoring systems: an examination of seven scores in an *in vivo* model of regional myocardial ischemia. Cardiovasc Res 22:656–665

Curtis MJ, Macleod BA, Walker MJA (1987) Models for the study of arrhythmia in myocardial ischaemia and infarction: the use of the rat. J Mol Cell Cardiol 19:399–419

Ellis CH (1956) Screening of drugs for antiarrhythmic activity. Ann NY Acad Sci 64:552–563

Frumin H, Kerin NZ, Rubenfire M (1989) Classification of antiarrhythmic drugs. J Clin Pharmacol 29:387–394

Grant (1992) On the mechanism of action of antiarrhythmic agents. Am Heart J 123:1130–1136

Harris AS (1950) Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. Circ Res 1:1318–1328

Harumi K, Tsutsumi T, Sato T, Sekiya S (1989) Classification of antiarrhythmic drugs based on ventricular fibrillation threshold. Am J Cardiol 64:10J–14J

Nattel S (1991) Antiarrhythmic drug classifications. A critical appraisal of their history, present status, and clinical relevance. Drugs 41:672–701

Nattel S (1993) Comparative mechanisms of action of antiarrhythmic drugs. Am J Cardiol 72:13F–17F

Podrid PJ, Mendes L, Beau SL, Wilson JS (1990) The oral antiarrhythmic drugs. Progr Drug Res 35:151–247

Ravens U (1992) Einteilungsprinzipien der Antiarrhythmika bei Herzrhythmusstörungen. Z Kardiol 81: Suppl 4:119–125

Rosen MR, Schwartz PJ (1991) The Sicilian Gambit. A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic mechanisms. Circulation 84:1831–1851

Sanguinetti MC (1992) Modulation of potassium channels by antiarrhythmic and antihypertensive drugs. Hypertension 19:228–236

Scholz H (1991) New classification of antiarrhythmic drugs. The modulated receptor hypothesis. New Trends Antiarrhythm 7:275–289

Scholz H (1994) Classification and mechanism of action of antiarrhythmic drugs. Fundam Clin Pharmacol 8:385–390

Sugimoto T, Murakawa Y, Toda I (1989) Evaluation of antifibrillatory effects of drugs. Am J Cardiol 64:33J–36J

Szekeres L (1979) Experimental models for the study of antiarrhythmic agents. In: Progress in Pharmacology Vol 2/4, pp 25–31, Gustav Fischer Verlag, Stuttgart

Szekeres L, Papp JG (1971) Production of experimental arrhythmias and methods for evaluating antiarrhythmic action. In: Experimental Cardiac Arrhythmias and Antiarrhythmic drugs. Académiai Kladó, Budapest, pp 24–92

Szekeres L, Papp JG (1975) Experimental cardiac arrhythmias. In: Schmier J, Eichler O (eds) Experimental Production of Diseases, Part 3, Heart and Circulation, Handbook of Experimental Pharmacology Vol XVI/3, Springer-Verlag New York Berlin Heidelberg, pp 131–182

The cardiac arrhythmia suppression trial (CAST) investigators (1990) Preliminary report: Effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. New Engl J Med 321:406–412

Vaughan-Williams EM (1970) Classification of antiarrhythmic drugs. In: Sandøe E, Flensted-Jensen E, OLesen KH (eds) Symposium on cardiac arrhythmias. Elsinore, Denmark, April 23–25, 1970, Publ. by AB Astra, Södertälje, Sweden, pp 449–472

Vaughan Williams EM (1975) Classification of antidysrhythmic drugs. Pharmacol Ther B1:115–138

Vaughan Williams EM (1984) A classification of antiarrhythmic actions reassessed after a decade of new drugs. J Clin Pharmacol 24:129–147

Vaughan Williams EM (1988) Classification of antiarrhythmic actions. In: Handbook of Experimental Pharmacology Vol 89:45–67, Springer, Heidelberg

Vaughan Williams EM (1991) Significance of classifying antiarrhythmic actions since the cardiac arrhythmia suppression trial. J Clin Pharmacol 31:123–135

Vaughan Williams EM (1992) Classifying antiarrhythmic actions: by facts or speculation. J Clin Pharmacol 32:964–977

Walker MJA, Curtis MJ, Hearse DJ, Campbell RWF, Janse MJ, Yellon DM, Cobbe SM, Coker SJ, Harness JB, Harron DWG, Higgins AJ, Julian DG, Lab MJ, Manning AS, Northover BJ, Parratt JR, Riemersma RA, Riva E, Russell DC, Sheridan DJ, Winslow E, Woodward B (1988) The Lambeth Conventions: guidelines for the study of arrhyth-

mias in ischemia, infarction, and reperfusion. Cardiovasc Res 22:447–455
Weirich J, Antoni H (1990) Differential analysis of the frequency-dependent effects of class 1 antiarrhythmic drugs according to periodical ligand binding: implications for antiarrhythmic and proarrhythmic activity. J Cardiovasc Pharmacol 15:998–1009
Weirich J, Antoni H (1991) Neue Aspekte zur frequenzabhängigen Wirkung von Klasse-1-Antiarrhythmika. Eine kritische Analyse der gebräuchlichen Subklassifikation. Z Kardiol 80:177–186
Wilson E (1984) Methods for detection and assessment of antiarrhythmic activity. Pharmacol Ther 24:401–433
Winbury MM (1956) Relation between atrial and ventricular anti-arrhythmic assay methods: rationale for a screening program. Ann NY Acad Sci 64:564–573
Woosley RL (1991) Antiarrhythmic drugs. Annu Rev Pharmacol Toxicol 31:427–455

A.5.0.2 Electrocardiography in Animals

PURPOSE AND RATIONALE

Recording of the electrocardiogram is an essential tool in the evaluation of anti-arrhythmic drugs (Johnston et al. 1983; Curtis and Walker 1986; Adaikan et al. 1992). Similar to the heart rate, the electrocardiogram is different between various species (Bazett 1920; Kisch 1953; Heise and Kimbel 1955; Beinfield and Lehr 1968; Budden et al. 1981; Driscoll 1981; Osborn 1981; Hayes et al. 1994). Many authors used the bipolar lead II between right foreleg and left hindleg, which is in line with the neutrally placed heart. Additionally, lead I (between right and left foreleg) stated to lie in the axis of the horizontal heart, and lead III (between left foreleg and left hindleg) in line with the vertical heart, may be used as well as unipolar leads (usually designed as V_1 to V_6) and the unipolar leads designed as aVL, aVR, and aVF. Out of several species being used the procedure for rats (Penz et al. 1992; Hayes et al. 1994) is described.

PROCEDURE

Male Sprague Dawley rats weighing 250–300 g are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbitone. The right jugular vein is cannulated for injections, while the left coronary artery is cannulated for recording blood pressure on a polygraph. The ECG is recorded using a Lead type II of configuration along the anatomical axis of the heart as determined by palpation. ECGs are recorded at a standard chart speed of 100 mm/s on a polygraph and simultaneously on a storage oscilloscope. Measurements of intervals are made on the chart recorder and from the memory trace of the monitor.

Since in the rat it is difficult to detect a T-wave that corresponds exactly with the T-wave seen in other species (Beinfeld and Lehr 1968; Driscoll 1981; Surawicz 1987) T-wave calculations are made on the basis of the repolarization wave that follows the QRS complex. The following variables are measured:

σ *T* = time for the depolarization wave to cross the atria, *P-R* interval, *QRS* interval, *Q-T* interval, and *RSh* (the height between the peak of R and S wave). The *RSh* magnitude is taken as a measure of the extent of S-wave depression as exerted by class I sodium channel blocking antiarrhythmics.

EVALUATION

Statistical analyses are based on ANOVA followed by Duncan's test for differences of means. In order to demonstrate the relationships between and drug effects, standard cumulative dose-response curves are constructed.

MODIFICATIONS OF THE METHOD

Osborne (1973, 1981) described a restraining device facilitating electrocardiogram recording in **conscious rats**.

Curtis and Walker (1986), Johnston et al. (1983) studied the responses to ligation of a coronary artery and the actions of antiarrhythmics in conscious rats.

Hayes et al. (1994) studied the ECG in **guinea pigs**, rabbits and primates.

Stark et al. (1989) described an epicardic surface and stimulation technique (SST-ECG) in Langendorff perfused guinea pig hearts.

Epicardial His bundle recordings in the guinea pig *in vivo* were described by Todt and Raberger (1992).

Chronic recording from the His bundle in awake nonsedated **dogs** was reported by Karpawich et al. (1983) and by Atlee et al. (1984).

Van de Water et al. (1989) reported a formula to correct the QT interval of the electrocardiogram in dogs for changes in heart rate.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-reentry and ventricular antifibrillatory studies in anesthetized dogs. The reentry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The pacing induced tachycardia that results from circus movements around the tricuspid ring was very persistent in duration and regular in cycle length. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

Weissenburger et al. (1991) developed an experimental model of the long QT syndrome in conscious dogs for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes."

Bauer et al. (2004) described pro- and antiarrhythmic effects of fast cardiac pacing in a canine model of acquired long QT syndrome.

Holter monitoring in conscious dogs was described by Krumpl et al. (1989a, b).

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Baboons and **monkeys** *(Macaca sp.)* were used by Adaikan et al. (1992).

REFERENCES AND FURTHER READING

Adaikan G, Beatch GN, Lee TL, Ratnam SS, Walker MJA (1992) Antiarrhythmic actions of Tedisamil: Studies in rats and primates. Cardiovasc Drugs Therapy 6:345–352

Atlee JL, Dayer AM, Houge JC (1984) Chronic recording from the His bundle in the awake dog. Basic Res Cardiol 79:627–638

Bauer A, Donahue JK, Voss F, Becker R, Kraft P, Senges JC, Kelemn K, Katus HA, Schoels W (2004) Pro- and antiarrhythmic effects of fast cardiac pacing in a canine model of acquired long QT syndrome. Naunyn Schmiedebergs Arch Pharmacol 369:447–454

Bazett HC (1920) An analysis of the time-relations of electrocardiograms. Heart 7:353–370

Beinfield WH, Lehr D (1968) QRS-T variations in the rat electrocardiogram. Am J Physiol 214:197–204

Budden R, Buschmann G, Kühl UG (1981) The rat ECG in acute pharmacology and toxicology. In: Budden R, Detweiler DK, Zbinden G (eds) The Rat Electrocardiogram in Pharmacology and Toxicology. Pergamon Press, Oxford, New York, pp 41–81

Coker SJ (1989) The anesthetized rabbit as a model for ischemia- and reperfusion-induced arrhythmias: Effects of quinidine and bretylium. J Pharmacol Meth 21:263–279

Curtis MJ, Walker MJA (1986) The mechanisms of action of the optical enantiomers of verapamil against ischaemia-induced arrhythmias in the conscious rat. Br J Pharmac 89:137–147

Driscoll P (1981) The normal rat electrocardiogram. In: Budden R, Detweiler DK., Zbinden G (eds) The Rat Electrocardiogram in Pharmacology and Toxicology. Pergamon Press, Oxford, New York, pp 1–14

Hayes E, Pugsley MK, Penz WP, Adaikan G, Walker MJA (1994) Relationship between QaT and RR intervals in rats, guinea pigs, rabbits and primates. J Pharmacol Toxicol Meth 32:201–207

Heise E, Kimbel KH (1955) Das normale Elektrokardiogramm der Ratte. Z Kreislaufforsch 44:212–221

Johnston KM, McLeod BA, Walker JMA (1983) Responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics. Can J Physiol Pharmacol 61:1340–1353

Karpawich PP, Gillette PC, Lewis RM, Zinner A, McNamara DG (1983) Chronic epicardial His bundle recordings in awake nonsedated dogs: a new method. Am Heart J 105:16–21

Kisch B (1953) The heart rate and the electrocardiogram of small animals. Exp Med Surg 11:117–130

Krumpl G, Todt H, Schunder-Tatzber S, Raberger G (1989a) Holter monitoring in conscious dogs. Assessment of arrhythmias occurring during ischemia and in the early reperfusion phase. J Pharmacol Meth 22:77–91

Krumpl G, Todt H, Schunder-Tatzber S, Raberger G (1989b) Holter monitoring in conscious dogs. Assessment of arrhythmias occurring in the late reperfusion phase after coronary occlusion. J Pharmacol Meth 22:93–102

Osborne BE (1973) A restraining device facilitating electrocardiogram recording in rats. Lab Anim 7:185–188

Osborn BE (1981) The electrocardiogram (ECG) of the rat. In: Budden R, Detweiler DK., Zbinden G (eds) The Rat Electrocardiogram in Pharmacology and Toxicology. Pergamon Press, Oxford, New York, pp 15–28

Penz W, Pugsley M, Hsieh MZ, Walker MJA (1992) A new ECG measure (RSh) for detecting possible sodium channel blockade *in vivo* in rats. J Pharmacol Meth 27:51–58

Stark G, Stark U, Tritthart HA (1989) Assessment of the conduction of the cardiac impulse by a new epicardic surface and stimulation technique (SST-ECG) in Langendorff perfused mammalian hearts. J Pharmacol Meth 21:195–209

Surawicz B (1987) The QT interval and cardiac arrhythmias. Ann Rev Med 38:81–90

Todt H, Raberger G (1992) Epicardial His bundle recordings in the guinea pig *in vivo*. J Pharmacol Toxicol Meth 27:191–195

Van de Water A, Verheyen J, Xhonneux R, Reneman RS (1989) An improved method to correct the QT interval of the electrocardiogram for changes in heart rate. J Pharmacol Meth 22:207–217

Weissenburger J, Chezalviel F, Davy JM, Lainée P, Guhennec C, Penin E, Engel F, Cynober L, Motté G, Cheymol G (1991) Methods and limitations an experimental model of the long QT syndrome. J Pharmacol Meth 26:23–42

Wu KM, Hunter TL, Proakis AG (1990) A dual electrophysiologic test for atrial antireentry and ventricular antifibrillatory studies. Effects of bethanidine, procainamide and WY-48986. J Pharmacol Meth 23:87–95

A.5.0.3 Aconitine Antagonism in Rats

PURPOSE AND RATIONALE

The plant alkaloid aconitine persistently activates sodium channels. Infusion of aconitine in the anesthetized rat causes ventricular arrhythmias. Drugs considered to have anti-arrhythmic properties can be tested in aconitine-intoxicated rats.

PROCEDURE

Male Ivanovas rats weighing 300–400 g are used. The animals are anesthetized by intraperitoneal injection of 1.25 g/kg urethane. Five µg/kg aconitine dissolved in 0.1 N HNO_3 is administered by continuous infusion into the saphenous vein of 0.1 ml/min and the ECG in lead II is recorded every 30 seconds. The test compound is injected orally or intravenously at a screening dose of 3 mg/kg 5 min before the start of the aconitine infusion. Eight–ten animals are used per compound.

EVALUATION

The anti-arrhythmic effect of a test compound is measured by the amount of aconitine/100 g animal (duration of infusion) which induces

- ventricular extrasystoles
- ventricular tachycardia
- ventricular fibrillation
- and death.

Higher doses of aconitine in the treated group as compared to an untreated control group are an indication of anti-arrhythmic activity.

Statistical significance between the groups is assessed by the Student's *t*-test.

The scores are allotted for the intensity and the duration of the effect relative to the efficacy of standard compounds.

Standard data:

- Procainamid, 5 mg/kg i.v. and lidocaine, 5 mg/kg, i.v. lead to an increase in LD_{100} by 65% (corresponds to LD_{100} of approximately 9 µg/100 g).

CRITICAL ASSESSMENT OF THE METHOD

Aconitine – antagonism *in vivo* has been proven as a valuable screening method for anti-arrhythmic activity.

MODIFICATIONS OF THE METHOD

Scherf (1947) studied the auricular tachycardia caused by aconitine administration in **dogs**.

Scherf et al. (1960) provoked atrial flutter and fibrillation in anesthetized dogs by application of a few crystals of aconitine or delphinine to the surface of the right atrium in the appendix area near the head of the sinus node.

Mc Leod and Reynold (1962) induced arrhythmia by aconitine in the isolated **rabbit** atrium.

Nwangwu et al. (1977) used aconitine as arrhythmogenic agent for screening of anti-arrhythmic agents in **mice**.

Yamamoto et al. (1993) used urethane-anesthetized rats under artificial respiration with tubocurarine pretreatment. After thoracotomy and incision of the pericardium, a piece of filter paper soaked with aconitine solution was applied to the right atrium. Test drugs were applied by continuous i.v. infusion. In addition to ECG lead II, intra-atrial ECG was monitored.

Aconitine-antagonism in conscious mice as screening procedure has been recommended by Dadkar and Bhattacharya (1974) and in anesthetized mice by Winslow (1980).

Nakayama et al. (1971) described the topical application of aconitine in a small cup placed on the right atrium of dogs to induce supraventricular arrhythmias.

A method using the **cat** has been developed by Winslow (1981).

Other Arrhythmogenic Agents

In addition to the aconitine model Vaille et al. (1992) demonstrated the selectivity of a $CaCl_2$ continuous infusion screening method in rats for the evaluation of antiarrhythmic calcium antagonists.

A mouse chloroform model was recommended by Lawson (1968).

Vargaftig et al. (1969) induced ventricular fibrillation in mice by inhalation of chloroform.

Papp et al. (1967) proposed the experimental $BaCl_2$-arrhythmia as a quantitative assay of anti-arrhythmic drugs.

Al-Obaid et al. (1998) used calcium chloride-induced arrhythmias for anti-arrhythmic activity evaluation in anesthetized male rats. Cardiac arrhythmias were induced by a single intravenous injection of 10% $CaCl_2$ (50 mg/kg). The induced arrhythmias were then analyzed for magnitude of initial bradycardia, onset, incidence and duration of the induced fibrillations. After the induction of the arrhythmia, the animal was allowed to recover completely (15–20 min) and the test compound was injected in different doses intravenously. The effect of the test compound on the basal heart rate was then examined and the percentage change in the heart rate was calculated. Seven min later, the arrhythmogenic dose of $CaCl_2$ was readministered and the effect of the treatment on the induced arrhythmia parameters was evaluated as percentage change in the measured parameters or as protection or non-protections against the induced fibrillations.

Tripathi and Thomas (1986) described a method for the production of ventricular tachycardia in the rat and guinea pig by exposing the animals to benzene vapors for 2 min followed by an intravenous adrenaline injection.

Arrhythmias could be induced by changing the medium of cultured rat heart muscle cells (Wenzel and Kloeppel 1978).

In isolated rat hearts ventricular fibrillation was induced by isoprenaline and a catechol-O-methyl transferase inhibitor at high perfusion temperature (Sono et al. 1985).

Takei (1994) described experimental arrhythmia in guinea pigs induced by grayanotoxin-I, a biologically active diterpenoid from the plant family of Ericaceae.

REFERENCES AND FURTHER READING

Al-Obaid AM, El-Subbagh HI, Al-Shabanah OA, Mahran MA (1998) Synthesis and biological evaluation of new cyclopenteno[b]-thiophene derivatives as local anesthetic and antiarrhythmic agents. Pharmazie 53:24–28

Bazzani C, Genedani S, Tagliavini S, Bertolini A (1989) Putrescine reverses aconitine-induced arrhythmia in rats. J Pharm Pharmacol 41:651–653

Brooks RR, Carpenter JF, Jones SM, Gregory CM (1989) Effects of dantrolene sodium in rodent models of cardiac arrhythmia. Eur J Pharmacol 164:521–530

Dadkar NK, Bhattacharya BK (1974) A rapid screening procedure for antiarrhythmic activity in the mouse. Arch Int Pharmacodyn Ther 212:297–301

Lawson JW (1968) Antiarrhythmic activity of some isoquinoline derivatives determined by a rapid screening procedure in the mouse. J Pharmacol Exp Ther 160:22–31

Lu HR, De Clerk F (1993) R 56 865, a Na^+/Ca^{2+}-overload inhibitor, protects against aconitine-induced cardiac arrhythmias *in vivo*. J Cardiovasc Pharmacol 22:120–125

Nakayama K, Oshima T, Kumakura S, Hashimoto K (1971) Comparison of the effects of various β-adrenergic blocking agents with known antiarrhythmic drugs on aconitine-arrhythmia produced by the cup method. Eur J Pharmacol 14:9–18

Nwangwu PU, Holcslaw TL, Stohs JS (1977) A rapid *in vivo* technique for preliminary screening of antiarrhythmic agents in mice. Arch Int Pharmacodyn 229:219–226

Papp G, Szekeres L, Szmolenszky T (1967) The effects of quinidine, ajmaline, papaverine and adrenergic beta-receptor inhibitors in experimental $BaCl_2$-arrhythmia developed for the quantitative assay of antiarrhythmic drugs. Acta Phys Acad Sci Hung 32:365–375

Paróczai M, Kárpáti E, Solti F (1990) The effect of bisaramil on experimental arrhythmias. Pharmacol Res 22:463–480

Scherf D (1947) Studies on auricular tachycardia caused by aconitine administration. Proc Soc Exp Biol Med 64:233–239

Scherf D, Blumenfeld S, Taner D, Yildiz M (1960) The effect of diphenylhydantoin (Dilantin) on atrial flutter and fibrillation provoked by focal application of aconitine or delphinine. Am Heart J 60:936–947

Sono K, Akimoto Y, Magaribuchi T, Kurahashi K, Fujiwara M (1985) A new model of ventricular fibrillation induced by isoprenaline and catechol-O-methyl transferase inhibitor at high perfusion temperature in isolated rat hearts. J Pharmacol Meth 14:249–254

Takei (1994) Grayanotoxin-I induced experimental arrhythmia in guinea pig. J Aichi Med Univ Assoc 22:495–512

Tripathi RM, Thomas GP (1986) A simple method for the production of ventricular tachycardia in the rat and guinea pig. J Pharmacol Meth 15:279–282

Vaille A, Scotto di Tella AM, Maldonado J, Vanelle P (1992) Selectivity of a $CaCl_2$ continuous infusion screening method in rats. Meth Find Exp Clin Pharmacol 14:183–187

Vargaftig BV, Coignet JL, Walmetz JL, Lefort J (1969) A critical evaluation of three methods for the study of adrenergic beta-blocking and anti-arrhythmic agents. Eur J Pharmacol 6:49–55

Wenzel DG, Kloeppel JW (1978) Arrhythmias induced by changing the medium of cultured rat heart muscle cells: a model for assessment of antiarrhythmic agents. J Pharmacol Meth 1:269–276

Winslow E (1980) Evaluation of antagonism of aconitine-induced dysrhythmias in mice as a method of detecting and assessing antidysrhythmic activity. Br J Pharmac 71:615–622

Winslow E (1981) Hemodynamic and arrhythmogenic effects of aconitine applied to the left atria of anesthetized cats. Effects of amiodarone and atropine. J Cardiovasc Pharmac 3:87–100

Yamamoto T, Hosoki K, Karasawa T (1993) Anti-arrhythmic effects of a new calcium antagonist, Monopetil, AJ-2615, in experimental arrhythmic models. Clin Exper Pharmacol Physiol 20:497–500

A.5.0.4 Digoxin-Induced Ventricular Arrhythmias in Anesthetized Guinea Pigs

PURPOSE AND RATIONALE

Overdose of cardiac glycosides, such as digoxin, induces ventricular extrasystoles, ventricular fibrillation, and finally death. The occurrence of these symptoms can be delayed by anti-arrhythmic drugs.

PROCEDURE

Male guinea pigs (Marioth strain) weighing 350–500 g are anesthetized with 35 mg/kg pentobarbital sodium intraperitoneally. Trachea, one jugular vein and one carotid artery are catheterized. Positive pressure ventilation is applied with a respiratory pump (Rhema GmbH, Germany) at 45 breaths/min. The carotid artery is used for monitoring systemic blood pressure via a pressure transducer. Digoxin is infused into the jugular vein with a perfusion pump (ASID BONZ PP 50) at a rate of 85 µg/kg in 0.266 ml/min until cardiac arrest. The electrocardiogram (lead III) is recorded with subcutaneous steel-needle electrodes (Hellige 19).

Treated groups ($n = 5$–10 animals) receive the test drug either orally 1 h or intravenously 1 min prior to the infusion. The control group ($n =$ at least 5 animals) receives the digoxin infusion only. The period until the onset of ventricular extrasystoles, ventricular fibrillation, and cardiac arrest is recorded. The total amount of infused digoxin (µg/kg) to induce ventricular fibrillation is calculated. Standard drugs are lidocaine (3 mg/kg i.v.) or ramipril (1 mg/kg p.o.).

EVALUATION

Using Student's *t*-test the doses of digoxin needed to induce ventricular extrasystoles, or ventricular fibrillation, or cardiac arrest, respectively, after treatment with anti-arrhythmic drugs are compared statistically with controls receiving digoxin only.

REFERENCES AND FURTHER READING

Brooks RR, Carpenter JF, Jones SM, Gregory CM (1989) Effects of dantrolene sodium in rodent models of cardiac arrhythmia. Eur J Pharmacol 164:521–530

Dörner J (1955) Zur Frage der Beziehungen zwischen Strophanthintoxicität und der Größe der Coronardurchblutung. Arch exp Path Pharmakol 226:152–162

Lindner E (1963) Untersuchungen über die flimmerwidrige Wirkung des N-(3′-phenyl-propyl-(2′))-1,1-diphenylpropyl-(3)-amins (Segontin). Arch Int Pharmacodyn 146:485–500

Linz W, Schölkens BA, Kaiser J, Just M, Bei-Yin Q, Albus U, Petry P (1989) Cardiac arrhythmias are ameliorated by local inhibition of angiotensin formation and bradykinin degradation with the converting-enzyme inhibitor ramipril. Cardiovasc Drugs Ther 3:873–882

Windus H (1952) Die Beeinflussung der Glykosidwirkung am Herzen durch coronargefässwirksame Medikamente. Klin Wschr 30:215–217

A.5.0.5
Strophanthin or Ouabain Induced Arrhythmia

PURPOSE AND RATIONALE

Acute intoxication with the cardiac glycoside strophanthin K induces ventricular tachycardia and multifocal ventricular arrhythmias in dogs. This can be used as a test model to evaluate the effect of potential antiarrhythmic drugs on ventricular arrhythmias.

PROCEDURE

Male or female dogs of either sex weighing approximately 20 kg are used. The animals are anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium. Two peripheral veins are cannulated for the administration of the arrhythmia-inducing substance (*V. brachialis*) and the test compound (*V. cephalica antebrachii*). For intraduodenal administration of the test drug, the duodenum is cannulated. Electrocardiogram is registered with needle electrodes from lead II. Heart frequency is derived from R-peaks of ECG. Two–three animals are used for one compound.

Strophanthin K is administered by continuous i.v.-infusion at a rate of 3 µg/kg/min. Thirty–fourty min later, signs of cardiac glycoside intoxication appear leading to ventricular tachycardia or to multifocal ventricular arrhythmias. When this state is achieved, the strophanthin infusion is terminated. When the arrhythmias are stable for 10 min, the test substance is administered intravenously in doses between 1.0 and 5.0 mg/kg or intraduodenally in doses between 10 and 30 mg/kg.

ECG II recordings are obtained at times: -0.5, 1, 2, 5 and 10 min following administration of test drug.

For i.v. administration: A test compound is considered to have an anti-arrhythmic effect if the extrasystoles immediately disappear. If the test compound does not show a positive effect, increasing doses are administered at 15 min-intervals. If the test substance does reverse arrhythmias, the next dose is administered after the reappearance of stable arrhythmias.

For i.d. administration: A test compound is considered to have a definite anti-arrhythmic effect if the extrasystoles disappear within 15 min. The test drug is considered to have "no effect" if it does not improve strophanthin intoxication within 60 min following drug administration.

EVALUATION

Evaluation of the therapeutic effect of a drug is difficult and somewhat arbitrary since there is no clear-cut correlation between effectiveness of a test compound and duration of its effect, i. e. return to normal ECGs. The standard drugs ajmaline, quinidine and lidocaine re-establish normal sinus rhythm at doses of 1 and 3 mg/kg (i.v.) and 10 mg/kg (i.d.). Arrhythmias are eliminated for 20 min (i.v.) and for > 60 min (i.d.) following drug administration.

MODIFICATIONS OF THE METHOD

Ettinger et al. (1969) used arrhythmias in dogs induced by ouabain to study the effects of phentolamine in arrhythmia.

Garrett et al. (1964) studied the antiarrhythmic activity of *N,N*-diisopropyl-*N*'-diethylaminoethylurea hydrochloride in anesthetized dogs with arrhythmias induced by ouabain, aconitine or acetylcholine. Furthermore, ultra-low frequency ballistocardiograms with ECG registration were performed in dogs.

Raper and Wale (1968) studied the effects on ouabain- and adrenaline-induced arrhythmias in **cats**.

Kerr et al. (1985) studied the effects of a vasodilator drug on ouabain-induced arrhythmias in anesthetized dogs.

A modified method for the production of cardiac arrhythmias by ouabain in anesthetized cats was published by Rao et al. (1988).

Brooks et al. (1989) infused ouabain intravenously to **guinea pigs** and determined the onset of ventricular extrasystoles and of fibrillation.

Thomas and Tripathi (1986) studied the effects of α-adrenoreceptor agonists and antagonists with different affinity for α_1- and α_2-receptors on ouabain-induced arrhythmias and cardiac arrest in guinea pigs.

Krzeminski (1991) and Wascher et al. (1991) used of ouabain-induced arrhythmia in guinea pigs for the evaluation of potential antidysrhythmic agents.

Al-Obaid et al. (1998) used ouabain-induced arrhythmias in anesthetized **Wistar rats** for evaluation of cyclopenteno[b]thiophene derivatives as antiarrhythmic agents.

REFERENCES AND FURTHER READING

Al-Obaid AM, El-Subbagh HI, Al-Shabanah OA, Mahran MA (1998) Synthesis and biological evaluation of new cyclopenteno[b]thiophene derivatives as local anesthetic and antiarrhythmic agents. Pharmazie 53:24–28

Brooks RR, Miller KE, Carpenter JF, Jones SM (1989) Broad sensitivity of rodent arrhythmia models to class I, II, III, and IV antiarrhythmic agents. Proc Soc Exp Biol Med 191:201–209

Dörner J (1955) Zur Frage der Beziehungen zwischen Strophanthintoxicität und Größe der Coronardurchblutung. Arch exper Path Pharmakol 226:152–162

Duce BR, Garberg L, Johansson B (1967) The effect of propranolol and the dextro and laevo isomers of H 56/28 upon ouabain-induced ventricular tachycardia in unanaesthetized dogs. Acta Pharmacol Toxicol 25: Suppl 2:41–49

Ettinger S, Gould L, Carmichael JA, Tashjian RJ (1969) Phentolamine: use in digitalis-induced arrhythmias. Am Heart J 77:636–640

Garrett J, Gonçalves Moreira M, Osswald W, Guimarães S (1964) Antiarrhythmic activity of N,N-diisopropyl-N′-diethylamino-ethylurea hydrochloride. J Pharmacol Exp Ther 143:243–251

Kerr MJ, Wilson R, Shanks RG (1985) Suppression of ventricular arrhythmias after coronary artery ligation by Pinacidil, a vasodilator drug. J Cardiovasc Pharmacol 7:875–883

Krzeminski T (1991) A rapid *in vivo* technique for the screening of potential anti-dysrhythmic agents. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany, pp 131–135

Rao TS, Seth SD, Nayar U, Manchanda SC (1988) Modified method for the production of cardiac arrhythmias by ouabain in anesthetized cats. J Pharmacol Meth 20:255–263

Raper C, Wale J (1968) Propranolol, MJ-1999 and Ciba-39089-Ba in ouabain and adrenaline induced cardiac arrhythmias. Eur J Pharmac 4:1–12

Thomas GP, Tripathi RM (1986) Effects of α-adrenoreceptor agonists and antagonists on ouabain-induced arrhythmias and cardiac arrest in guinea pig. Br J Pharmac 89:385–388

Wascher TC, Dittrich P, Kukovetz WR (1991) Antiarrhythmic effects of two new propafenone related drugs. A study on four animals models of arrhythmia. Arzneim Forsch/Drug Res 41:119–124

A.5.0.6 Ventricular Fibrillation Electrical Threshold

PURPOSE AND RATIONALE

The use of anti-arrhythmic drugs in the treatment of ventricular arrhythmias aims to prevent the development of ventricular fibrillation. Several electrical stimulation techniques have been used to measure ventricular fibrillation threshold such as single pulse stimulation, train of pulses stimulation, continuous 50-Hz stimulation and sequential pulse stimulation.

PROCEDURE

Adult dogs weighing 8–12 kg are anesthetized with sodium pentobarbital (35 mg/kg) and ventilated with air using a Harvard respiratory pump. Systolic arterial pressure is monitored and body temperature maintained by a thermal blanket. The chest is opened by a midline sternotomy and the heart suspended in a pericardial cradle. The sinus node is crushed and a 2.0 mm diameter Ag-AgCl stimulating electrode is embedded in a Teflon disc sutured to the anterior surface of the left ventricle. The heart is then driven by 3-ms square anodal constant current pulses for 400 ms of the basic cycle and is prematurely stimulated by one 3-ms test stimulus through the driving electrode. Electrical stimulation is programmed by a digital stimulator. A recording electrode is placed on the surface of each ventricle. A silver plate is implanted under the skin in the right femoral region as indifferent electrode. Lead II of the body surface electrocardiogram is monitored. To determine ventricular fibrillation threshold (VFT), a 0.2- to 1.8-second train of 50-Hz pulses is delivered 100 ms after every eighteenth basic driving stimulus. The current intensity is increased from the diastolic threshold in increments of 10 µA to 1.0 mA or until ventricular fibrillation occurs. The minimal current intensity of the pulse train required to induce sustained ventricular fibrillation is defined as the VFT. When ventricular fibrillation occurs, the heart is immediately defibrillated and allowed to recover to control conditions for 15 to 20 min. Anti-arrhythmic drugs are administered through the femoral vein.

EVALUATION

Ventricular fibrillation threshold (VFT) is determined before and after administration of test drugs at given time intervals. The mean values of 10 experiments are compared using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Marshall et al. (1981) and Winslow (1984) suggested to determine VFT in the pentobarbitone anesthetized rat.

Wu et al. (1989) recommended a conscious dog model for re-entrant atrial tachycardia.

Wu et al. (1990) described a dual electrophysiologic test for atrial anti-re-entry and ventricular antifibrillatory studies in dogs. The re-entry portion of the model was created surgically by a Y-shaped crushing around the tissue between the superior and inferior vena cava and tissue parallel to the AV groove. The antifibrillatory activities were assessed by determination of the ventricular fibrillation threshold using a train-stimuli method.

A chronically prepared rat model of electrically induced arrhythmias was described by Walker and Beatch (1988).

REFERENCES AND FURTHER READING

Burgess MJ, Williams D, Ershler P (1977) Influence of test site on ventricular fibrillation threshold. Am Heart J 94:55–61

Harumi K, Tsutsumi T, Sato T, Sekiya S (1989) Classification of antiarrhythmic drugs based on ventricular fibrillation threshold. Am J Cardiol 64:10J–14J

Jaillon P, Schnittger I, Griffin JC, Winkle RA (1980) The relationship between the repetitive extrasystole threshold and the ventricular fibrillation threshold in the dog. Circ Res 46:599–605

Marshall RJ, Muir AW, Winslow E (1981) Comparative antidysrhythmic and hemodynamic effects of orally or intravenously administered mexiletine and Org 6001 in the anesthetized rat. Br J Pharmacol 74:381–388

Murakawa Y, Toda I, Nozaki A, Kawakubo K, Sugimoto T (1989) Effects of antiarrhythmic drugs on the repetitive extrasystole threshold and ventricular fibrillation threshold. Cardiology 76:58–66

Papp JG, Szekeres L (1968) Analysis of the mechanism of adrenaline actions on ventricular vulnerability. Eur J Pharmacol 3:5–26

Sugimoto T, Murakawa Y, Toda I (1989) Evaluation of antifibrillatory effects of drugs. Am J Cardiol 64:33J–36J

Vanremoortere E, Wauters E (1986) Fibrillation threshold curves and anti-arrhythmic drugs. Arch Int Pharmacodyn 176:476–479

WalkerMJA, Beatch GN (1988) Electrically induced arrhythmias in the rat. Proc West Pharmacol Soc 31:167–170

Wilson E (1984) Methods for detection and assessment of antiarrhythmic activity. Pharmacol Ther 24:401–433

Wu KM, Proakis AG, Hunter TL, Shanklin JR (1989) Effects of AHR-12234 on cardiac transmembrane action potentials, *in situ* cardiac electrophysiology and experimental models of arrhythmia. Arch Int Pharmacodyn 301:131–150

Wu KM, Hunter TL, Proakis AGl (1990) A dual electrophysiologic test for atrial anti-re-entry and ventricular antifibrillatory studies. J Pharmacol Meth 23:87–95

A.5.0.7
Coronary Artery Ligation, Reperfusion Arrhythmia and Infarct Size in Rats

PURPOSE AND RATIONALE

Coronary artery ligation in anesthetized rats results in arrhythmias and myocardial infarction. Following occlusion of the left main coronary artery, very marked ventricular dysrhythmias occur. Electrocardiogram is recorded during ligation and subsequent reperfusion. The amount of infarcted tissue is measured by means of p-nitro-blue tetrazolium chloride-staining in myocardial sections. The model is used to test drugs with potential anti-arrhythmic activities.

PROCEDURE

Groups of 8–10 male Sprague-Dawley rats weighing 350–400 g are used. The animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. The trachea is intubated to allow artificial ventilation (Starling pump). A catheter is placed in an external jugular vein for administration of test compounds. Peripheral blood pressure is recorded from the common carotid artery using a pressure transducer and a polygraph. The chest is opened by left thoracotomy at the forth intercostal space. After opening the pericard, the heart is exteriorized by gentle pressure on the chest walls and a thin silk thread (Ethicon 1.5 metric, 4–0) attached to an atraumatic needle is placed around the left coronary artery about 2–3 mm distal of the origin of the left coronary artery for later ligation. From that point on, the animal is ventilated with room air using a stroke volume of 1 ml/100 g body weight at a rate of 54 strokes/min. The heart is then placed back in the chest cavity. Any animal in which this procedure itself produces dysrhythmias or a sustained fall in mean arterial blood pressure to less than 70 mm Hg has to be discarded from the study.

After an equilibration time of approx. 45 min, the test substance or the vehicle (control) is administered by intravenous injection. Five min later, the ligature at the left coronary artery is closed either for 15 or 90 min (in case infarct-size is assessed) and subsequently reperfused for 30 min. For oral application, the test compounds are dissolved or suspended in the vehicle 30 min before occlusion. Peripheral blood pressure and ECG lead II are recorded continuously during the whole experiment. Rectal temperature is maintained at 38°C. The numbers of ventricular premature beats (VPB), ventricular tachycardia (VT) and ventricular fibrillation (VF) are counted in the occlusion and reperfusion periods and evaluated according to the guidelines of the Lambeth Convention (Walker et al. 1988).

Preparation to Determine Infarct Size

At the end of the reperfusion period, the animal is sacrificed with an overdose of pentobarbital sodium, the heart is dissected and cut into transversal sections (approx. 1 mm thick) from the apex to the base. The slices are stained with p-nitro-blue tetrazolium chloride solution (0.25 g/L p-nitro-blue tetrazolium chloride in Sörensen phosphate buffer, containing 100 mM D, L-maleate) in order to visualize the infarct tissue (blue/violet-stained healthy tissue, unstained necrotic tissue). The slices are photographed on color transparency film for the determination of infarct area. Left ventricle and infarct area are measured by planimetry from projections of all slices with the exclusion of the apex and the slice containing the ligature.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
  - peripheral blood pressure [mm Hg]
  - heart rate [beats/min]
  - pressure rate index (PRI) (BPs × HR) [mm Hg × beats/1000]
- arrhythmias
  - ventricular extrasystoles (= premature ventricular contractions) (PVC)
    - percent animals with PVC
    - number of PVC/5 or 30 min
  - ventricular tachycardia (VT) (VT defined as any run of seven or more consecutive ventricular extrasystoles)
    - percent animals with VT
    - duration [s] of VT/5 or 30 min
  - ventricular fibrillation (VF)
    - percent animals with VF
    - duration [s] of VF/5 or 30 min
- infarct size (area)

The different characteristics are evaluated separately and compared with a positive control (5 mg/kg nicainoprol i.v.).

Changes of parameters in drug-treated animals are compared to vehicle control values.

Statistical significance is assessed by the Student's *t*-test.

MODIFICATIONS OF THE METHOD

Leprán et al. (1983) placed a loose silk loop around the left coronary artery and passed the thread through a cylinder shaped polyethylene tube outside the thorax. The rats were allowed to recover from primary surgery. The loose ligature was tightened 7–10 days thereafter and arrhythmias recorded by ECG tracings.

Johnston et al. (1983) described the responses to ligation of a coronary artery in conscious rats and the actions of anti-arrhythmics.

As reported in Sect. A.3.1.2 and A.3.1.3, the isolated heart according to LANGENDORFF and the isolated working rat heart preparation can be used for ligation experiments inducing arrhythmias. Lubbe et al. (1978) reported ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart as a model for assessment of anti-fibrillatory action of anti-arrhythmic agents.

Bernier et al. (1986) described reperfusion-induced arrhythmias in the isolated perfused rat heart. The isolated rat heart was perfused according the LANGENDORFF-technique. A ligature was placed around the left anterior descending coronary artery close to its origin. The arterial occlusion was maintained for 10 min followed by reperfusion. Test compounds were included in the perfusion medium. With epicardial ECG-electrodes the number of premature ventricular complexes, the incidence and duration of ventricular fibrillation, and the incidence of ventricular tachycardia were recorded.

Abraham et al. (1989) tested antiarrhythmic properties of tetrodotoxin against occlusion-induced arrhythmias produced by ligation of the left anterior descending coronary artery in the rat.

MacLeod et al. (1989) tested a long acting analogue of verapamil for its actions against arrhythmias induced by ischemia and reperfusion in conscious and anesthetized rats, as well as for effects on epicardial intracellular action potentials.

Harper et al. (1993) found that the inhibition of Na^+/H^+ exchange preserves viability, restores mechanical function, and prevents the pH paradox in reperfusion injury to rat neonatal myocytes.

Scholz et al. (1993, 1995) reported protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion in rats.

Likewise, Yasutake et al. (1994) found protection against reperfusion-induced arrhythmias in rats by intracoronary infusion of a Na^+/H^+ exchange inhibitor.

Aye et al. (1997) tested the effects of a Na^+/H^+ exchange inhibitor on reperfusion ventricular arrhythmias in rat hearts.

Ferrara et al. (1990) studied the effect of flecainide acetate on reperfusion- and barium-induced ventricular tachyarrhythmias in the isolated perfused rat heart by monitoring heart rate, coronary flow rate, left ventricular systolic pressure, dp/dt_{max}, and the voltage of the epicardial electrogram.

Heterogeneity of ventricular remodeling after acute myocardial infarction in rats has been reported by Capasso et al. (1992).

Bellemin-Baurreau et al. (1994) described an *in vitro* method for evaluation of antiarrhythmic and antiischemic agents by using programmed electrical stimulation of the isolated rabbit heart after ligation of the left ventricular branch of the coronary artery and a reperfusion period of 15 min.

The use of the rat in models for the study of arrhythmias in myocardial ischemia and infarction has been reviewed by Curtis et al. (1987).

Black and Rodger (1996), Black (2000) reviewed the methods used to study experimental myocardial ischemic and reperfusion injury in various animal species.

Linz et al. (1997) reported that in isolated rat hearts with ischemia-reperfusion injuries, perfusion with bradykinin reduces the duration and incidence of ventricular fibrillations, improves cardiodynamics, reduces release of cytosolic enzyme, end preserves energy-rich phosphate and glycogen stores.

Mulder et al. (1998) studied the effects of chronic treatment with calcium antagonists in rats with chronic heart failure induced by coronary artery ligation.

The effect of antihypertensive agents on cardiac and vascular remodelling was discussed by Mallion et al. (1999).

REFERENCES AND FURTHER READING

Abraham S, Beatch GN, MacLeod BA, Walker MJA (1989) Antiarrhythmic properties of tetrodotoxin against occlusion-induced arrhythmias in the rat: a novel approach to the study of antiarrhythmic effects of ventricular sodium channel blockade. J Pharmacol Exp Ther 251:1166–1173

Aye NN, Xue YX, Hashimoto K (1997) Antiarrhythmic effects of cariporide, a novel Na^+-H^+ exchange inhibitor, on reperfusion ventricular arrhythmias in rat hearts. Eur J Pharmacol 339:121–127

Bellemin-Baurreau J, Poizot A, Hicks PE, Armstrong JM (1994) An *in vitro* method for evaluation of antiarrhythmic and antiischemic agents by using programmed electrical stimulation of rabbit heart. J Pharmacol Toxicol Meth 31:31–40

Bernier M, Hearse DJ, Manning AS (1986) Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. Circ Res 58:331–340

Black SC, Rodger IW (1996) Methods for studying experimental myocardial ischemic and reperfusion injury. J Pharmacol Toxicol Meth 35:179–190

Black SC (2000) *In vivo* models of myocardial ischemia and reperfusion injury. Application to drug discovery and evaluation. J Pharmacol Toxicol Meth 43:153–167

Brooks RR, Carpenter JF, Jones SM, Gregory CM (1989) Effects of dantrolene sodium in rodent models of cardiac arrhythmia. Eur J Pharmacol 164:521–530

Capasso JM, Li P, Zhang X, Anversa P (1992) Heterogeneity of ventricular remodeling after acute myocardial infarction in rats. Am J Physiol 262 (Heart Circ Physiol 31):H486–H495

Clark C, Foreman MI, Kane KA, McDonald FM, Parratt JR (1980) Coronary artery ligation in anesthetized rats as a method for the production of experimental dysrhythmias and for the determination of infarct size. J Pharmacol Meth 3:357–368

Colatsky Th (1989) Models of myocardial ischemia and reperfusion injury: Role of inflammatory mediators in determining infarct size. Pharmacological Methods in the Control of Inflammation, pp 283–320, Alan R. Liss, Inc

Curtis MJ, Macleod BA, Walker MJA (1987) Models for the study of arrhythmias in myocardial ischaemia and infarction: the use of the rat. J Mol Cell Cardiol 19:399–419

Ferrara N, Abete P, Leosco D, Caccese P, Orlando M, Landino P, Sederino S, Tedeschi C, Rengo F (1990) Effect of flecainide acetate on reperfusion- and barium-induced ventricular tachyarrhythmias in the isolated perfused rat heart. Arch Int Pharmacodyn 308:104–114

Harper IS, Bond JM, Chacon E, Reece JM, Herman B, Lemasters JJ (1993) Inhibition of Na^+/H^+ exchange preserves viability, restores mechanical function, and prevents the pH paradox in reperfusion injury to rat neonatal myocytes. Basic Res Cardiol 88:430–442

Harris N, Kane KA, Muir AW, Winslow E (1982) Influences of hypothermia, cold, and isolation stress on the severity of coronary artery ligation-induced arrhythmias in rats. J Pharmacol Meth 7:161–171

Harris S (1950) Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. Circ Res 1:1318–1328

Johnston KM, MacLeod BA, Walker MJA (1983) Responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics. Can J Physiol Pharmacol 61:1340–1353

Krzeminski T (1991) Reperfusion – induced arrhythmias in anaesthetized rats; The essential model for the study of potential anti-dysrhythmic and cytoprotective drugs according to the LAMBETH conventions. 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany, pp 125–130

Leprán I, Koltai M, Siegmund W, Szekeres L (1983) Coronary artery ligation, early arrhythmias, and determination of the ischemic area in conscious rats. J Pharmacol Meth 9:219–230

Linz W, Wiemer G, Schölkens BA (1997) Beneficial effects of bradykinin on myocardial energy metabolism and infarct size. Am J Cardiol 80:118A–123A

Lubbe WF, Daries PS, Opie LH (1978) Ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart: a model for assessment of antifibrillatory action of antiarrhythmic agents. Cardiovasc Res 12:212–220

Mallion JM, Baguet JP, Siche JP, Tremel F, de Gaudemaris R (1999) Cardiac and vascular remodelling: effect of antihypertensive agents. J Human Hypertens 13 [Suppl 1]:S35–S41

Manning AS, Hearse DJ (1984) Review: Reperfusion-induced arrhythmias: Mechanism and prevention. J Mol Cell Cardiol 16:497–518

MacLeod BA, Moult M, Saint KM, Walker MJA (1989) The antiarrhythmic efficacy of anipamil against occlusion and reperfusion arrhythmias. Br J Pharmacol 98:1165–1172

Martorana PA, Linz W, Göbel H, Petry P, Schölkens BA (1987) Effects of nicainoprol on reperfusion arrhythmia in the isolated working heart and on ischemia and reperfusion arrhythmia and myocardial infarct size in the anesthetized rat. Eur J Pharmacol 143:391–401

Mulder P, Richard V, Thuillez C (1998) Different effects of calcium antagonists in a rat model of heart failure. Cardiology 89, Suppl 1:33–37

Paróczai M, Kárpáti E, Solti F (1990) The effect of bisaramil on experimental arrhythmias. Pharmacol Res 22:463–480

Schölkens BA, Linz W, König W (1988) Effects of the angiotensin converting enzyme inhibitor, ramipril, in isolated ischaemic rat heart are abolished by a bradykinin antagonist. J Hypertens 6, Suppl 4:S25–S28

Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Schölkens BA (1992) Effects of Na^+/H^+ exchange inhibitors in cardiac ischaemia. J Mol Cell Cardiol 24:731–740

Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Schölkens BA (1993) Hoe 694, a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. Br J Pharmacol 109:562–568

Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Schölkens BA (1993) Hoe 694, a new Na^+/H^+

exchange inhibitor and its effects in cardiac ischaemia. Br J Pharmacol 109:562–568
Scholz W, Albus U, Counillon L, Gögelein H, Lang HJ. Linz W, Weichert A, Schölkens BA (1995) Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc Res 29:260–268
Selye H, Bajusz E, Grasso S, Mendell P (1960) Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11:398–407
Sun W, Wainwright CL (1994) The potential antiarrhythmic effects of exogenous and endogenous bradykinin in the ischaemic heart *in vivo*. Coron Artery Dis 5:541–550
Uematsu T, Vozeh S, Ha HR, Hof RP, Follath F (1986) Coronary ligation-reperfusion arrhythmia models in anesthetized rats and isolated perfused rat hearts. Concentration-effect relationships of lidocaine. J Pharmacol Meth 16:53–61
van Gilst WH, de Graeff PA, Wesseling H, de Langen CDJ (1986) Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: A comparison of captopril, enalapril, and HOE 489. J Cardiovasc Pharmacol 8:722–728
Walker MJA, Curtis MJ, Hearse DJ, Campbell RWF, Janse MJ, Yellon DM, Cobbe SM, Coker SJ, Harness JB, Harron DWG, Higgins AJ, Julian DG, Lab MJ, Manning AS, Northover BJ, Parratt JR, Riemersma RA, Riva E, Russell DC, Sheridan DJ, Winslow E, Woodward B (1988) The Lambeth Conventions; guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. Cardiovasc Res 22:447–455
Yasutake M, Ibuki C, Hearse DJ, Avkiran M (1994) Na+/H+ exchange and reperfusion arrhythmias: protection by intracoronary infusion of a novel inhibitor. Am J Physiol:H2430–H2440

A.5.0.8
Ventricular Arrhythmia After Coronary Occlusion

A.5.0.8.1
Ventricular Fibrillation After Coronary Occlusion and Reperfusion in Anesthetized Dogs

PURPOSE AND RATIONALE

Coronary artery occlusion in anesthetized dogs is accompanied by an increase in heart rate, heart contractility, left ventricular end-diastolic pressure, and blood pressure as well as by ventricular arrhythmias. During a subsequent reperfusion period, a high percentage of control animals die from ventricular fibrillation. Drugs with potential protective effects are tested which reduce both hemodynamic and electrical changes.

PROCEDURE

Dogs of either sex weighing 20–25 kg are used. Anesthesia is induced by intravenous injection of 30 mg/kg thiobutobarbital sodium and maintained by i.v. administration of 20 mg/kg chloralose and 250 mg/kg urethane followed by subcutaneous administration of 2 mg/kg morphine. The animals are placed in the right lateral position. Respiration is maintained through a tracheal tube using a positive pressure respirator (Bird Mark 7). A peripheral vein (saphenous vein) is cannulated for the administration of test compound. The ECG is recorded continuously in lead II (Einthoven).

Preparation for Hemodynamic Measurements

For recording of peripheral systolic and diastolic blood pressure, the cannula of a femoral artery is connected to a pressure transducer (Statham P 23 DB). For determination of left ventricular pressure (LVP), a Millar microtip catheter (PC 350) is inserted via the left carotid artery. Left ventricular end-diastolic pressure (LVEDP) is measured on a high-sensitivity scale; heart rate (HR) is determined from the LVP wave form. Myocardial contractility is measured as the rate of rise of LVP (dp/dt max). The sum of ST-segment elevations is calculated from five values of the peripheral limbs in ECG lead II. The pressure-rate index ($PRI = BPs \times HR$) serves as a measure of oxygen consumption.

Experimental Course

The heart is exposed through a left thoracotomy between the fourth and fifth intercostal space, the pericard is opened and the left anterior descending coronary artery (LAD) is prepared. A silk suture is placed around the LAD, just below the first diagonal branch. After an equilibration period of approx. 45 min., the test substance or the vehicle (controls) is administered as an intravenous bolus. Twenty min later, the ligature at the coronary artery is closed for 90 min. During the occlusion period, the test compound or the vehicle (controls) are given by continuous infusion. After release of the coronary obstruction, the animal is monitored for a 30 min reperfusion period. All parameters are recorded during the whole experiment. At the end of the test, surviving animals are sacrificed by an overdose of pentobarbital sodium.

EVALUATION

The following parameters are evaluated:

- mortality
- hemodynamics
- arrhythmias
  - ventricular extrasystoles (= premature ventricular contractions) (PVC)
  - percent animals with PVC
  - number of PVC/5 or 30 min

- ventricular tachycardia (VT) (VT defined as any sequence of seven or more consecutive ventricular extrasystoles)
- duration [s] of VT/5 or 30 min
- ventricular fibrillation (VF)
- percent animals with VF

The different characteristics are evaluated separately. Changes of parameters in drug-treated animals are compared to vehicle controls. Statistical significance of the differences is calculated by means of the Student's *t*-test.

Standard data:

Mortality: In an representative experiment, 10 out of 12 of control animals died from ventricular fibrillation during the 30 min reperfusion period. One out of 8 molsidomine-treated animals died and the death was also from ventricular fibrillation during the reperfusion phase. (Molsidomine was given as a continuous infusion of 0.5 mg/kg/ml/min during the occlusion period; controls received saline).

MODIFICATIONS OF THE METHOD

Varma and Melville (1963) described ventricular fibrillation induced by coronary occlusion during hypothermia in **dogs**.

Wilkerson and Downey (1978) described a technique for producing ventricular arrhythmias in dogs through coronary occlusion by an embolus (glass beads) being introduced into the coronary circulation via a rigid cannula which is inserted through the carotid artery.

Weissenburger et al. (1991) described a model in dogs suitable for screening the bradycardia-dependent proarrhythmic effects of drugs and for studying the electrophysiology of "torsades de pointes".

Coker (1989) recommended the anesthetized **rabbit** as a model for ischemia- and reperfusion-induced arrhythmias.

Thiemermann et al. (1989) described a rabbit model of experimental myocardial ischemia and reperfusion. Drugs were administered by intravenous infusion 5 min after the occlusion of the left anterior-lateral coronary artery and continued during the 60 min occlusion and subsequent 3 h reperfusion periods.

Hendrikx et al. (1994) reported that the Na^+-H^+ exchange inhibitor HOE 694 improves post-ischemic function and high-energy phosphate resynthesis and reduced Ca^{2+} overload in the isolated perfused rabbit heart.

Barrett et al. (1997) described a method of recording epicardial monophasic action potentials and ischemia-induced arrhythmias following coronary artery ligation in intact rabbits.

Naslund et al. (1992) described a closed chest model in *pigs*. Occlusion was induced in pentobarbitone anesthetized, mechanically ventilated **pigs** by injection of a 2 mm ball into a preselected coronary artery. Reperfusion was achieved by retraction of the ball via an attached filament.

D'Alonzo et al. (1994) evaluated the effects of potassium channel openers on pacing- and ischemia-induced ventricular fibrillation in anesthetized pigs.

Sack et al. (1994) described the effects of a Na^+/H^+ antiporter inhibitor on post-ischemic reperfusion in pig heart.

Premaratne et al. (1995) used a **baboon** open chest model of myocardial ischemia and reperfusion. Baboons underwent occlusion of the left anterior descending coronary artery for 2 h. Fifteen min after occlusion, the treated group received hyaluronidase i.v. over a 10-min period. The ischemic period was followed by 22 h of reperfusion. At the end of the reperfusion period, the hearts were excised and the perfusion bed at risk for infarction was determined by infusion of a microvascular dye.

REFERENCES AND FURTHER READING

Barrett TC, MacLeod BA, Walker MJA (1997) A model of myocardial ischemia for the simultaneous assessment of electrophysiological changes and arrhythmias in intact rabbits. J Pharmacol Toxicol Meth 37:27–36

Coker SJ (1989) Anesthetized rabbit as a model for ischemia- and reperfusion-induced arrhythmias. Effects of quinidine and bretylium. J Pharmacol Meth 21:263–279

Colatsky TJ (1989) Models of myocardial ischemia and reperfusion injury: Role of inflammatory mediators in determining infarct size. In: Pharmacological Methods in the Control of Inflammation. Alan R. Liss, Inc., pp 283–320

D'Alonzo AJ, Hess TA, Darbenzio RB, Sewter JC, Conder ML, McCullough JR (1994) Effects of cromakalim or pinacidil on pacing- and ischemia-induced ventricular fibrillation in the anesthetized pig. Basic Res Cardiol 89:163–176

Hendrikx M, Mubagwa K, Verdonck F, Overloop K, Van Hecke P, Vanstapel F, Van Lommel A, Verbeken E, Laurweryns J, Flameng W (1994) New Na^+-H^+ exchange inhibitor HOE 694 improves postischemic function and high-energy phosphate resynthesis and reduced Ca^{2+} overload in isolated perfused rabbit heart. Circulation 89:2787–2798

Krumpl G, Todt H, Schunder-Tatzber S, Raberger G (1990) Programmed electrical stimulation after myocardial infarction and reperfusion in conscious dogs. J Pharmacol Meth 23:155–169

Linz W, Schölkens BA (1992) Role of bradykinin in the cardiac effects of angiotensin-converting enzyme inhibitors. J Cardiovasc Pharmacol 20(Suppl 9):S83–S90

Linz W, Wiemer G, Gohlke P, Unger T, Schölkens BA (1994) The contribution of bradykinin to the cardiovascular actions of ACE inhibitors. In Lindpaintner K, Ganten D (eds) The Cardiac Renin Angiotensin System. Futura Publ Co., Inc., Armonk, NY, pp 253–287

Martorana PA, Mogilev AM, Kettenbach B, Nitz RE (1983) Effect of molsidomine on spontaneous ventricular fibrillation following myocardial ischemia and reperfusion in the dog. In: Chazov E, Saks V, Rona G (eds) Advances in Myocardiology. Plenum Publishing Comp., New York, Vol. 4, pp 605–613

Martorana PA, Kettenbach B, Breipohl G, Linz W, Schölkens BA (1990) Reduction of infarct size by local angiotensin-converting enzyme inhibition is abolished by a bradykinin antagonist. Eur J Pharmacol 182:395–396

Naslund U, Haggmark S, Johansson G, Pennert K, Reiz S, Marklund SL (1992) Effects of reperfusion and superoxide dismutase on myocardial infarct size in a closed chest pig model. Cardiovasc Res 26:170–178

Premaratne S, Watanabe BI, LaPenna WF, McNamara JJ (1995) Effects of hyaluronidase on reducing myocardial infarct size in a baboon model of ischemia-reperfusion. J Surg Res 58:205–210

Sack S, Mohri M, Schwarz ER, Arras M, Schaper J, Ballagi-Pordány G, Scholz W, Lang HJ, Schölkens BA, Schaper W (1994) Effects of a new Na^+/H^+ antiporter inhibitor on postischemic reperfusion in pig heart. J Cardiovasc Pharmacol 23:72–78

Thiemermann C, Thomas GR, Vane JR (1989) Defibrotide reduces infarct size in a rabbit model of experimental myocardial ischaemia and reperfusion. Br J Pharmacol 97:401–408

Varma DR, Melville KI (1963) Ventricular fibrillation induced by coronary occlusion during hypothermia in dogs and the effects of quinidine, quinacrine and oxytocin. Canad J Biochem Physiol 41:511–517

Weissenburger J, Chezalviel F, Davy JM, Lainée P, Guhennec C, Penin E, Engel F, Cynober L, Motté G, Cheymol G (1991) Methods and limitations of an experimental model of long QT syndrome. J Pharmacol Meth 26:23–42

Wilkerson DR, Downey JM (1978) Ventricular arrhythmias produced by coronary artery occlusion in closed-chest dogs. J Pharmacol Meth 1:39–44

A.5.0.8.2 Harris Dog Model of Ventricular Tachycardia

PURPOSE AND RATIONALE

In 1950, Harris found that the mortality in dogs after coronary occlusion with a 2-stage ligation procedure was lower than with 1-stage ligation. The left descending coronary artery is partially occluded for 30 min after which time total ligation is performed. Under these conditions arrhythmias develop within 4–7 h, reach a peak between 24 and 48 h and abate within 3–5 days.

PROCEDURE

Surgical Procedure

Dogs of either sex are anesthetized by intravenous injection of methohexitone sodium (10 mg/kg), an endotracheal tube is inserted, and anesthesia maintained with halothane. The heart is exposed through an incision in the fourth or fifth intercostal space. The anterior descending branch of the left coronary artery is dissected free below its second branch and ligated in two stages. Two ligatures are placed around the artery and a 21 gauge needle. The first ligature is tied round the artery and the needle, which is then removed. Thirty min later, the second ligature is tied tightly round the artery. The chest is closed in layers 30 min after the second ligature has been tied, and the dog is allowed to recover.

Test Procedure

Further observations are made when the dogs are conscious, e. g., 22–24 h after ligation of the coronary artery. The dogs are positioned to lie on their side and remain in this position throughout the experiment. Mean blood pressure is recorded from a catheter placed in the femoral artery. Lead II and aV_L of the electrocardiogram and blood pressure are continuously recorded for a control period of 30 min before and during drug administration. Drugs are administered either by injection or by continuous infusion via a hind leg vein.

EVALUATION

The number of sinus and ectopic beats are counted for each successive 5-min period. Beats with a distinct P wave preceding a mean frontal QRS vector of normal duration are counted as sinus in origin; all others are denoted as ectopic.

MODIFICATIONS OF THE METHOD

The model which resembles late arrhythmias occurring in postinfarction patients has been used with modifications by many authors (e. g., Kerr et al. 1985; Reynolds and Brown 1986; Gomoll 1987; Garthwaite et al. 1989; Krumpl et al. 1989a, b; Trolese-Mongheal et al. 1985, 1991; Spinelli et al. 1991).

Methods for producing experimental complete atrioventricular block in dogs were described and reviewed by Dubray et al. (1983) and by Boucher and Duchene-Marullaz (1985).

REFERENCES AND FURTHER READING

Boucher M, Duchene-Marullaz P (1985) Methods for producing experimental complete atrioventricular block in dogs. J Pharmacol Meth 13:95–107

Clark BB, Cummings JR (1956) Arrhythmias following experimental coronary occlusion and their response to drugs. Ann NY Acad Sci 64:543–551

Dubray C, Boucher M, Paire M, Duchene-Marullaz P (1983) A method for determining the atrial effective refractory period in the unanaesthetized dog. J Pharmacol Meth 9:157–164

Garthwaite SM, Hatley FR, Frederik LG, Cook C (1989) Efficacy and plasma concentrations of SC-36602 in canine models of ventricular arrhythmia. J Cardiovasc Pharmacol 13:218–226

Gomoll AW (1987) Assessment of drug effects on spontaneous and induced ventricular arrhythmias in a 24-h canine infarction model. Arzneim Forsch/Drug Res 37:787–794
Harris AS (1950) Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. Circ Res 1:1318–1328
Kerr MJ, Wilson R, Shanks RG (1985) Suppression of ventricular arrhythmias after coronary artery ligation by Pinacidil, a vasodilator drug. J Cardiovasc Pharmacol 7:875–883
Krumpl G, Todt H, Schunder-Tatzber S, Raberger G (1989a) Holter monitoring in conscious dogs. Assessment of arrhythmias occurring during ischemia and in the early reperfusion phase. J Pharmacol Meth 77–91
Krumpl G, Todt H, Schunder-Tatzber S, Raberger G (1989b) Holter monitoring in conscious dogs. Assessment of arrhythmias occurring in the late reperfusion phase after coronary occlusion. J Pharmacol Meth 22:92–102
Menken U, Wiegand V, Bucher P, Meesmann W (1979) Prophylaxis of ventricular fibrillation after acute coronary occlusion by chronic beta-adrenoceptor blockade with atenolol. Cardiovasc Res 13:588–594
Reynolds RD, Brown BS (1986) Antiarrhythmic activity of flestolol, a novel ultra-short-acting β-adrenoceptor antagonist, in the dog. Eur J Pharmacol 131:55–66
Spinelli W, Hoffman B, Hoffman BF (1991) Antiarrhythmic drug action in the Harris dog model of ventricular tachycardia. J Cardiovasc Electrophysiol 2:21–33
Trolese-Mongheal Y, Trolese JF, Lavarenne J, Duchene-Marullaz P (1985) Use of experimental myocardial infarct to demonstrate arrhythmogenic activity of drugs. J Pharmacol Meth13:225–234
Trolese-Mongheal Y, Barthelemy J, Trolese JF, Duchene-Marullaz P (1991) Time course of spontaneous ventricular arrhythmias following acute coronary occlusion in the dog. J Pharmacol Meth 26:125–137
Vegh A, Papp JG, Parratt J (1994) Attenuation of the antiarrhythmic effects of ischaemic preconditioning by blockade of bradykinin B_2 receptors. Br J Pharmacol 113:1167–1172

A.5.0.8.3 Protection against Sudden Coronary Death

PURPOSE AND RATIONALE

The group of Lucchesi described an experimental dog model to test protection against sudden coronary death (Patterson et al. 1982; Uprichard et al. 1989a, b; Chi et al. 1990a, b, 1991; Kitzen et al. 1990; Black et al. 1991, 1993).

Surgical Preparation

Purpose-bred male mongrel dogs weighing 14–22 kg are anesthetized with 30 mg/kg pentobarbital i.v. The dogs are ventilated with room air through a cuffed endotracheal tube and a Harvard respirator. A cannula is inserted in the left external jugular vein. A left thoracotomy is performed between the fourth and fifth ribs, and the heart is exposed and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) is isolated at the tip of the left atrial appendage, and the left circumflex coronary artery (LCX) is isolated ~1 cm from its origin. After a 20-gauge hypodermic needle has been placed on the LAD, a ligature is tied around the artery and the needle. The needle is then removed, resulting in critical stenosis of the vessel. The LAD is perfused for 5 min in the presence of the critical stenosis. Ischemic injury of the anterior ventricular myocardium is achieved by 2-h occlusion of the LAD by a silicon rubber snare. The vessel is reperfused after 2 h in the presence of the critical stenosis. During the period of LAD reperfusion, an epicardial bipolar electrode (1-mm silver posts, 3-mm interelectrode separation) is sutured on the left atrial appendage for subsequent atrial pacing. A bipolar plunge electrode (25-gauge stainless steel, 5 mm long, 3 mm separation) is sutured on the interventricular septum, adjacent to the occlusion site and overlying the right ventricular outflow tract (RVOT). Two similar stainless steel bipolar plunge electrodes are sutured to the left ventricular (LV) wall: one at the distribution of the LAD distal to the occlusion (infarct zone, IZ), and the second in the distribution of the LCX (non-infarct zone, NZ). A 30-gauge silver-coated copper wire electrode is passed through the wall and into the lumen of the LCX and sutured to the adjacent surface of the heart. Silver disc electrodes are implanted subcutaneously for ECG monitoring. The surgical incision is closed and the animals are allowed to recover.

Drug Treatment

The animals are treated after the recovery period during the 3 days of programmed electrical stimulation either with the test drug or with the solvent.

Electrophysiologic Studies and Programmed Electrical Stimulation

Programmed electrical stimulation (PES) is performed between days 3 and 5 after induction of anterior myocardial infarction by occlusion/perfusion of the LAD. Animals are studied while conscious and unsedated. Heart rate, ECG intervals and other electrophysiologic parameters (for details see original publications) are determined before PES is started. Premature ventricular stimuli are introduced in the region of the right ventricular outflow tract. The extra stimuli are triggered from the R-wave of the ECG, and the R-S_2 coupling interval is decreased from 350 ms until ventricular refractoriness occurs. At this time, double and triple ventricular extra stimuli are introduced during sinus rhythm. Ventricular tachyarrhythmias are defined as 'non- sustained', if five or more repetitive ventricular responses are initiated reproducibly, but terminated spontaneously. Ventricular tachyarrhythmias are

defined as 'sustained', if they persist for at least 30 s or, in the event of hemodynamic compromise, require ventricular burst pacing for their termination.

Sudden Cardiac Death

A direct anodal 15 µA current from a 9-V nickel-cadmium battery is passed through a 250 Ohm resistor and applied to the electrode in the lumen of the left circumflex coronary artery. The cathode of the battery is connected to a s.c. implanted disc electrode. Lead II ECG is recorded for 30 s every 15 min on a cardiocassette recorder. After 24 h of constant anodal current or development of ventricular fibrillation, the animals are sacrificed, the hearts are excised and the thrombus mass in the LCX is removed and weighed. The heart is sectioned transversely and incubated for 15 min at 37°C in a 0.4% solution of tetrazolium triphenyl chloride for identification of infarcted areas. Time of onset of ventricular ectopy and of lethal arrhythmia is provided from recordings of the cardiocassette.

EVALUATION

Non-sustained and sustained tachyarrhythmias are evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Sudden coronary death is one of the leading causes of death in developed countries. These facts warrant the use of complicated models in higher animals for search of active drugs.

MODIFICATIONS OF THE METHOD

Schwartz et al. (1984) described an experimental preparation for sudden cardiac death in dogs. The animals were chronically instrumented and studied 1 month after an anterior myocardial infarction. A balloon catheter around the circumflex coronary artery was inflated to produce acute myocardial ischemia and the occlusion was maintained for 2 min. Several days later, the animals were subjected to a submaximal stress on a motor-driven treadmill for 12–18 min. During the last minute of exercise the left coronary artery was occluded, the treadmill stopped, and the occlusion was maintained for a second minute.

Schwartz et al. (1988) analyzed the baroreceptor reflexes in conscious dogs with and without a myocardial infarction to get insights in the mechanisms of sudden death.

Cahn and Cervoni (1990) reviewed of the use of animal models of sudden cardiac death for drug development.

Pak et al. (1997) found that canine tachycardia-induced cardiomyopathy is a useful model for studying mechanisms and therapy of sudden cardiac death in heart failure. Adamson et al. (1994) performed a longitudinal study in dogs at high and low risk for sudden death and found an unexpected interaction between β-adrenergic blockade and heart rate variability before and after myocardial infarction.

Basso et al. (2004) recommended arrhythmogenic right ventriculopathy causing sudden death in boxer dogs as an animal model of human disease.

REFERENCES AND FURTHER READING

Adamson PB, Huang MH, Vanoli E, Foreman RD, Schwartz PJ, Hull SS (1994) Unexpected interaction between β-adrenergic blockade and heart rate variability before and after myocardial infarction. A longitudinal study in dogs at high and low risk for sudden death. Circulation 90:976–982

Basso C, Fox PR, Meurs KM, Towbin JA, Spier AW, Calabrese F, Maron BJ, Thiene G (2004) Arrhythmogenic right ventriculopathy causing sudden death in boxer dogs. A new animal model of human disease. Circulation 109:1180–1185

Black SC, Chi L, Mu DX, Lucchesi RR (1991) The antifibrillatory actions of UK 68,798, a class III antiarrhythmic agent. J Pharm Exp Ther 258:416–423

Black SC, Butterfield JL, Lucchesi BR (1993) Protection against programmed stimulation-induced ventricular tachycardia and sudden coronary death by NE-10064, a class III antiarrhythmic drug. J Cardiovasc Pharmacol 22:810–818

Cahn PS, Cervoni P (1990) Current concepts and animal models of sudden cardiac death for drug development. Drug Dev Res 19:199–207

Chi L, Mu DX, Driscoll EM, Lucchesi BR (1990a) Antiarrhythmic and electrophysiologic actions of CK-3579 and sematilide in a conscious canine model of sudden coronary death. J Cardiovasc Pharmacol 16:312–324

Chi L, Uprichard ACG, Lucchesi BR (1990b) Profibrillatory actions of pinacidil in a conscious canine model of sudden coronary death. J Cardiovasc Pharmacol 15:452–464

Chi L, Mu DX, Lucchesi BR (1991) Electrophysiology and antiarrhythmic actions of E-4031 in the experimental animal model of sudden coronary death. J Cardiovasc Pharmacol 17:285–295

Kitzen JM, Chi L, Uprichard ACG, Lucchesi BR (1990) Effects of combined thromboxane synthase inhibition/thromboxane receptor antagonism in two models of sudden cardiac death in the canine: limited role of thromboxane. J Cardiovasc Pharmacol 16:68–80

Pak PH, Nuss HB. Tunin RS, Kaab S, Tomaselli GF, Marban E, Kass DA (1997) Repolarization, abnormalities, arrhythmia and sudden death in canine tachycardia-induced cardiomyopathy. J Am Coll Cardiol 30:576–584

Patterson E, Holland K, Eller BT, Lucchesi BR (1982) Ventricular fibrillation resulting from a site remote from previous myocardial infarction. A conscious canine model for sudden coronary death. Am J Cardiol 50:1414–1423

Schwartz PJ, Billman GE, Stone HL (1984) Autonomic mechanisms in ventricular fibrillation induced by myocardial ischemia during exercise in dogs with healed myocardial infarction. An experimental preparation for sudden cardiac death. Circulation 69:790–800

Schwartz PJ, Vanoli EV, Stramba-Badiale M, de Ferrari GM, Billman GE, Foreman RD (1988) Autonomic mechanisms and sudden death. New insights from analysis of baroreceptor reflexes in conscious dogs with and without a myocardial infarction. Circulation 78:969–979

Uprichard ACG, Chi L, Kitzen JM, Lynch JJ, Frye JW, Lucchesi BR (1989a) Celiprolol does not protect against ventricular tachycardia or sudden death in the conscious canine: a comparison with pindolol in assessing the role of intrinsic sympathomimetic activity. J Pharm Exp Ther 251:571–577

Uprichard ACG, Chi L, Lynch JJ, Frye JW, Driscoll EM, Frye JW, Lucchesi BR (1989b) Alinidine reduces the incidence of ischemic ventricular fibrillation in a conscious canine model, a protective effect antagonized by overdrive atrial pacing. J Cardiovasc Pharmacol 14:475–482

A.5.0.8.4 Ventricular Fibrillation Induced by Cardial Ischemia During Exercise

PURPOSE AND RATIONALE

Billman and his group developed methods to evaluate antiarrhythmic drugs for their activity in cardiovascular parameters in an exercise-plus-ischemia test.

PROCEDURE

Surgical Preparation

Mongrel dogs, weighing 15.4 to 19.1 kg, are anesthetized and instrumented to measure left circumflex CBF, left ventricular pressure and ventricular electrogram (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984). The animals are given Innovar Vet (0.02 mg/kg fentanyl citrate and 1 mg/kg hydroperidol i.v.) as a preanesthetic, whereas a surgical plane of anesthesia is induced with sodium pentobarbital (10 mg/kg i.v.). A left thoracotomy is made in the fourth intercostal space, and the heart is exposed and supported by a pericardial cradle. A 20-MHz pulsed Doppler flow transducer and a hydraulic occluder are placed around the left circumflex artery. A pair of insulated silver-coated wires are sutured to the epicardial surface of both the left and right ventricles. These electrodes are used for ventricular pacing or to record a ventricular electrogram from which HR is determined using a Gould Biotachometer (Gould Instruments, Cleveland, OH). A precalibrated solid-state pressure transducer (Konigsberg Instruments, Pasadena, CA) is inserted into the left ventricle via a stab wound in the apical dimple. Finally, a two-stage occlusion of the left anterior descending coronary artery is performed approximately one third the distance from the origin to induce an anterior wall myocardial infarction. This vessel is partially occluded for 20 min and then tied off. All leads from the cardiovascular instrumentation are tunneled under the skin to exit on the back of the animal's neck. A transdermal fentanyl patch that delivers 75 µg/h for 72 h is placed on the back of the neck (secured with adhesive tape) to decrease postoperative discomfort. In addition, bupivacaine HCl, a long-acting local anesthetic, is injected to block the intercostal nerves (i. e., pain fibers) in the area of the incision. Each animal is placed on prophylactic antibiotic therapy (amoxicillin 500 mg p.o.) three times daily for 7 days. The animals are placed in an "intensive care" setting for the first 24 h and placed on antiarrhythmic therapy (Billman and Hamlin 1996; Billman et al. 1993, 1997; Schwartz et al. 1984).

Exercise-Plus-Ischemia Test

The studies begin 3 to 4 weeks after the production of the myocardial infarction. The animals are walked on a motor-driven treadmill and trained to lie quietly without restraint on a laboratory table during this recovery period. Susceptibility to VF is then tested. The animals run on a motor-driven treadmill while workload is increased every 3 min for a total of 18 min. The protocol begins with a 3-min warm-up period, during which the animals run at 4.8 km/h at 0% grade. The speed is increased to 6.4 km/h, and the grade is increased every 3 min as follows: 0%, 4%, 8%, 12% and 16%. During the last minute of exercise, the left circumflex coronary artery is occluded, the treadmill is stopped and the occlusion is maintained for 1 additional min (total occlusion time, 2 min). Large metal plates (diameter, 11 cm) are placed across the animal's chest so that electrical defibrillation can be achieved with minimal delay but only after the animal is unconscious (10–20 s after VF begin). The occlusion is immediately released if VF occur.

The animals then receive one or more of the following treatments:

1 the exercise-plus-ischemia test is repeated after pretreatment with the standard drug glibenclamide (1.0 mg/kg i.v.). The drug is injected in a cephalic vein; 3 min before exercise begins.
2 The exercise-plus-ischemia test is repeated after pretreatment with the test drug
3 Finally, a second control (saline) exercise plus ischemia test is performed 1 week after the last drug test. At least 5 days are intermitted between drug treatments. Drugs are given in a random order.

Refractory Period Determination

On a subsequent day, the effective refractory period is determined using a Medtronic model 5325 programmable stimulator, both at rest and during myocardial ischemia. The heart is paced for 8 beats (S_1; in-

trastimulus interval, 300 ms; pulse duration, 1.8 ms at twice-diastolic threshold of ~6 mA). The intrastimulus interval is progressively shortened between the last paced beat and a single extrastimulus (S_2). The refractory period represents the shortest interval capable of generating a cardiac response and is measured using either the left or right ventricular electrodes. This procedure is completed within 30 s. Once the control values are determined, refractory period measurements are repeated after the standard drug glibenclamide (1.0 mg/kg i.v.), or the test drug. After the completion of these studies, refractory period is determined during myocardial ischemia (2-min occlusion of the left circumflex coronary artery) ~60 s after the onset of the coronary occlusion.

Reactive Hyperemia Studies

The K_{ATP} has been implicated in vascular regulation, particularly CBF (Aversano et al. 1991; Belloni and Hintze 1991; Daut et al. 1990). Therefore, the effects of standard and test drug on the response to brief interruptions in CBF are also evaluated. Animals are placed on a laboratory table, and the left circumflex coronary is occluded three or four times for 15 s. At least 2 min (or until CBF had returned to preocclusion base line) elapse between occlusions. The occlusions are then repeated 5 min after standard and test drug. On the subsequent day, the studies are repeated with the drug that had not been given the previous day.

EVALUATION

All hemodynamic data are recorded on a Gould model 2800S eight-channel recorder (Cleveland, OH) and a Teac model MR-30 FM tape recorder (Tokyo, Japan). Coronary blood flow is measured with a University of Iowa Bioengineering flowmeter model 545 C-4 (Iowa City, IA). The rate of change of left ventricular pressure [$d(LVP)/dt$] is obtained by passing the left ventricular pressure through a Gould differentiator that has a frequency response linear to > 300 Hz. The data are averaged over the past 5 s of each exercise level. The coronary occlusion data are averaged over the last 5 s before and at the 60-s line point (or VF onset) after occlusion onset. The total area between the peak CBF and return to base line is measured for each 15-s occlusion, and the percent repayment is calculated. The reactive hyperemia response to each occlusion is then averaged to obtain one value for each animal. The data are then analyzed using analysis of variance for repeated measures. When the F ratio is found to exceed a critical value ($P < 0.05$), Scheffe's test is used to compare the mean values. The effects of the drug intervention on arrhythmia formation are determined using a χ^2 test with Yates' correction for continuity. All data are reported as mean ± SEM. Cardiac arrhythmias, PR interval and QT interval are evaluated at a paper speed of 100 mm/s. QT interval is corrected for HR using Bazett's method.

CRITICAL ASSESSMENT OF THE METHOD

Tests combining coronary constriction with physical exercise may resemble most closely the situation in coronary patients.

REFERENCES AND FURTHER READING

Aversano T, Ouryang P, Silverman H (1991) Blockade of the ATP-sensitive potassium channel modulates reactive hyperemia in the canine coronary circulation. Circ Res 69:618–622

Belloni FI, Hintze TH (1991) Glibenclamide attenuates adenosine-induced bradycardia and vasodilation. Am J Physiol 261:H720–H727

Billman GE (1994) Role of ATP sensitive potassium channel in extracellular potassium accumulation and cardiac arrhythmias during myocardial ischaemia. Cardiovasc Res 28:762–769

Billman GE, Hamlin RL (1996) The effects of mifebradil, a novel calcium channel antagonist on ventricular arrhythmias induced by ischemia and programmed electrical stimulation. J Pharmacol Exp Ther 277:1517–1526

Billman GE, Avendano CE, Halliwill JR, Burroughs JM (1993) Effects of the ATP-dependent potassium channel antagonist, glyburide, on coronary blood flow and susceptibility to ventricular fibrillation in unanesthetized dogs. J Cardiovasc Pharmacol 21:197–204

Billman GE, Castillo LC, Hensley J, Hohl CM, Altschuld RA (1997) Beta$_2$-adrenergic receptor antagonists protect against ventricular fibrillation: *in vivo* and *in vitro* evidence for enhanced sensitivity to beta$_2$-adrenergic stimulation in animals susceptible to sudden death. Circulation 96:1914–1922

Billman GE, Englert HC, Schölkens BA (1998) HMR 1883, a novel cardioselective inhibitor of the ATP-sensitive potassium channel. Part II: Effects on susceptibility to ventricular fibrillation induced by myocardial ischemia in conscious dogs. J Pharmacol Exp Ther 286:1465–1473

Collins MN, Billman GE (1989) Autonomic response to coronary occlusion in animals susceptible to ventricular fibrillation. Am J Physiol 257 (Heart Circ Physiol 26): H1886–H1889

Daut J, Meier-Rudolph W, von Beckenrath N, Merke G, Gunther K, Godel-Meinen I (1990) Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. Science 247:1341–1344

Schwartz PJ, Billman GE, Stone HL (1984) Autonomic mechanisms in ventricular fibrillation induced by myocardial ischemia during exercise in dogs with healed myocardial infarction. Circulation 69:790–800

A.5.0.9 Experimental Atrial Fibrillation

A.5.0.9.1 Atrial Fibrillation by Atrial Pacing in Dogs

PURPOSE AND RATIONALE

Morillo et al. (1995) published a model of sustained atrial fibrillation by chronic rapid atrial pacing in dogs. Halothane-anesthetized mongrel dogs underwent insertion of a transvenous lead at the right atrial appendage that was continuously paced at 400 beats/min for 6 weeks. Two-dimensional echocardiography was performed to assess the effects of rapid atrial pacing on atrial size. Atrial vulnerability was defined as the ability to induce sustained repetitive atrial responses during programmed electrical stimulation. Effective refractory period (ERP) was measured at two endocardial sites of the right atrium. Sustained atrial fibrillation (AV) was defined as AF $\geq$15 min. In animals with sustained AF, 10 quadripolar epicardial electrodes were surgically attached to the left and right atria. The local atrial fibrillatory cycle length (AFCL) was measured in a 20-s window. Marked biatrial enlargement was documented after 6 weeks of continuous rapid atrial pacing. An increase in atrial area of at least 40% was necessary to induce sustained AF.

More studies using this method were performed by Gaspo et al. (1997a, 1997b), Yue et al. (1997), Sun et al. (1998), and Nattel and Li (2000).

Shiroshita-Takeshita et al. (2004) studied the effect of drugs on atrial fibrillation promotion by atrial-tachycardia remodeling on dogs.

PROCEDURE

Mongrel dogs were anesthetized with ketamine (5.3 mg/kg i.v.), diazepam (0.25 mg/kg i.v.) and halothane (1.5%). Unipolar leads were inserted through jugular veins into the right ventricular apex and the right atrial appendage and connected to pacemakers (Medtronic) in subcutaneous pockets in the neck. A bipolar electrode was inserted into the right atrium for stimulation and recording during serial electrophysiological study. AV block was created by radiofrequency ablation of control ventricular response during atrial tachypacing. The right ventricular pacemaker was programmed to 80 beats/min.

After 24 h for recovery, a baseline closed-chest serial electrophysiological study was performed under ketamine/diazepam/isoflurane anesthesia, and then atrial tachypacing (400 beats/min) was initiated. The closed-chest electrophysiological study was repeated at 2, 4, and 7 days of atrial tachypacing, and a final open-chest electrophysiological study was performed on day 8 under morphine-chloralose anesthesia.

Results of atrial tachypacing in drug-treated dogs were compared with results of dogs without treatment (controls).

Study Protocol

Dogs were anesthetized and ventilated mechanically. The atrial pacemaker was deactivated and a right atrium appendage effective refractory period was measured at basic lengths of 150, 200, 250, 300, and 360 ms with 10 basic stimuli (S_1) followed by a premature extra-stimulus (S_2) with 5-ms decrements. The longest S_1-S_2 failing to capture defined the effective refractory period. AF was induced by atrial burst pacing at 10 Hz and 4 times threshold current. To estimate mean AF duration in each dog, AF was induced 10 times if AF duration was $<$20 min and 5 times if AF lasted 20–30 min and then averaged. If AF lasted longer than 30 min, it was considered sustained and was terminated by DC cardioversion. A 20-min rest period was then allowed before continuing measurements.

For open-chest electrophysiological studies, dogs were anesthetized and ventilated mechanically. A femoral artery and both femoral veins were cannulated for pressure monitoring and drug administration. A median sternotomy was performed, and bipolar electrodes were hooked to the right atrial and left atrial appendages for recording and stimulation. A programmable stimulator (Digital Cardiovascular Instruments) was used to deliver twice-threshold currents. Five silicon sheets containing 240 bipolar electrodes were sutured onto the atrial surface (Fareh et al. 2001). Atrial effective refractory periods were measured as multiple basic cycle lengths in the right and left atrial appendages and at basic cycle length 300 ms in six additional sites: right and left atrium posterior wall, right and left atrium inferior wall, and right and left atrium Bachmann's bundle. Atrial fibrillation vulnerability was determined as the percentage of atrial sites at which AF could be induced by single extra-stimuli.

EVALUATION

Data are presented as mean $\pm$ SEM. Multiple-group comparisons were obtained by ANOVA. AF duration data were analyzed after logarithmic transformation. Bonferroni-corrected *t* tests were used to evaluate individual-mean differences.

MODIFICATIONS OF THE METHOD

Verheule et al. (2004) described a canine model of atrial fibrillation due to chronic atrial dilatation.

Courtemanche et al. (1999) and Ramirez et al. (2000) published mathematical models of fibrillation-induced electrical remodeling and of canine atrial action potentials.

Pinto and Boyden (1999) reviewed electrical remodeling in ischemia and infarction.

Cabo and Boyden (2003) performed a computational analysis of electrical remodeling of the epicardial border zone in the canine infarcted heart.

Sakabe et al. (2004) reported that enalapril prevents perpetuation of atrial fibrillation by suppressing atrial fibrosis and over-expression of connexin43 in a canine model of atrial pacing-induced left ventricular dysfunction.

Baartscheer et al. (2005) induced combined volume and pressure overload in New Zealand white **rabbits**. In a first surgical procedure, volume overload was produced by rupture of the aortic valve until pulse pressure increased by about 100%, and after 3 weeks, pressure overload was created by suprarenal abdominal aortic constriction of 50%. In these animals, chronic inhibition of the Na^+/H^+ exchanger attenuated cardiac hypertrophy and prevented cellular remodeling in heart failure.

Using **cultured atrial myocytes** (HL-1 cells), Yang et al. (2005) found that rapid field stimulation (300 beats/min) causes electrical remodeling.

REFERENCES AND FURTHER READING

Baartscheer A, Schumacher CA, van Borren MMGJ, Belterman CNW, Coronel R, Opthof T, Fiolet JWT (2005) Chronic inhibition of Na^+/H^+-exchanger attenuates cardiac hypertrophy and prevents cellular remodelling in heart failure. Cardiovasc Res 65:83–92

Cabo C, Boyden PA (2003) Electrical remodelling of the epicardial border zone in the canine infracted heart: a computational analysis. Am J Physiol 284:H372–H384

Courtemanche M, Ramirez RJ, Nattel S (1999) Ionic targets for drug therapy and atrial-fibrillation-induced electrical remodelling: insights from a mathematical model. Cardiovasc Res 42:477–489

Fareh S, Bardeau A, Nattel S (2001) Differential efficacy of L- and T-type calcium channel blockers in preventing tachycardia-induced atrial remodelling in dogs. Cardiovasc Res 49:762–770

Gaspo R, Bosch RF, Bou-Abboud E, Nattel S (1997a) Tachycardia-induced changes in Na^+ current in a chronic dog model of atrial fibrillation. Circ Res 81:1045–1052

Gaspo R, Bosch RF, Talajic M, Nattel S (1997b) Functional mechanisms underlying tachycardia-induced sustained atrial fibrillation in a chronic dog model. Circulation 96:4027–4035

Morillo CA, Klein GJ, Jones DL, Guiraudon CM (1995) Chronic rapid atrial pacing. Structural, functional, and electrophysiological characteristics of a new model of sustained atrial fibrillation. Circulation 91:1588–1595

Nattel S, Li D (2000) Ionic remodelling in the heart. Pathophysiological significance and therapeutic opportunities for atrial fibrillation. Circ Res 87:440–447

Pinto JMB, Boyden PA (1999) Electrical remodelling in ischemia and infarction. Cardiovasc Res 42:284–297

Ramirez RJ, Nattel S, Courtmanche M (2000) Mathematical analysis of canine atrial action potentials: rate, regional factors, and electrical remodelling. Am J Physiol 279:H1767–H1785

Sakabe M, Fujiki A, Nishida K, Sugao M, Nagasawa H, Tsuneda T, Mizumaki K, Inoue H (2004) Enalapril prevents perpetuation of atrial fibrillation by suppressing atrial fibrosis and over-expression of connexion43 in a canine model of atrial pacing-induced left ventricular dysfunction. J Cardiovasc Pharmacol 43:851–859

Shiroshita-Takeshita A, Schram G, Lavoie J, Nattel S (2004) Effect of simvastatin and antioxidant vitamins on atrial fibrillation promotion by atrial-tachycardia remodelling on dogs. Circulation 110:2313–2319

Sun H, Gaspo R, Leblanc N, Nattel S (1998) Cellular mechanisms of atrial dysfunction caused by sustained atrial tachycardia. Circulation 98:719–727

Verheule S, Wilson E, Banthia S, Everett TH, Shanbhag S, Sih HJ, Olgin J (2004) Direction-dependent conduction abnormalities on a canine model of atrial fibrillation due to chronic atrial dilatation. Am J Physiol 287:H634–H644

Yang Z, Shen W, Rottman JN, Wikswo JP, Murray KT (2005) Rapid stimulation causes electrical remodelling in cultured atrial myocytes. J Mol Cell Cardiol 38:299–308

Yue L, Feng J, Gaspo R, Li GR, Wang Z, Nattel S (1997) Ionic remodelling underlying action potential changes in a canine model of atrial fibrillation. Circ Res 81:512–525

A.5.0.9.2 Atrial Fibrillation in Chronically Instrumented Goats

PURPOSE AND RATIONALE

Atrial fibrillation is the most common tachyarrhythmia in humans. It causes palpitations, decreased cardiac output, heart failure and systemic thromboembolism, and is associated with significant mortality. Wijffels et al. (1995) demonstrated in a chronically instrumented conscious goat model, that episodes of atrial fibrillation may be self-perpetuating ("Atrial fibrillation begets atrial fibrillation") and have suggested that there may be a purely electrophysiological explanation (termed atrial electrical remodeling) for the increase of atrial fibrillation with time. This model has been extensively used to study pathophysiological mechanism and the influence of drugs (Wijffels et al. 1997, 1999, 2000; Allessie et al. 1998; Tieleman et al. 1999; Duytschaever et al. 2000, 2005; Garratt and Fynn 2000; Van der Velden 2000a, 2000b; Veloso 2001; Brendel and Peukert 2003; Shan et al. 2004; Blaauw et al. 2004a).

Blaauw et al. (2004b) studied the efficacy and atrial selectivity of a blocker of the early ultrarapid compo-

nent of the delayed rectifier (I_{kur}) in remodeled atria of the goat.

PROCEDURE

Female goats weighing 52 ± 2 kg were used. According to the method of Duytschaever et al. (2001), Teflon-felt plaques with multiple electrodes were sutured onto the free wall of each atrium, Bachmann's bundle, and the left ventricle. All leads were tunnelled subcutaneously to the neck and exteriorized by four 30-pole connectors. Experiments were started 3–4 weeks after surgery. Atrial fibrillation was induced by a fibrillation pacemaker (Wijffels et al. 1995).

The atria were paced with biphasic stimuli of 2 ms duration and $4 \times$ threshold. The atrial effective refractory period was measured at the free wall of the right and left atria during regular pacing (interval, 400–200 ms). Single interpolated stimuli were applied after eight basic stimuli, starting within the refractory period. The longest interval that failed to capture the atria (2-ms increments) was taken as the atrial effective refractory period. Atrial conduction velocity was measured along Bachmann's bundle during right atrial pacing. The distance over which conduction velocity was measured ranged from 3.5 to 5 cm.

The length of the fibrillation waves was determined at the right atrial free wall by measuring the refractory period and conduction velocity during AF. The refractory period was measured by slow, fixed-rate pacing (1 Hz), resulting in a series of single, randomly coupled, premature stimuli. Local capture of AF was evidenced by radial spread of activation from the pacing site and a short delay between stimulus and response. For each coupling interval, the percentage of capture was determined. The shortest interval capturing the atrium $\geq$50% was taken as the refractory period. Conduction velocity was determined with a mapping electrode containing 5×6 electrodes (interelectrode distance, 4 mm) from the local conduction vectors within areas of 3×3 electrodes. At least 50 AF cycles were used to determine conduction velocity.

Inducibility of AF was measured at the right and left atria by single premature stimuli applied during regular pacing (400 ms). In case a premature beat induced a rapid irregular rhythm lasting > 1 s, AF was considered inducible. The AF cycle length was measured automatically by an algorithm detecting the negative intrinsic deflection of the fibrillation electrogram. A median value of 300 consecutive intervals was calculated. QT duration was measured during atrial pacing and persistent AF from either an epicardial electrogram or a precordial ECG. Because Bazett's formula cannot be applied during AF, another approach was used to correct QT duration. In each goat, the relationship between the RR interval and QT duration was determined during 20 s of AF. The RR-QT relationship after drug administration was compared with the normal RR-QT relation.

The electrophysiological effects of the test drug and a conventional class III drug were measured before and after 48 h (1–4 days) of AF. The drugs were infused intravenously over 1 h, during which time AF cycle length was monitored. After 30 min of infusion, refractory period, conduction velocity, median RR interval, and QT duration were measured. Successful cardioversion was defined as termination of AF within $\leq$1 h of drug administration.

EVALUATION

Differences between groups were evaluated by paired Student's *t*-test or by two-way repeated ANOVA with post hoc Bonferroni's *t*-test. McNemar's test was used to compare AF inducibility. Changes in corrected QT duration were calculated by the one-sample *t*-test. Differences were considered significant at $P < 0.05$. Results are presented as mean $\pm$ SEM.

REFERENCES AND FURTHER READING

Allessie MA, Wijffels MCEF, Dorland R (1998) Mechanisms of pharmacological cardioversion of atrial fibrillation by Class I drugs. J Cardiovasc Electrophysiol 9 [8 Suppl]:S69–S77

Blaauw Y, Beier N, van der Voort P, van Hunnik A, Schotten U, Allessie MA (2004a) Inhibitors of the Na^+/H^+ exchanger cannot prevent atrial electrical remodeling in the goat. J Cardiovasc Electrophysiol 15:440–446

Blaauw Y, Gögelein H, Tieleman RG, van Hunnik A, Schotten U, Allessie MA (2004) "Early" class III drugs for the treatment of atrial fibrillation. Efficacy and atrial selectivity of AVE0118 in remodeled atria of the goat. Circulation 110:1717–1724

Brendel J, Peukert S (2003) Blockers of the Kv1.5 channel for the treatment of atrial hypertension. Curr Med Chem Cardiovasc Hematol Agents 1:273–287

Duytschaever MF, Garratt CJ, Allessie MA (2000) Profibrillatory effects of verapamil but not of digoxin in the goat model of atrial fibrillation. J Cardiovasc Electrophysiol 11:1375–1385

Duytschaever MF, Mast F, Killian M, Blaauw Y, Wijffels M, Allessie M (2001) Methods for determining the refractory period and excitable gap during persistent atrial fibrillation in the goat. Circulation 104:947–962

Duytschaever MF, Blaauw Y, Allessie MA (2005) Consequences of atrial electrical remodeling for the anti-arrhythmic action of class IC and class III drugs. Cardiovasc Res 67:69–76

Garratt CJ, Fynn SP (2000) Atrial electrical remodeling and atrial fibrillation. Q J Med 93:563–565

Shan Z, van der Voort PH, Blaauw Y, Duytschaever M, Allessie MA (2004) Fractionation of electrograms and linking of activation during pharmacological cardioversion of persistent atrial fibrillation in the goat. J Cardiovasc Electrophysiol 15:572–580

Tieleman RG, Blaauw Y, Van Gelder IC, De Langen CDJ, de Kam PJ, Grandjean JG, Patberg KW, Bel KJ, Allessie MA, Crijns HJGM (1999) Digoxin delays recovery from tachycardia-induced electrical remodeling of the atria. Circulation 100:1836–1842

Van der Velden HMW, Ausma J, Rook MB, Hellemons AJCGM, van Veen TAAB, Allessie MA, Jongsma HJ (2000a) Gap junctional remodeling on relation to stabilization of atrial fibrillation in the goat. Cardiovasc Res 46:476–486

Van der Velden HMW, van der Zee, Wijffels MC, van Leuven C, Dorland R, Vos MA, Jongsma HJ, Allessie MA (2000b) Atrial fibrillation in the goat induces changes in monophasic action potential and mRNA expression of ion channels involved in repolarization. J Cardiovasc Electrophysiol 11:1262–1269

Veloso HH (2001) Electrophysiologic effects of digoxin in the goat model of atrial fibrillation and its clinical implications. J Cardiovasc Electrophysiol 12:735–736

Wijffels MCEF, Kirchhof CJHJ, Dorland R, Allessie MA (1995) Atrial fibrillation begets atrial fibrillation. A study in awake chronically instruments goats. Circulation 92:1954–1968

Wijffels MCEF, Kirchhof CJHJ, Dorland R, Power J, Allessie MA (1997) Electrical remodeling due to atrial fibrillation in chronically instrumented conscious goats. Roles of neurohumoral changes, ischemia, atrial stretch, and high rate of electrical activation. Circulation 96:3710–3720

Wijffels MCEF, Kirchhof CJHJ, Dorland R, Allessie MA (1999) Pharmacological cardioversion of chronic atrial fibrillation in the goat by class IA, IC, and III drugs: a comparison between hydroquinidine, cibenzoline, flecainide, and *d*-sotalol. J Cardiovasc Electrophysiol 10:178–193

Wijffels MCEF, Dorland R, Mast F, Allessie MA (2000) Widening the excitable gap during pharmacological cardioversion of atrial fibrillation in the goat. Effects of cibenzoline, hydroquinidine, flecainide, and *d*-sotalol. Circulation 102:260–267

A.5.0.9.3 Influence on Ultrarapid Delayed Rectifier Potassium Current in Pigs

PURPOSE AND RATIONALE

Blockade of cardiac potassium channels and the resulting prolongation of repolarization and refractoriness is the mode of action of class III antiarrhythmic drugs. In the human heart, the ultrarapid delayed rectifier potassium current (I_{Kur}) was identified in atrial, but not in ventricular tissue. It appears to contribute to action potential repolarization (Wang et al. 1993; Li et al. 1996) in the atrium but not the ventricle. The molecular correlate of the human cardiac ultrarapid delayed rectifier potassium current seems to be the Kv1.5 protein (Fedida et al. 1993; Wang et al. 1993; Feng et al. 1997, 1998).

Since prolongation of ventricular repolarization seems to be invariably associated with proarrhythmia (early afterdepolarizations leading to torsades de pointes arrhythmia) as shown with available potassium channel blockers (class III drugs) such as the highly selective and potent I_{Kr}-channel blocker dofetilide (Torp-Pedersen et al. 1999), blockade of a cardiac current that is exclusively present in the atria is highly desirable as it is expected to be devoid of ventricular proarrhythmic effects. Therefore, the atrial Kv1.5 channel is a highly attractive target in the search for new and safer atrial antiarrhythmic drugs (Nattel and Singh 1999).

In a series of studies, Wirth and Knobloch (2001), Knobloch et al. (2002, 2004), and Wirth et al. (2003) investigated electrophysiological and antiarrhythmic effects of I_{Kur} channel blockers on left versus right pig atrium *in vivo* in comparison with I_{Kr} blockers in pigs.

PROCEDURE

Surgery

Castrated male pigs (24–30 kg) of the German Landrace were premedicated with 3 ml Rompun 2% i.m. (xylacine HCL, 23.3 mg/ml = 3 mg/kg i.m.) and 6 ml Hostaket (ketamine HCl, 115 mg/ml = 20 mg/kg i.m.) and anesthetized with an i.v. bolus of 5 ml Narcoren (pentobarbital, 160 mg/ml = 25–30 mg/kg i.v.) followed by a continuous intravenous infusion of 12–17 mg/kg per h pentobarbital. Animals were ventilated with room air and oxygen by a respirator (ABV-Intensiv; Stephan, Gackenbach, Germany). After a left thoracotomy the lung was retracted, the pericardium incised and the heart suspended in a pericardial cradle. Bipolar body surface ECG was recorded using subcutaneous needle electrodes in the classical lead II or lead III arrangement.

Atrial Effective Refractory Period Measurements

Atrial effective refractory period (ERP) measurements at different basic cycle lengths (BCL 240/300/400 ms) were performed (Wirth and Knobloch 2001). Atrial responses to the pacing procedure were visualized via monophasic action potential (MAP) from the left and right atrium as will be described below. A conditioning train of ten basic stimuli (S1) at twice-diastolic pacing threshold was followed by a diastolic extrastimulus (S2, pulse duration 1 ms) starting about 30 ms above the expected ERP with a 5-ms decrement (UHS 20, universal heart stimulator; Biotronik, Berlin, Germany). The longest coupling interval unable to elicit a propagated atrial response was taken as the atrial ERP.

MAP Recording Sites and Atrial Pacing Electrodes

Left atrial ERP was measured via a MAP pacing catheter (EP Technologies, Model 1675; Boston Scientific, La Garenne-Colombes, France), which was fixed in each pig in the middle of the left atrial free wall in

an approximately perpendicular position by a holding device (Yuan et al. 1994). The tip of the MAP pacing catheter was covered by a sponge. Programmed stimulation was performed by the MAP pacing catheter.
Right atrial MAP for ERP measurement was taken from the endocardium of the right atrium also via a steerable MAP pacing catheter. The catheter, inserted via the V. femoralis, was used for atrial stimulation too. Its position in the right atrium was checked by palpitation and by the typical atrial MAP morphology and duration and, additionally, by short rapid atrial pacing at a BCL of 240 ms, which the ventricle was not able to follow 1:1 as indicated by frequent *p*-waves dissociated from the QRS complex. There were no significant differences between endocardial and epicardial ERP measurements in the free walls of either atrium at baseline and after drug.

Left Atrial Vulnerability

During the ERP measurement procedure the mere S2-extrastimulus, which followed the ten conditioning S1 stimuli during the ERP-measurement procedure, frequently triggered runs of atrial tachycardia in the left, not the right, atrium. The occurrence of tachycardias was primarily unintended, but then exploited as a parameter for the judgement of the antiarrhythmic efficacy of compounds (referred to as left atrial vulnerability). Whether or not a run of S2-triggered atrial tachyarrhythmia occurred during the ERP-measurement procedure at a given BCL was noted. The occurrences of triggered tachyarrhythmias were summed up for the three BCLs tested over three time points (during a 30-min period before or after a drug). Thus, the maximal occurrence of S2-tachyarrhythmias during the control or drug period in an individual animal was nine.

Drugs

Drugs were dissolved in polyethyleneglycol (PEG) 400 (Riedel-de Haen, Seelze, Germany) and administered i.v. over 5 min in a volume of 3 ml. Vehicle was injected at least 30 min before each drug. For each drug a separate group of pigs was used.

EVALUATION

All data were presented as means ± SEM. Two-way ANOVA for repeated measures followed by Student's *t*-test was used for the calculation of statistically significant differences between left and right atrial ERP prolongations at the three basic cycle lengths and the inhibition of left atrial vulnerability. A value of $P < 0.05$ was accepted as significant. The longest ERP at each pacing rate after drug administration was taken and expressed as absolute or percent increase from vehicle control. Interatrial difference in refractoriness was calculated as the difference between the left and right atrial ERP.

REFERENCES AND FURTHER READING

Fedida D, Wible B, Wang Z, Fermini B, Faust F, Nattel S, Brown AM (1993) Identity of a novel delayed rectifier current from human heart with a cloned K^+ channel current. Circ Res 73:210–216

Feng J, Wible B, Li GR, Wang Z, Nattel S (1997) Antisense oligodeoxynucleotides directed against Kv1.5 mRNA specifically inhibit ultrarapid delayed rectifier K^+ current in cultured adult human atrial myocytes. Circ Res 80:572–579

Feng J, Xu D, Wang Z, Nattel S (1998) Ultrarapid delayed rectifier current inactivation in human atrial myocytes: properties and consequences. Am J Physiol 275:H1717–H1725

Knobloch K, Brendel J, Peukert S, Rosenstein B, Busch AE, Wirth KJ (2002) Electrophysiological and antiarrhythmic effects of the novel I_{Kur} channel blockers, S9947 and S20951, on left vs. right pig atrium in vivo in comparison with the I_{Kr} blockers dofetilide, azimilide, d,l-sotalol and ibutilide. Naunyn-Schmiedebergs Arch Pharmacol 366:482–487

Knobloch K, Brendel J, Rosenstein B, Bleich M, Busch AE, Wirth KJ (2004) Atrial-selective antiarrhythmic actions of novel I_{Kur} vs. I_{Kr}, I_{Ks}, and I_{Kach} class Ic drugs and β-blockers in pigs. Med Sci Monit 10:BR221–BR228

Li GR, Feng J, Wang Z, Fermini B, Nattel S (1996) Adrenergic modulation of ultrarapid delayed rectifier K^+ current in human atrial myocytes. Circ Res 78:903–915

Nattel S, Singh BN (1999) Evolution, mechanisms, and classification of antiarrhythmic drugs: focus on class III actions. Am J Cardiol 84:11R–19R

Torp-Pedersen C, Moller M, Bloch-Thomsen PE, Kober L, Sandoe E, Egstrup K, Agner E, Carlsen J, Videbaek J, Marchant B, Camm AJ (1999) Dofetilide in patients with congestive heart failure and left ventricular dysfunction. N Engl J Med 341:857–865

Wang Z, Fermini B, Nattel S (1993) Sustained depolarization-induced outward current in human atrial myocytes: evidence for a novel delayed rectifier K^+ current similar to Kv1.5 cloned channel currents. Circ Res 73:1061–1076

Wirth KJ, Knobloch K (2001) Differential effects of dofetilide, amiodarone, and class Ic drugs on left and right atrial refractoriness and left atrial vulnerability in pigs. Naunyn-Schmiedebergs Arch Pharmacol 363:166–174

Wirth KJ, Paehler T, Rosenstein B, Knobloch K, Maier T, Frenzel J, Brendel J, Busch AE, Bleich M (2003) Atrial effects of the novel K^+-channel-blocker AVE0118 in anesthetized pigs. Cardiovasc Res 60:298–306

Yuan S, Blomstrom-Lundqvist C, Olsson SB (1994) Monophasic action potentials: concepts to practical applications. J Cardiovasc Electrophysiol 5:287–308

A.5.0.10 Characterization of Anti-Arrhythmic Activity in the Isolated Right Ventricular Guinea Pig Papillary Muscle

PURPOSE AND RATIONALE

According to Vaughan-Williams (1970) anti-arrhythmic drugs are divided into 4 different classes depending on their mode of action. Class I anti-arrhythmic

agents decrease the upstroke velocity of the action potential through blockade of Na^+ channels. Class II drugs block β-receptors. Class III anti-arrhythmic agents prolong action potential duration, presumably through blockade of K^+ channels. Class IV anti-arrhythmic agents inhibit the slow calcium influx during the plateau of the action potential through Ca^{2+} channel blockade. These electrophysiological actions also have functional manifestations, e. g., Na^+ channel blockade decreases excitability, K^+ channel blockade lengthens refractory period, and Ca^{2+} channel blockade decreases tension of cardiac muscle. A simple and accurate non-microelectrode method is necessary to identify and classify potential anti-arrhythmic drugs into the classes I, III, and IV. In right ventricular guinea pig papillary muscle developed tension (DT), excitability (EX), and effective refractory period (ERP) are measured.

PROCEDURE

Guinea pigs of either sex weighing 200–400 g are stunned, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed into a container of prewarmed, preoxygenated physiologic solution and the pericardium, atria, and other tissues are removed. The heart is then pinned to a dissection dish, and the right ventricle is opened. The tendinous end of the papillary muscle is ligated with a silk thread, and the chordae tendinae are freed from the ventricle. The opposite end of the papillary muscle is then cut free close to the ventricular wall. The non-ligated end of the papillary muscle is clamped into a tissue holder, the end of which is a leucite block containing platinum wire field electrodes.

The preparation is transferred to a tissue bath containing 75 ml of a physiological salt solution that is gassed continuously with 95% O_2/5% CO_2 and maintained at a temperature of 35°C and a pH of 7.4. The silk thread is used to connect the muscle to a Grass FT03C force transducer. An initial resting tension of 1 g is established. Muscles are field stimulated to contract isometrically. The stimulus duration is 1 ms, the frequency 1 Hz, and the voltage twice threshold. Pulses are delivered with the use of a Grass S88 constant voltage stimulator, and developed tension is recorded with the use of a polygraph recorder. The preparation is equilibrated in this manner for 90 min with bath solution changes every 15 min. Control measurements of the force-frequency curve, stimulus strength-duration curve and the effective refractory period are made following the 75 min bath exchange, i. e., during the last 15 min of equilibration.

The force-frequency curve is obtained by measuring developed tension over a range of stimulation frequencies (0.3, 0.5, 0.8, 1.0, and 1.2 Hz). The tissue is contracted for 90 s at each of these frequencies with a brief period of stimulation at 1.5 Hz inserted between increments. The purpose of the 1.5 Hz insert is to keep "pacing history" constant as well as to minimize progressive, nonspecific depression during the lower frequency stimulation series. Both pre- and postdrug developed tension (at each frequency) are expressed as a percentage of the predrug developed tension at 1 Hz. The percent change in post treatment (versus pretreatment) developed tension at 1 Hz is used to quantitate an agent's inotropic effect.

The stimulus strength-duration curves are determined by varying the stimulus duration (0.1, 0.4, 0.8, 1.0, 1.5, 3.0, and 3.4 ms) and finding the threshold voltage that produced a 1:1 correspondence between stimulus and response at each duration. The degree of shift in the strength-duration curve is measured by computing the area between the pre- and post-treatment curves. The boundaries for the area are determined by the first (x-axis parallel) and the last (x-axis perpendicular) durations and by lines from the origin to the second and fourth durations.

Effective refractory period (ERP) is measured at 1 Hz using twin pulse stimuli. After every 8–10 pulses, a second delayed stimulus (S_2) identical to the basic drive pulse (S_1) is introduced. This procedure is repeated, shortening the delay (S_1–S_2) by 5 ms increments. The value of the ERP is taken as the longest delay (S_1–S_2) for which there is a single response to twin pulses. The change in ERP is computed as the difference (ms) between the pre- and post-treatment ERP values.

At the conclusion of the 90 min predrug equilibration period, an aliquot of the test drug designed to achieve the desired final concentration is added to the bath. The tissue must equilibrate for 1 h in the drug solution before postdrug measurements of the force-frequency curve, stimulus strength-duration curve, and effective refractory period are obtained.

EVALUATION

The changes in effective refractory period (ERP) (post treatment minus pretreatment), the degree of shift in the strength-duration curve (geometrical area between pre- and post-treatment curves), and the percent changes in post treatment developed tension at 1 Hz are calculated. The results of these calculations are used to classify the compound as a class I, III, or IV anti-arrhythmic agent on the basis of its effect on de-

veloped tension, excitability, and effective refractory period. An upward and right shift of the strength-duration curve (decrease in excitability) is characteristic for a class I anti-arrhythmic agent, such disopyramide. Selective prolongation of effective refractory period is characteristic for class III anti-arrhythmic agents, such as sotalol. Depression of developed tension, and/or flattening or reversal of the force-frequency curve is characteristic for a class IV anti-arrhythmic agent, such as verapamil.

CRITICAL ASSESSMENT OF THE METHOD

The model of the electrically stimulated isolated guinea pig papillary muscle is a simple method to classify anti-arrhythmic agents. Some drugs have multiple actions and, therefore, belong in more than one class. For further characterization analysis of the action potential is necessary.

MODIFICATIONS OF THE METHOD

O'Donoghue and Platia (1991) recommended the use of monophasic action potential recordings for the evaluation of anti-arrhythmic drugs.

Shibuya et al. (1993) studied the effects of the local anesthetic bupivacaine on contraction and membrane potential in isolated canine right ventricular papillary muscles. From analysis of action potential it is concluded that at low concentrations contraction is depressed mainly due to a Na^+ channel block, whereas at high concentrations also Ca^{2+} channels may be blocked.

Kodama et al. (1992), Maryuama et al. (1995) studied the effects of potential antiarrhythmics on maximum upstroke velocity and duration of action potential in isolated right papillary muscles of guinea pigs as well as the influence of these agents on single ventricular myocytes.

Borchard et al. (1982) described a method for inducing arrhythmias or asystolia by the application of 50 Hz alternating current (ac) to electrically driven isolated left atria and right papillary muscles of the guinea pig. An increase in driving frequency from 1 to 3 Hz effected a significant reduction of the threshold of ac-arrhythmia in guinea pig papillary muscle, but no change in atria. A decrease in temperature from 31°C to 25°C and an increase in Ca^{2+} from 1.25 to 5 mmol/l elevated the threshold for ac-arrhythmia and -asystolia. Fast sodium channel inhibitors increased threshold of ac-arrhythmia in left atria and papillary muscles, whereas the slow channel inhibitor verapamil was ineffective in concentrations up to 6 µmol/l.

REFERENCES AND FURTHER READING

Arnsdorf MF, Wasserstrom JA (1986) Mechanism of action of antiarrhythmic drugs: A matrical approach. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan HE (eds) The Heart and Cardiovascular System. Scientific Foundations. Vol 2. Raven Press, New York, pp 1259–1316

Borchard U, Bösken R, Greef K (1982) Characterization of antiarrhythmic drugs by alternating current induced arrhythmias in isolated heart tissues. Arch Int Pharmacodyn 256:253–268

Hackett AM, McDonald SJ, Schneider P, Schweingruber F, Garthwaite SM (1990) Simple *in vitro* method to characterize anti-arrhythmic agents. J Pharmacol Meth 23:107–116

Kodama I, Suzuki R, Honjio H, Toyama J (1992) Electrophysiological effects of diprafenone, a dimethyl congener of propafenone on guinea pig ventricular cells. Br J Pharmacol 107:813–820

Maruyama K, Kodama I, Anno T, Suzuki R, Toyama J (1995) Electrophysiological effects of Ro 22–9194, a new antiarrhythmic agent, on guinea pig ventricular cells. Br J Pharmacol 114:19–26

O'Donoghue S, Platia EV (1991) Monophasic action potential recordings: evaluation of antiarrhythmic drugs. Progr Cardiovasc Dis 34:1–14

Sanguinetti MC, Jurkiewitz NK (1990) Two components of cardiac delayed rectifier K^+ current. J Gen Physiol 96:195–215

Shibuya N, Momose Y, Ito Y (1993) Effects of bupivacaine on contraction and membrane potential in isolated canine papillary muscles. Pharmacology 47:158–166

Vaughan-Williams EM (1970) Classification of antiarrhythmic drugs. In: Sandøe E, Flensted-Jensen E, OLesen KH (eds) Symposium on cardiac arrhythmias. Elsinore, Denmark, April 23–25, 1970, Publ. by AB Astra, Södertälje, Sweden, pp 449–472

A.5.0.11
Action Potential and Refractory Period in Isolated Left Ventricular Guinea Pig Papillary Muscle

PURPOSE AND RATIONALE

Intracellular action potential in the left ventricular guinea pig papillary muscle is recorded after electrical stimulation. The stimulation frequency is varied in order to determine the refractory period. Resting potential, upstroke velocity, duration of action potential, threshold, refractory period and contractile force can be measured *in vitro*. Compounds which affect the duration of the effective refractory period may have anti-arrhythmic or pro-arrhythmic effects. In addition, the inotropic effect (positive or negative) of the test compound is determined.

PROCEDURE

Guinea pigs of either sex (Marioth strain) weighing 250–300 g are sacrificed by stunning, the carotid arteries are severed, and the thoracic cage is opened immediately. The heart is removed, placed in a container of prewarmed, pre-oxygenated Ringer solution, and the pericardium and the atria are trimmed away. The

left ventricle is opened and the two strongest papillary muscles removed. They are fixed between a suction electrode for electrical stimulation and a force transducer for registration of contractions. Ringer solution oxygenated with carbogen (95% O_2/5% CO_2) at a temperature of 36°C is used.

A standard micro electrode technique is applied to measure the action potential via a glass micro electrode containing 3 M KCl solution, which is inserted intracellularly. The papillary muscle is stimulated with rectangular pulses of 1 V and of 1 ms duration at an interval of 500 ms. The interval between two stimuli is variable in order to determine refractory periods. The intensity of the electrical current is just below the stimulation threshold.

The intracellular action potential is amplified, differentiated for registration of upstroke velocity (dV/dt) (Hugo Sachs micro electrode amplifier), together with the contraction force displayed on an oscilloscope (Gould digital storage oscilloscope OS 4000), and recorded (Gould 2400 recorder).

The effects on fast sodium channels as well as on calcium channels can be studied. The former requires measurement of the normal action potential and the latter the slow action potential obtained at 30 mM K^+. To estimate the relative refractory periods, the second stimuli are set in decremental intervals until contraction ceases. Relative refractory period is defined as the minimum time interval of two stimuli at which each of the stimuli is answered by a contraction. The stimulation threshold is also measured.

After an equilibrium time of 30 min the test compound is added. After 15 and 30 min the following parameters are compared with the predrug values:

- Resting potential mV
- upstroke velocity V/s
- duration of action potential ms
- stimulation threshold V
- refractory period ms
- contraction force mg

The organ bath is flushed thoroughly between two consecutive applications of increasing test drug doses.

EVALUATION

Contractile force [mm] and relative refractory period [ms] are determined before and after drug administration. $ED_{25\,ms}$- and $ED_{50\,ms}$-values are determined. ED_{25ms} or ED_{50ms} is defined as the concentration of test drug in the organ bath at which the relative refractory period is reduced or prolonged by 25 ms or 50 ms.

Since many anti-arrhythmic agents possess additionally negative inotropic effects, changes in the force of contraction are also determined.

ED_{50} values are calculated from log-probit analyses. Scores are allotted relative to the efficacy of standard compounds (lidocaine, propranolol, quinidine).

The following changes are indicators for anti-arrhythmic activity:

- increase of stimulation threshold
- decrease of upstroke velocity
- prolongation of action potential
- increase of refractory period.

Upstroke velocity and duration of action potential are used for **classification purposes**.

MODIFICATIONS OF THE METHOD

Tande et al. (1990) studied the electromechanical effects of a class III anti-arrhythmic drug on guinea pig and rat papillary muscles and atria using conventional microelectrode technique.

Shirayama et al. (1991) studied with a similar technique the electrophysiological effects of sodium channel blockers in isolated guinea pig left atria.

Dawes (1946) described a method of examining substances acting on the refractory period of cardiac muscle using isolated rabbit auricles.

The same method was recommended as first step of a screening program for quinidine-like activity by Schallek (1956).

Wellens et al. (1971) studied the decrease of maximum driving frequency of isolated guinea pig auricles after antiarrhythmic drugs and beta-blockers.

Salako et al. (1976) recorded electropotentials along the conducting system after stimulation of the proximal part of the His bundle in rabbits.

Brown (1989), Wu et al. (1989), Gwilt et al. (1991a, b) measured *in vitro* transmembrane action potential in Purkinje fibers and endocardial ventricular muscles from dogs.

Voltage clamp techniques in isolated cardiac myocytes from guinea pigs have been used by Wettwer et al. (1991).

Nygren et al. (2004) described heterogeneity of action potential durations in isolated mouse left and right atria recorded using voltage-sensitive dye mapping.

REFERENCES AND FURTHER READING

Adaniya H, Hiraoka M (1990) Effects of a novel class III anti-arrhythmic agent, E-4031, on reentrant tachycardias in rabbit right atrium. J Cardiovasc Pharmacol 15:976–982

Brown BS (1989) Electrophysiological effects of ACC-9358, a novel class I antiarrhythmic agent, on isolated canine

Purkinje fibers and ventricular muscle. J Pharmac Exp Ther 248:552–558
Dawes GS (1946) Synthetic substitutes for quinidine. Br J Pharmacol 1:90–112
Gwilt M, Dalrymple HW, Burges RA, Blackburn KJ, Dickinson RP, Cross PE, Higgins AJ (1991a) Electrophysiologic properties of UK-66,914, a novel class III antiarrhythmic agent. J Cardiovasc Pharmacol 17:376–385
Gwilt M, Arrowsmith JE, Blackburn KJ, Burges RA, Cross PE, Dalrymple HW, Higgins AJ (1991b) UK-68–798: A novel, potent and highly selective class III antiarrhythmic agent which blocks potassium channels in cardiac cells. J Pharmacol Exp Ther 256:318–324
Linz W, Schölkens BA, Kaiser J, Just M, Bei-Yin Q, Albus U, Petry P (1989) Cardiac arrhythmias are ameliorated by local inhibition of angiotensin formation and bradykinin degradation with the converting-enzyme inhibitor ramipril. Cardiovasc Drugs Ther 3:873–882
Nygren A, Lomax AE, Giles WR (2004) Heterogeneity of action potential durations in isolated mouse left and right atria recorded using voltage-sensitive dye mapping. Am J Physiol 287:H26134–H2643
Salako LA, Vaughan Williams EM, Wittig JH (1976) Investigations to characterize a new anti-arrhythmic drug, ORG 6001, including a simple test for calcium antagonism. Br J Pharmacol 57:251–262
Schallek W (1956) A screening program for quinidine-like activity. Arch Int Pharmacodyn 105:221–229
Shirayama T, Inoue D, Inoue M, Tatsumi T, Yamahara Y, Asayama J, Katsume H, Nakegawa M (1991) Electrophysiological effects of sodium channel blockers on guinea pig left atrium. J Pharm Exp Ther 259:884–893
Tande PM, Bjørnstad, Yang T, Refsum H (1990) Rate-dependent class III antiarrhythmic action, negative chronotropy, and positive inotropy of a novel I_k blocking drug, UK-68,798: potent in guinea pig but no effect in rat myocardium. J Cardiovasc Pharmacol 16:401–410
Wellens D, Dessy F, de Klerk L (1971) Antiarrhythmic drugs and maximum driving frequency of isolated guinea pig auricles. Arch Int Pharmacodyn 190:411–414
Wettwer E, Scholtysik G, Schaad A, Himmel H, Ravens U (1991) Effects of the new class III antiarrhythmic drug E-4031 on myocardial contractility and electrophysiological parameters. J Cardiovasc Pharmacol 17:480–487
Wu KM, Proakis AG, Hunter TL, Shanklin JR (1989) Effects of AHR-12234 on cardiac transmembrane action potentials, *in situ* cardiac electrophysiology and experimental models of arrhythmia. Arch Int Pharmacodyn 301:131–150

A.6 Methods to Induce Cardiac Hypertrophy and Insufficiency

Animal models of cardiac hypertrophy and insufficiency have been reviewed by Hasenfuss (1988), Muders and Elsner (2000), and Vanoli et al. (2004).

REFERENCES AND FURTHER READING

Hasenfuss G (1988) Animal models of human cardiovascular disease, heart failure and hypertrophy. Cardiovasc Res 39:60–76
Muders F, Elsner F (2000) Animal models of chronic heart failure. Pharmacol Res 41:605–612
Vanoli E, Bacchini S, Panigada S, Pentimalli F, Adamson PB (2004) Experimental models of heart failure. Eur Heart J Suppl 6 (Suppl F):F7–F15

A.6.0.1 Cardiac Hypertrophy and Insufficiency in Rats

A.6.0.1.1 Aortic Banding in Rats

PURPOSE AND RATIONALE

Blood flow restriction of the aorta in rats induces not only hypertension but also cardiac hypertrophy within several weeks. Angiotensin converting enzyme inhibitors, even at subantihypertensive doses, but not other antihypertensive drugs, inhibit cardiac hypertrophy (Linz et al. 1991; Schölkens et al. 1991; Gohlke et al. 1992; Linz 1992a; Linz et al. 1996; Bruckschlegel et al. 1995; Ogawa et al. 1998).

PROCEDURE

Male Sprague Dawley rats weighing 270–280 g are fasted 12 h before surgery. Anesthesia is induced by i.p. injection of 200 mg/kg hexobarbital. The abdomen is shaved, moistened with a disinfectant and opened by a cut parallel to the linea alba. The intestine is moistened with saline and placed in a plastic cover to prevent desiccation. The aorta is prepared free from connective tissue above the left renal artery and underlaid with a silk thread. Then, a cannula no. 1 (0.9×40 mm) is placed longitudinally to the aorta and both aorta and cannula are tied. The cannula is removed, leaving the aortic lumen determined by the diameter of the cannula. The intestine is placed back into the abdominal cavity with the application of 5.0 mg rolitetracycline (Reverin). In sham-operated controls no banding is performed. The skin is closed by clipping.

The animals are treated once daily over a period of 6 weeks with doses of the ACE-inhibitor or other antihypertensive drugs found previously effective to lower blood pressure in rats. At the end of the experiment blood pressure is measured under hexobarbital anesthesia (200 mg/kg i.p.) via indwelling catheters in the left carotid artery. Blood pressure measurement in conscious rats with the convential tail-cuff method is not possible due to the large pressure difference across the ligature. Therefore, only one measurement at the end of the study is possible. The hearts are removed, rinsed in saline until free of blood and gently blotted to dryness. Total cardiac mass is determined by weighing on an electronic balance to the nearest 0.1 mg. The atria and all adjacent tissues are trimmed off and the weight of the left ventricle including the septum as well as the

remaining cardiac tissue representing the right ventricle are determined separately. Weights are calculated per 100 g body weight.

EVALUATION

Total cardiac mass, weight of left and right ventricle of treated rats are compared with operated controls and sham-operated controls.

MODIFICATIONS OF THE METHOD

Uetmasu et al. (1989) described a simple method for producing graded aortic insufficiencies in rats and subsequent development of cardiac hypertrophy. Selective perforation of the right cup of the aortic valve or in combination with that of the left valve cup was performed using a plastic rod inserted from the right common carotid artery. Hypertrophy of the heart, but no hypertension or cardiac insufficiency, was observed.

Similar methods were used by Yamazaki et al. (1989) to study the alterations of cardiac adrenoceptors and calcium channels subsequent to aortic insufficiency, by Umemura et al. (1992) to study baroreflex and β-adrenoceptor function and by Ishiye et al. (1995) to study the effects of an angiotensin II antagonist on the development of cardiac hypertrophy due to volume overload.

Hyperplastic growth response of vascular smooth muscle cells in the thoracic aorta was found following induction of acute hypertension in rats by aortic coarctation by Owens and Reidy (1985). Changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with aortic banding were studied by Feldman et al. (1993). Muders et al. (1995) produced aortic stenosis in rats by placing a silver clip (inner diameter 0.6 mm) on the ascending aorta. Schunkert et al. (1995) studied alteration of growth responses in established cardiac pressure overload hypertrophy in rats with aortic banding. **Prevention of cardiac hypertrophy after aortic banding** by ACE inhibitors probably mediated by bradykinin could be shown (Linz et al. 1989, 1992a, b, 1993, 1994; Linz and Schölkens 1992; Schölkens et al. 1991; Weinberg et al. 1994).

Weinberg et al. (1997) studied the effect of angiotensin AT_1 receptor inhibition on hypertrophic remodelling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis.

REFERENCES AND FURTHER READING

Bruckschlegel G, Holmer SR, Jandeleit K, Grimm D, Muders F, Kromer EP, Riegger GA, Schunkert H (1995) Blockade of the renin-angiotensin system in cardiac pressure-overload hypertrophy in rats. Hypertension 25:250–259

Feldman AM, Weinberg EO, Ray PE, Lorell BH (1993) Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with aortic banding. Circ Res 73:184–192

Gohlke P, Stoll M, Lamberty V, Mattfeld T, Mall G, van Even P, Martorana P, Unger T (1992) Cardiac and vascular effects of chronic angiotensin converting enzyme inhibition at subantihypertensive doses. J Hypertens 10 (Suppl 6):S141–S145

Ishiye M, Umemura K, Uematsu T, Nakashima M (1995) Effects of losartan, an angiotensin II antagonist, on the development of cardiac hypertrophy due to volume overload. Biol Pharm Bull 18:700–704

Linz W, Schölkens BA (1992) A specific B_2-bradykinin receptor antagonist Hoe 140 abolishes the antihypertrophic effect of ramipril. Br J Pharmacol 105:771–772

Linz W, Schölkens BW, Ganten D (1989) Converting enzyme inhibition specifically prevents the development and induces regression of cardiac hypertrophy in rats. Clin Exper Hypert. Theory and Practice A11 (7):1325–1350

Linz W, Henning R, Schölkens BA (1991) Role of angiotensin II receptor antagonism and converting enzyme inhibition in the progression and regression of cardiac hypertrophy in rats. J Hypertens 9 (Suppl 6):S400–S401

Linz W, Schaper J, Wiemer G, Albus U, Schölkens BW (1992a) Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats. Br J Pharmacol 107:970–975

Linz W, Wiemer G, Schölkens BA (1992b) Contribution of bradykinin to the cardiovascular effects of ramipril. J Cardiovasc Pharmacol 22 (Suppl 9):S1–S8

Linz W, Wiemer G, Schölkens BA (1993) Bradykinin prevents left ventricular hypertrophy in rats. J Hypertension 11 (Suppl 5):S96–S97

Linz W, Wiemer G, Gohlke P, Unger T, Schölkens BA (1994) The contribution of bradykinin to the cardiovascular actions of ACE inhibitors. In Lindpaintner K, Ganten D (eds) The Cardiac Renin Angiotensin System. Futura Publ Co., Inc., Armonk, NY, pp 253–287

Linz W, Wiemer G, Schmidts HL, Ulmer W, Ruppert D, Schölkens BA (1996) ACE inhibition decreases postoperative mortality in rats with left ventricular hypertrophy and myocardial infarction. Clin Exper Hypertension 18:691–712

Muders F, Kromer EP, Bahner U, Elsner D, Ackermann B, Schunkert H, Plakovits M, Riegger GAJ (1995) Central vasopressin in experimental aortic stenosis in the rat. Cardiovasc Res 29:416–421

Ogawa T, Linz W, Schölkens BA, de Bold AJ (1998) Regulation of aortic atrial natriuretic factor and angiotensinogen in experimental hypertension. J Cardiovasc Pharmacol 32:1001–1008

Owens GK, Reidy MA (1985) Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation. Circ Res 57:695–705

Schölkens BA, Linz W, Martorana PA (1991) Experimental cardiovascular benefits of angiotensin-converting enzyme inhibitors: Beyond blood pressure reduction. J Cardiovasc Pharmacol 18 (Suppl 2):S26–S30

Schunkert T, Weinberg EO, Bruckschlegel G, Riegger AJ, Lorell BH (1995) Alteration of growth responses in established cardiac pressure overload hypertrophy in rats with aortic banding. J Clin Invest 96:2768–2774

Uetmatsu T, Yamazaki T, Matsuno H, Hayashi Y, Nakashima M (1989) A simple method for producing graded aortic insufficiencies in rats and subsequent development of cardiac hypertrophy. J Pharmacol Meth 22:249–257

Umemura K, Zierhut W, Quast U, Hof RP (1992) Baroreflex and β-adrenoceptor function are diminished in rat cardiac hypertrophy due to volume overload. Basic Res Cardiol 87:263–271

Weinberg EO, Min Ae Lee, Weigner M, Lindpaintner K, Bishop SP, Benedict CR, Ho KKL, Douglas PS, Chafizadeh E, Lorell BH (1997) Angiotensin AT_1 receptor inhibition: Effects on hypertrophic remodelling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis. Circulation 95:1592–1600

Weinberg EO, Schoen FJ, George D, Kagaya Y, Douglas PS, Litwin SE, Schunkert H, Benedict CR, Lorell BH (1994) Angiotensin-converting enzyme inhibition prolongs survival and modifies the transition to heart failure in rats with pressure overload hypertrophy due to ascending aortic stenosis. Circulation 90:1410–1422

Yamazaki T, Uematsu T, Mizuno A, Takiguchi Y, Nakashima M (1989) Alterations of cardiac adrenoceptors and calcium channels subsequent to production of aortic insufficiency in rats. Arch Int Pharmacodyn Ther 299:155–168

A.6.0.1.2 Chronic Heart Failure in Rats

PURPOSE AND RATIONALE

Rat models of heart failure were reviewed by Muders and Elsner (2000). Chronic heart failure can be induced in rats by occlusion of coronary arteries. One of the first reports was by Selye et al. (1960). More recent reports are by Pfeffer et al. (1979), Hodsman et al. (1988), Van Veldhuisen et al. (1994, 1995), Kajstura et al. (1994), Gómez et al. (1997), Liu et al. (1997a, 1997b), and Jadavo et al. (2005).

Itter et al. (2004) described a model of chronic heart failure (CHF) in spontaneously hypertensive rats.

PROCEDURE

Study Design

Adult male 4-month-old SHR/NHsd and WKY/NHsd rats (Harlan Sprague Dawley, Winkelmann, Germany) weighing 250–300 g were used. Cardiovascular failure was induced by permanent (8 weeks) occlusion of the left coronary artery 2 mm distal to the origin from the aorta resulting in a large infarction of the free left ventricular wall.

Eight weeks after surgery, parameters indicating CHF were measured. Cardiac hypertrophy, function, and geometric properties were determined by the "working heart" mode and *in vivo* determinations by MRI and heart weight. Hydroxyproline/proline ratio was measured as an indicator of heart fibrosis.

Surgery

The rats were anesthetized with a mixture of ketamine/xylazine (35/2 mg/kg) i.p. The left ventrolateral thorax was shaved and prepared to create a disinfected surgical access area. When a stable anesthesia was achieved the animals were placed on a small animal operation table, intubated and ventilated with room air using a small animal ventilator (KTR-4, Hugo Sachs Elektronik, March-Hugstedten, Germany). The level of anesthesia was deemed as adequate following loss of the pedal withdrawal reflex and absence of the palpebral reflex. The tidal volume was adjusted at 3–5 ml and the ventilation rate was 40 breaths/min. Left thoracotomy was performed via the third intercostal space. The heart was exposed and the pericardium opened. The left main coronary artery was ligated with Perma-Hand silk 4–0 USP (Ethicon, Norderstedt, Germany) near its origin at the aorta (2 mm distal to the edge of the left atrium). Ligation resulted in infarction of the free left ventricular wall. Ligation was deemed successful when the anterior wall of the left ventricle turned pale. At this point the lungs were hyperinflated by increasing the positive endexpiratory pressure, and the chest was closed. The rats were placed on a heating pad. They were continuously monitored until they start moving in their cages. To avoid ventricular arrhythmias, lidocaine (2mg/kg i.m.) was given before surgery. The sham procedure consisted of opening the pericardium and placing a superficial suture in the epicardium of the LV. To prevent acute lung edema, the rats received furosemide 2 mg/kg twice daily for 3 days via the drinking water.

Measurements at the End of the Study

Before killing the animals 8 weeks after MI, non-invasive sequential nuclear magnetic resonance (NMR) measurements of heart geometric properties were done. Thereafter the animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (Heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved (stage III 3, reflexes absent), the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The right carotid artery was cannulated with a polyethylene catheter to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure and heart rate over a stable time course of 10 min.

A transverse laparotomy and a right anterolateral thoracotomy were performed, and the heart was rapidly removed for the evaluation of its function in the working heart mode. Thereafter the heart weight, and

the left and right ventricular weights were determined. For infarct size determination the left ventricle was sectioned transversely into four slices from the apex to the base. The infarct size was determined by planimetry and expressed as a percentage of LV mass. Lung weight and further lung histology sections were evaluated. Hydroxyproline/proline ratio was determined in paraffin-embedded slices of the left ventricle.

Magnetic Resonance Imaging

The animals were monitored by MRI at day 7 and day 42 post-MI. The rats were anesthetized with a mixture of 1% halothane and 30/70 N_2O/oxygen with a specially manufactured rat mask. The fully anesthetized rats (phase III 3) were placed on a cradle made of Plexiglas in a supine position. Respiration and ECG were monitored continuously. MRI experiments were performed according to Rudin et al. (1991). The images were acquired by a spinecho sequence SE (500/20), the field of view was 50 mm, the image resolution was 256×256 pixels with a dimension of 0.2×0.2 mm. Four adjacent transverse slices were recorded; slice thickness was 1.5 mm. Before the acquisition of data, a coronary pilot scan was measured for adequate positioning of the transverse slices. MRI data acquisition was gated to the cardiac cycle by a Physiograd SM 785 MR monitoring system (Bruker, Karlsruhe, Germany). Two sets of transverse images were acquired, one at end-systole and another at end-diastole. End-diastole was defined as the image obtained 8 ms after the onset of the R wave of the ECG, corresponding to the largest cavity area. End-systole was defined as the image with the smallest LV cavity area. The image analysis was done using Bruker software (Karlsruhe, Germany). The parameters of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), septum size, infarct size, ejection fraction (EF), left ventricular chamber diameter (r) and circumference were measured. EF was estimated in percentage terms by the subtraction LVEDV–LVESV. After the procedure the rats were ventilated with oxygen, the mask was replaced, and they were brought back into their cages. They were monitored until they started moving in the cage.

Blood Pressure/Heart Rate

The animals were anesthetized with pentobarbitone (180 mg/kg i.p.) and subsequently heparinized (Heparin sodium 500 IU/100 g body weight i.p.). Once stable anesthesia was achieved, the animals were connected to an artificial respirator via a PE tube inserted into the trachea and ventilated with room air. The right carotid artery was cannulated with a polyethylene catheter. The catheter was connected to a PLUGSYS measuring system (Hugo Sachs Elektronik, March-Hugstedten, Germany) to monitor mean blood pressure, systolic blood pressure, diastolic blood pressure and heart rate over a stable time course of 10 min.

Working Heart

For the final investigations, the heart of the anesthetized rat was rapidly removed and immersed in physiological buffer chilled to 4°C. The aorta was dissected free and mounted onto a cannula (internal diameter: 1.4 mm) attached to a perfusion apparatus. The hearts were perfused according to the method of Langendorff with an oxygenated (95% O_2/5% CO_2) noncirculating Krebs–Henseleit solution of the following compositions (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4$, 1.64; $NaHCO_3$, 24.88; KH_2PO_4, 1.18; glucose, 5.55; and Na-pyruvate, 2.0 at a perfusion pressure of 60 mmHg. Any connective tissue, thymus or lung was carefully removed. A catheter placed into the pulmonary artery drained the coronary effluent perfusate that was collected for the determination of coronary flow and venous pO_2 measurements. The left atrium was cannulated via an incision of the left auricle. All pulmonary veins were ligated close to the surface of the atria. When a tight seal with no leaks had been established and after a 15-min equilibration period, the hearts were switched into the working mode, using a filling pressure (preload) of 12 mmHg in WKY/NHsd and 18 mmHg in SH rats. The afterload pressure was 60 mmHg in WKY/NHsd and 80 mmHg in SH rats. After validation of the basis parameters the afterload pressure was enhanced in a cumulative manner from an additional 20 mmHg to 140 mmHg. Thereafter the isovolumetric maxima were determined by enhancing the preload pressure in steps of 5 mmHg to 30 mmHg. Flow and pressure signals for computation were obtained from the PLUGSYS-measuring system. Computation of data was performed with a sampling rate of 500 Hz, averaged every 2 s, using the software Aquire Plus V1.21f (PO-NE-MAH, Hugo Sachs Elektronik, March-Hugstedten, Germany).

Determination of Infarct Size

After the evaluation of the external heart work, the total heart weight, the left and right ventricular weights were determined. The left ventricle was then sectioned transversely into four slices from the apex to the base. Eight pictures were taken of each rat heart, two from each slice. Total infarct size was determined by planimetry of the projected and magnified slices.

The area of infarcted tissue as well as the intact myocardium of each slice were added together and averaged. The infarcted fraction of the left ventricle was calculated from these measurements and expressed as a percentage of the LV mass. The left ventricular perimeter, diameter, infarct scar length, as well as wall thickness and infarct wall thinning were determined as well. According to Pfeffer et al. (1985) and Pfeffer and Pfeffer (1987), rats with infarct sizes < 20% and > 40% were excluded from the study.

Lung Histological Determination

After lung weight determination, the organ was immersed in 4% formalin (pH 7.0–7.5; 0.1 M). The lung was cut into small pieces, dehydrated and embedded in paraffin. Hematoxylin and eosin (HE) sections were evaluated by light microscopy.

Hydroxyproline/Proline Ratio

After embedding, the rest of the fixed left ventricular tissue was freeze-dried. Proline and hydroxyproline was then analyzed according to the method of López de León and Rojkind (1985) and the ratio of both were calculated.

REFERENCES AND FURTHER READING

The data are given as mean ± SEM. Statistics were performed using the SAS system statistics package (SAS Institute, Cary, N.C., USA) with a sequential rejection *t*-test.

MODIFICATIONS OF THE METHOD

Jain et al. (2000) studied the effects of angiotensin II receptor blockade after coronary ligation and exercise training on treadmill in rats.

Medvedev and Gorodetskaya (1993) induced heart failure in rats by microembolization of coronary vessels with 15-µm plastic microspheres.

Katona et al. (2004) found that selective sensory denervation by capsaicin aggravates adriamycin-induced cardiomyopathy in rats.

A simple and rapid method of developing high output heart failure and cardiac hypertrophy in rats by producing **aortacaval shunts** was described by Garcia and Diebold (1990). Rats weighing 180–200 g were anesthetized with 30 mg/kg i.p. pentobarbitone. The vena cava and the abdominal aorta were exposed by opening the abdominal cavity via a midline incision. The aorta was punctured at the union of the segment two-thirds caudal to the renal artery and one-third cephalic to the aortic bifurcation with an 18-gauge disposable needle. The needle was advanced into the aorta, perforating its adjacent wall and penetrating in the vena cava. A bulldog vascular clamp was placed across the aorta caudal to the left renal artery. Once the aorta was clamped, the needle was fully withdrawn and a drop of cyanoacrylate glue was used to seal the aorta-punctured point. The clamp was removed 30 s later. The patency of the shunt was verified visually by swelling vena cava and the mixing of arterial and venous blood. The peritoneal cavity was closed with silk thread stitches and the skin with metallic clips. Rats with aorta-caval shunts developed cardiac hypertrophy with significantly higher absolute and relative heart weights.

Other studies with aortocaval shunts in rats were published by Flaim et al. (1979) and Liu et al. (1991).

Isoyama et al. (1988) studied myocardial hypertrophy after creating aortic insufficiency in rats.

Terlink et al. (1998) studied ventricular dysfunction in rats with diffuse isoproterenol-induced myocardial necrosis.

Sabbah et al. (1991) described a model of chronic heart failure produced by multiple sequential coronary microembolizations in **dogs.**

REFERENCES AND FURTHER READING

Flaim SF, Minteer WJ, Nellis SH, Clark DP (1979) Chronic arteriovenous shunt: evaluation of a model for heart failure in rat. Am J Physiol 236:H698–H704

Garcia R, Diebold S (1990) Simple, rapid, and effective method of producing aortacaval shunts in the rat. Cardiovasc Res 24:430–432

Gómez AM, Valdivia HH, Cheng H, Lederer MR, Santana LF, Cannell MB, McCune SA, Altschuld REA, Lederer WJ (1997) Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. Science 276:800–806

Hodsman GP, Kohzuki M, Howes LG, Sumithran E, Tsunoda K, Johnston CI (1988) Neurohumoral responses to chronic myocardial infarction in rats. Circulation 78:376–381

Itter G, Jung W, Juretschke P, Schölkens BA, Linz W (2004) A model of chronic heart failure in spontaneous hypertensive rats (SHR). Lab Anim 38:138–148

Isoyama S, Grossman W, Wei JY (1988) The effect of myocardial adaptation to volume overload in the rat. J Clin Invest 81:1850–1857

Jadavo S, Huang C, Kirshenbaum L, Karmazyn M (2005) NHE-1 inhibition improves impaired mitochondrial permeability transition and respiratory function during postinfarction remodelling in the rat. J Mol Cell Cardiol 38:135–143

Jain M, Liao R, Ngoy S, Whittaker P, Apstein CS, Eberli FR (2000) Angiotensin II receptor blockade attenuates the deleterious effects of exercise training on post-MI ventricular remodelling in rats. Cardiovasc Res 46:66–72

Kajstura J, Zhang X, Reiss K, Szoke E, Li P, Lagrasta C, Cheng W, Darzynkiewicz Z, Olivetti G, Anversa P (1994) Myocyte cellular hyperplasia and myocytes cellular hypertrophy contribute to chronic ventricular remodelling in coronary artery narrowing-induced cardiomyopathy in rats. Circ Res 74:383–400

Katona M, Boros K, Sántha P, Ferdinandy P, Dux M, Jancsó G (2004) Selective sensory denervation by capsaicin aggra-

vates adriamycin-induced cardiomyopathy in rats. Naunyn-Schmiedebergs Arch Pharmacol 370:436–443
Liu Z, Hilbelink DR, Crockett WB, Gerdes AM (1991) Regional changes in hemodynamics and cardiomyocyte size in rats with aortacaval fistulas. 1. Developing and established hypertrophy. Circ Res 69:52–58
Liu YH, Yang XP, Sharov VG, Nass O, Sabbah HN, Peterson E, Carretero OA (1997a) Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. J Clin Invest 99:1926–1935
Liu YH, Yang XP, Nass O, Sabbah HN, Petersen E, Carretero OA (1997b) Chronic heart failure induced by coronary artery ligation in Lewis inbred rats. Am J Physiol 272 (2 Pt2):H722–H727
López de León A, Rojkind M (1985) A simple method for collagen and total protein determination in formalin-.fixed paraffin-embedded sections. J Histochem Cytochem 33:737–743
Medvedev OS, Gorodetskaya EA (1993) Systemic and regional hemodynamic effects of perindopril in experimental heart failure. Am Heart J 126(3 Pt2):764–769
Muders F, Elsner F (2000) Animal models of chronic heart failure. Pharmacol Res 41:605–612
Pfeffer MA, Pfeffer JM, Fishbein MC, Fletcher PJ, Spadaro J, Kloner RA, Braunwald E (1979) Myocardial infarct size and ventricular function in rats. Circ Res 44:593–512
Pfeffer MA, Pfeffer JM (1987) Ventricular enlargement and reduced survival after myocardial infarction. Circulation 7 [Suppl. IV]:93–97
Pfeffer MA, Pfeffer JM, Steinberg C, Finn P (1985) Survival after an experimental myocardial infarction: beneficial effects of long-term therapy with captopril. Circulation 72:406–412
Rudin M, Pedersen B, Umemura K, Zierhut W (1991) Determination of rat heart morphology and function *in vivo* in two models of cardiac hypertrophy by means of magnetic resonance imaging. Basic Res Cardiol 8:165–74
Sabbah HN, Stein PD, Kono T, Gheorghiade M, Levine TB, Jafri S, Hawkins ED, Goldstein S (1991) A canine model of chronic heart failure produced by multiple sequential coronary microembolizations. Am J Physiol 260(4 Pt 2):H1379–H1384
Selye H, Bajusz E, Grasso S, Mendell P (1960) Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11:398–407
Terlink JR, Pfeffer JM, Pfeffer MA (1998) Effect of left ventricular sphericity on the evolution of ventricular dysfunction in rats with diffuse isoproterenol-induced myocardial necrosis. J Cardiac Fail 4:45–56
Van Veldhuisen DJ, van Gilst WH, de Smet BJ, de Graeff PA, Scholtens E, Buikema H, Girbes AR, Wesseling H, Lie KI (1994) Neurohumoral and hemodynamic effects of ibopamine in a rat model of chronic myocardial infarction and heart failure. Cardiovasc Drugs Ther 8:245–250
Van Veldhuisen DJ, Brodde OE, van Gilst WH, Schulze C, Hegeman H, Anthonio RL, Scholtens E, de Graeff PA, Wesseling H, Lie KI (1995) Relation between myocardial β-adrenoceptor density and hemodynamic and neurohumoral changes in a rat model of chronic myocardial infarction: effects of ibopamine and captopril. Cardiovasc Res 30:386–393

A.6.0.2
Cardiac Hypertrophy and Insufficiency in Mice

A.6.0.2.1
Cardiac Hypertrophy in Mice

PURPOSE AND RATIONALE

Rockman et al. (1991, 1993) developed a model of ventricular hypertrophy in the intact mouse by use of microsurgical techniques.

PROCEDURE

Eight-week-old adult mice weighing 18–22 g are anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine, 5 mg/kg xylazine, and 2.5 mg/kg morphine. Animals are placed under a dissecting microscope in the supine position and a midline cervical incision is made to expose the trachea and carotid arteries. After endotracheal intubation, the cannula is connected to a volume cycled rodent ventilator on supplemental oxygen with a tidal volume of 0.2 ml and a respiratory rate of 110 per min. Both left and right carotid arteries are cannulated with flame stretched PE50 tubing. Catheters are connected to modified P50 Statham transducers.

The chest cavity is entered in the second intercostal space at the left upper sternal border through a small incision and the thymus is gently deflected out of the field of view to expose the aortic arch. After the transverse aorta is isolated between the carotid arteries, it is constricted by a 7.0 nylon suture ligature against a 27-gauge needle, the latter being promptly removed to yield a constriction of 0.4 mm diameter and provide a reproducible transverse aortic constriction of 65–75%.

The hemodynamic effects of acute and chronic constriction are followed by monitoring the pressure gradient between the two carotid arteries in anesthetized animals. Systolic and mean arterial pressure at baseline, during total occlusion when the ligature is tied, and early (15 min) and late (7 days) after transverse aortic constriction are recorded. The increase in systolic pressure provides an adequate mechanical stimulus for the development of cardial hypertrophy.

To confirm myocardial hypertrophy, both sham-operated and aortic-constricted hearts are examined 7 days after operation. Hearts examined for *cell size* are perfused with 4% paraformaldehyde/1% glutaraldehyde through the apex, immersed in osmium tetroxide, dehydrated in graded alcohols, and embedded in araldite. Tissue blocks are sectioned at a thickness of 1 μm, mounted on slides, and stained with tolu-

idine blue. Cell areas are measured by manually tracing the cell outline on an imaging system connected to a computer.

At the end of the experiment, mice were sacrificed in anesthesia, heart excised and weighed, the atria and ventricles separately frozen in liquid nitrogen for Northern blot analysis. Total RNA is extracted by a single step extraction with guanidinium thiocyanate. The RNA is size fractionated by agarose gel electrophoresis, transferred to nylon membranes by vacuum blotting, and hybridized with the appropriate complementary DNA probes labeled with ^{32}P by random priming to a specific activity of $0.95–1.2 \times 10^6$ cpm/ng.

EVALUATION

Variables measured are expressed as mean $\pm$ SD. Statistical significance of differences between sham-operated and thoracic aortic-constricted animals is assessed by Student's *t*-test.

MODIFICATIONS OF THE METHOD

Dom et al. (1994) studied myosin heavy chain regulation and myocytes' contractile depression after LV hypertrophy in aortic-banded mice.

Okada et al. (2004) subjected mice to transverse aortic constriction. Echocardiographic analysis demonstrated cardiac hypertrophy and failure 1 and 4 weeks after surgery. Cardiac expression of endoplasmatic reticulum chaperones was significantly increased indicating that pressure overload by transverse aortic constriction induced prolonged endoplasmatic reticulum stress.

REFERENCES AND FURTHER READING

Dom GW, Robins J, Ball N, Walsh RA (1994) Myosin heavy chain regulation and myocytes contractile depression after LV hypertrophy in aortic-banded mice. Am J Physiol 267(1 Pt2):H400–H406

Okada KI, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, Hirata A, Fujita M, Nagamachi Y, Nakatani T, Yutani C, Ozawa K, Ogawa S, Tomoike H, Hori M, Kitakaze M (2004) Prolonged endoplasmatic reticulum stress in hypertrophic and failing heart after aortic constriction. Possible contribution of endoplasmatic reticulum stress to cardiac myocyte apoptosis. Circulation 110:705–712

Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, Ross J Jr, Chien KR (1991) Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an *in vivo* murine model of cardiac hypertrophy. Proc Natl Acad Sci USA 88:8277–8281

Rockman HA, Knowlton KU, Ross J Jr, Chien KR (1993) *In vivo* murine cardiac hypertrophy. A novel model to identify genetic signaling mechanisms that activate an adaptive physiological response. Circulation 87 (Suppl VII):VII-14–VII-21

A.6.0.2.2 Chronic Heart Failure in Mice

PURPOSE AND RATIONALE

Several authors reported the development of murine models of cardiac failure (Kaplan et al. 1994; Rockman et al. 1994; Balasubramaniam et al. 2004; Suzuki et al. 2004; Walther et al. 2004; Wang et al. 2004; Liao et al. 2005).

Xu et al. (2004) studied cardioprotection in mice with heart failure by dual inhibition of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP).

PROCEDURE

Mice with a targeted deletion of the B_2 kinin receptor gene or C57BL/6J mice at an age of 10–12 weeks were anesthetized with 50 mg/kg sodium pentobarbital i.p., intubated and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space; the lungs were retracted to expose the heart, and the pericardium was opened. The left anterior descending coronary artery was ligated with an 8–0 nylon suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was considered successful when the anterior wall of the left ventricle turned pale and an obvious ST segment elevation was observed. The lungs were inflated by increasing positive end-expiratory pressure and the thoracotomy site was closed. Sham-operated mice were subjected to the same procedure except that the suture around the left anterior coronary artery was not tied.

Systolic blood pressure was measured in conscious mice using a non-invasive computerized tail-cuff system. Cardiac geometry and function were evaluated with a Doppler echocardiographic system. LV diastolic dimension was measured and ejection fraction was calculated from

$[(LVAd - LVAs)/LVAd] \times 100$, where LVAd is LV diastolic area and LVAs is LV systolic area.

Four weeks after surgery, each strain was separated into one group treated with an ACE inhibitor, one group treated with a NEP inhibitor, one group treated with both inhibitors and one control group. All drugs were administered in drinking water for 20 weeks.

At the end of the study, all mice were anesthetized with pentobarbital and the heart stopped at diastole

by intraventricular injection of 15% KCl. The heart, lungs, and liver were weighed to assess hypertrophy and congestion. Infarct size was determined by Gomori trichrome staining and expressed as the ration of the infarcted portion to total LV circumference.

Sections (6 µm) from each slice were double-stained with fluorescein-labeled peanut agglutinin to delineate the myocyte cross-sectional area and interstitial space and rhodamine-labeled *Griffonia simplicifolia* lectin I to show the capillaries. To calculate interstitial collagen fraction, the total surface area (microscopic field), interstitial space (collagen plus capillaries), and area occupied by capillaries alone were measured by computer-assisted videodensometry.

After 20 weeks of treatment, plasma renin was measured.

EVALUATION

Data were expressed as mean ± SE. Mortality rates were compared using χ^2 tests. For the echo, blood pressure, heart weight, lung weight, infarct size, plasma renin concentration, and histology data, paired or two-sample tests using non-parametric methods were used to perform all comparisons of interest.

REFERENCES AND FURTHER READING

Balasubramaniam R, Chawla S, Mackenzie L, Schwiening CJ, Grace AA, Huang CLH (2004) Nifedipine and diltiazem suppress ventricular arrhythmogenesis and calcium release in mouse hearts. Pflügers Arch 449:150–158

Kaplan ML, Cheslow Y, Vikstrom K, Malhotra A, Geenen DL, Nakouzi A, Leinwand LA, Buttrik PM (1994) Cardiac adaptations to chronic exercise in mice. Am J Physiol 267(3 Pt2):H1167–H1173

Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Asanuma H, Minamino T, Tomoike H, Hori M, Kitakaze M (2005) Benidipine, a long-acting calcium channel blocker, inhibits cardiac remodeling in pressure-overloaded mice. Cardiovasc Res 65:879–888

Rockman HA, Ono S, Ross RS, Jones LR, Karimi M, Bhargava V, Ross J, Jr, Chien KR (1994) Molecular and physiological alterations in murine ventricular dysfunction. Proc Natl Acad Sci USA 91:2694–2698

Suzuki Y, Nakano K, Sugiyama M, Imagawa JI (2004) βARK1 inhibition improves survival in a mouse model of heart failure induced by myocardial infarction. J Cardiovasc Pharmacol 44:329–334

Walther T, Steendijk P, Westermann D, Hohmann C, Schulze K, Heringer-Walther S, Schultheiss HP, Tschöpe C (2004) Angiotensin deficiency in mice leads to dilated cardiomyopathy. Eur J Pharmacol 493:161–165

Wang D, Liu YH, Yang XP, Rhaleb NE, Xu J, Peterson E, Rudolph AE, Carretero OA (2004) Role of a selective aldosterone blocker in mice with chronic heart failure. J Cardiac Fail 10:67–73

Xu J, Carretero OA, Liu YH, Yang F, Shesley EG, Oja-Tebbe N, Yang XP (2004) Dual inhibition of ACE and NEP provides greater cardioprotection in mice with heart failure. J Cardiac Fail 10:83–89

A.6.0.2.3 Transgenic Mice and Heart Failure

PURPOSE AND RATIONALE

Several hundreds of papers on transgenic mice and heart failure are published. Only a few can be mentioned here.

Chien (1995) described cardiac muscle diseases in genetically engineered mice.

Edwards et al. (1996) described severe cardiomyopathy in transgenic mice overexpressing the skeletal muscle myogenic regulator *myf5*.

Arber et al. (1997) found that MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure.

Graham et al. (1997) described a mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoforms of the adenine nucleotide translocator.

Iwase et al. (1997) studied cardiomyopathy in transgenic mice induced by overexpression of the cardiac stimulatory G protein α subunit.

Knollmannn et al. (2000) reported remodeling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin.

Beggah et al. (2002) described reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes.

Verheule et al. (2004) found increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-β1.

Duncan et al. (2005) found that chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy.

Hartil and Charron (2005) reviewed mouse models where transgenic technology has been utilized to alter expression of genes involved in cardiac uptake and metabolism of either lipid or carbohydrate.

Hilfiker-Kleiner et al. (2005) reported that STAT3-knockout mice harboring a cardiomyocyte-restricted deletion of STAT3 showed enhanced susceptibility to cardiac injury caused by myocardial ischemia, systemic inflammation, or drug toxicity.

Sanbe et al. (2005) studied reversal of amyloid-induced heart disease in desmin-related cardiomyopathy.

REFERENCES AND FURTHER READING

Arber S, Hunter JJ, Ross J, Jr, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P (1997) MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 88:393–403

Beggah AT, Escoubert B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, Bocchi B, Peuchmaur M, Delcayre C (2002) Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. Proc Natl Acad Sci USA 99:7160–7165

Chien KR (1995) Cardiac muscle diseases in genetically engineered mice: evolution of molecular physiology. Am J Physiol 269:H755–H766

Duncan JG, Ravi R, Stull LB, Murphy AM (2005) Chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy. Am J Physiol 289:H1512–H1518

Edwards JG, Lyons GE, Micales BK, Malhotra A, Factor S, Leinwand LA (1996) Cardiomyopathy in transgenic *myf5* mice. Circ Res 78:379–387

Graham BH, Waymire KG, Cottrell B, Trounce IA, MacGregor GR, Wallace DC (1997) A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoforms of the adenine nucleotide translocator. Nat Genet 16:226–234

Hartil K, Charron MJ (2005) Genetic modification of the heart: transgenic modification of cardiac lipid and carbohydrate utilization. J Mol Cell Cardiol 39:581–593

Hilfiker-Kleiner D, Hilfiker A, Drexler H (2005) Many good reasons to have STAT3 in the heart. Pharmacol Ther 107:131–137

Iwase M, Uechi M, Vatner DA, Asai K, Shannon RP, Kudej RK, Wagner TE, Wight DC, Patrick TA, Ishikawa Y, Homcy CJ, Vatner SF (1997) Cardiomyopathy induced by cardiac Gs α overexpression. Am J Physiol 272 (1 Pt2):H585–H589

Knollmannn BC, Knollmann-Ritschel BEC, Weissman NJ, Jones LR, Morad M (2000) Remodelling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. J Physiol (Lond) 525.2:483–498

Sanbe A, Osinska H, Villa C, Gulick J, Klevitsky R, Glabe CG, Kayed R, Robbins J (2005) Reversal of amyloid-induced heart disease in desmin-related cardiomyopathy. Proc Natl Acad Sci USA 102:13592–13597

Verheule S, Sato T, Everett T, Engle SK, Otten D, Rubart-von der Lohe M, Nakajima HO, Nakajima H, Field LJ, Olgin FE (2004) Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-β1. Circ Res 94:1458–1465

A.6.0.3 Cardiac Insufficiency in Guinea Pigs

PURPOSE AND RATIONALE

Congestive heart failure in man is characterized by cardiac hypertrophy, peripheral edema, lung and liver congestion, dyspnea, hydrothorax and ascites. Effective treatment is achieved by cardiac glycosides. Based on techniques reported by Selye et al. (1960) a method was developed to induce congestive heart failure in guinea pigs with symptoms very close to human pathology (Vogel and Marx 1964; Vogel et al. 1965).

PROCEDURE

Male guinea pigs weighing 250–400 g are used. The fur at the ventral thorax is shaved and the skin disinfected. The animal is anesthetized with ether. The skin is cut with scissors on the left side at a length of 4 cm. The left musculus pectoralis is cut at the costal insertion and elevated. The forth intercostal space is opened with two blunted forceps. The heart is pressed against the opening with the left hand. The pericardium is opened with a fine forceps and pulled back to the basis of the heart. The beating heart is extruded from the thorax wound by pressure with the left hand on the right thorax wall. A ring-shaped clamp covered with a thin rubber tube is placed around the basis of the heart keeping the heart outside of the thorax without closing off the blood circulation. A thread soaked with diluted disinfectant solution is placed as a loop around the apex of the heart and tightened so that the apical third of both ventricles is tied off. The degree of tightening of the loop is essential. Complete interruption of blood supply to the apical third resulting in necrosis has to be avoided as well as the loop's slipping off. Technical skill is necessary to place the loop around the beating heart into the correct position. After removal of the clamp the heart is placed back, the incision between the forth and fifth costal rib closed and the musculus pectoralis placed over the wound. Intrathoracal air forming a pneumothorax is removed by pressure on both sides of the thorax. After application of an antibiotic emulsion the skin wound is closed. The surgical procedure has to be finished within a short period of time.

The animals develop symptoms of severe congestive heart failure with a death rate of 80% within 14 days. Lung weight and relative heart weight are significantly increased. Exudate in the thorax cavity and ascites amount between 3.5 and 7.5 ml with extreme values of 17.5 ml. Lung edema and liver congestion are found histologically. Peripheral edema and preterminal dyspnea and tachypnea are observed. When treated with various doses (0.1 to 100 µg/kg) of cardiac glycosides s.c. or i.m. over a period of 14 days the symptoms of cardiac insufficiency, e. g., volumes of transudate as well as death rate, are dose-dependent diminished.

EVALUATION

From survival rate, ED_{50} values of cardiac glycosides can be calculated which are in the same dosage range as therapeutic doses in man.

CRITICAL ASSESSMENT OF THE METHOD

The experimental model in guinea pigs reflects very closely the symptoms of cardiac insufficiency in man, e. g., lung congestion, hydrothorax, liver congestion, ascites, peripheral edema and cardiac hypertrophy. The

therapeutic potency of cardiac glycosides can be evaluated with this method. Additional factors being known to enhance the symptoms of congestive heart failure in man, like salt load and diphtheria toxin, further increase mortality and hydropic symptoms. The method can be used for special purposes, however, it needs considerable training and technical skill.

MODIFICATIONS OF THE METHOD

Siri et al. (1989, 1991) produced left ventricular hypertrophy in the guinea pig by gradually increasing ventricular afterload. A mildly constricting band was placed around the ascending aorta of very young guinea pigs (225–275 g). With growth to 500–1000 g, left ventricular systolic pressure increased and ventricular hypertrophy developed. Only some of the animals developed dyspnea and severe ventricular dysfunction.

Kiss et al. (1995) studied the effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure in guinea pigs. The descending aorta was banded for 4 and 8 weeks in adult guinea pigs.

Tweedle et al. (1995) assessed subrenal banding of the abdominal aorta as a method of inducing cardiac hypertrophy in the guinea pig.

Pfeffer et al. (1987) induced myocardial infarction in **rats** by ligation of the left coronary artery and found hemodynamic benefits and prolonged survival with long-term captopril therapy.

Acute ischemic left ventricular failure can be induced in anesthetized **dogs** by repeated injections of plastic microspheres into the left coronary artery (see A.3.2.4).

Huang et al. (1997) created congestive heart failure in **sheep** by selective sequential intracoronary injection of 90 µm microspheres under 1.5% isofluorane injection.

REFERENCES AND FURTHER READING

Huang Y, Kawaguchi O, Zeng B, Carrington RAJ, Horam CJ, Yuasa T, Abdul-Hussein N, Hunyor SN (1997) A stable ovine congestive heart failure model. A suitable substrate for left ventricular assist device assessment. ASAIO J 43:M408–M413

Kiss E, Ball NA, Kranias EG, Walsh RA (1995) Differential changes in cardiac phospholamban and sarcoplasmic reticular Ca^{2+}-ATPase protein levels. Effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure. Circ Res 77:759–764

Pfeffer JM, Pfeffer MA, Braunwald E (1987) Hemodynamic benefits and prolonged survival with long-term captopril therapy in rats with myocardial infarction and heart failure. Circulation 75:I-149–I-155

Selye H, Bajusz E, Grasso S, Mendell P (1960) Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11:398–407

Siri FM, Nordin C, Factor SM, Sonnenblick E, Aronson R (1989) Compensatory hypertrophy and failure in gradual pressure-overloaded guinea pig heart. Am J Physiol 257(3 Pt2):H1016–H1024

Siri FM, Krueger J, Nordin C, Ming Z, Aronson RS (1991) Depressed intracellular calcium transients and contraction in myocytes from hypertrophied and failing guinea pig hearts. Am J Physiol 261 (2 Pt2):H514–H530

Tweedle D, Henderson CG, Kane KA (1995) Assessment of subrenal banding of the abdominal aorta as a method of inducing cardiac hypertrophy in the guinea pig. Cardioscience 6:115–119

Vogel HG, Marx KH (1964) Untersuchungen an der experimentellen hydropischen Herzinsuffizienz des Meerschweinchens. Naunyn Schmiedeberg's Arch Path Pharm 247:337

Vogel HG, Marx KH, Ther L (1965) Über die experimentelle hydropische Herzinsuffizienz am Meerschweinchen. Operationstechnik, Einfluß zusätzlicher Faktoren und Effekt von Herzglykosiden. Arzneim Forsch/Drug Res 15:542–548

A.6.0.4 Cardiomyopathic Syrian Hamster

PURPOSE AND RATIONALE

Cardiomyopathy in Syrian hamsters has been described by Bajusz et al. (1966), Bajusz and Lossnitzer (1968), Bajusz (1969), Bajusz et al. (1969a, b), Homburger and Bajusz (1970), Gertz (1972). The disease originates from an autosomal, recessively transmissible disorder, which leads to degenerative lesions in all striated muscles and in particular in the myocardium. Histopathological changes consist of myocytolytic necrosis followed by fibrosis and calcification. The evolution of the cardiomyopathic disease can be characterized by five distinct phases: A prenecrotic stage, in which no pathology is evident, a time of active myocytolysis and cellular necrosis, a phase of fibrosis and calcium deposition, an overlapping period of reactive hypertrophy of the remaining viable myocytes, and a final stage of depressed myocardial performance and failure.

PROCEDURE

The model of cardiomyopathy in Syrian hamsters has been used by several authors. One has to note, that several strains of cardiomyopathic hamsters have been used: strain Bio 53:58 by Capasso et al. (1989, 1990) and by Chemla et al. (1992, 1993), strain BIO 14.6 by Tapp et al. (1989) and by Sen et al. (1990), strain CHF 146 CM by van Meel et al. (1989) and by Haleen et al. (1991), strain BIO82.62 by ver Donck et al. (1991), strain J-2-N by Kato et al. (1992), strain CHF 147 by Desjardins et al. (1989), Hanton et al. (1993).

Various experimental protocols have been described. Most authors use survival rate and heart weight as end point (e. g., van Meel et al. 1989; ver Donck et al. 1991; Hanton et al. 1993). Generally, the experiments are started with animals at an age of 120 to 200 days.

Capasso et al. (1989, 1990) studied the mechanical and electrical properties of cardiomyopathic hearts of Syrian hamsters using isolated left ventricular posterior papillary muscles.

Tapp et al. (1989) tested stress-induced mortality in cardiomyopathic hamsters by five consecutive daily 2-h periods supine immobilizations at 4°C.

Sen et al. (1990) tested the inotropic and calcium kinetic effects of calcium channel agonists and antagonists in primary cultures of isolated cardiac myocytes.

Haleen et al. (1991) tested the effects of an angiotensin converting enzyme inhibitor not only on survival, but also on left ventricular failure in the isolated Langendorff heart by measurement of left ventricular end-diastolic pressure, dP/dt_{max} and mean coronary flow.

Dixon et al. (1997) tested the effect of an AT_1 receptor antagonist on cardiac collagen remodelling in the cardiomyopathic Syrian hamster.

In addition to the effects on left ventricular papillary muscles strips, Chemla et al. (1992) tested the effects on diaphragm contractility in the cardiomyopathic Syrian hamster.

Whitmer et al. (1988) and Kuo et al. (1992) tested sarcolemmal and sarcoplasmatic reticulum calcium transport in the cardiomyopathic Syrian hamster.

Nigro et al. (1997) identified the Syrian hamster cardiomyopathy gene.

Tanguay et al. (1997) tested the coronary and cardiac sensitivity to a vasoselective benzothiazepine-like calcium antagonist in isolated, perfused failing hearts of Syrian hamsters.

Bilate et al. (2003) recommended the Syrian hamster as a model for the dilated cardiomyopathy of Chagas' disease. Female hamsters were infected via the intraperitoneal route with *Trypanosoma cruzi* Y strain blood trypomastigotes. Survival was monitored, echocardiography was performed after 4 and 12 months, and histopathological examinations were carried out at the end of the study period.

CRITICAL ASSESSMENT OF THE METHOD

Positive effects of various drugs have been found in the cardiomyopathic hamster, such as cardiac glycosides, inotropic compounds, beta-blockers, calcium antagonists, and ACE-inhibitors. The specificity of the effects has to be challenged.

MODIFICATIONS OF THE METHOD

The **tight-skin (TSK) mouse** is a genetic model of pulmonary emphysema connected with right ventricular hypertrophy (Martorana et al. 1990; Gardi et al. 1994).

REFERENCES AND FURTHER READING

Bajusz E (1969) Hereditary cardiomyopathy: a new disease model. Am Heart J 77:686–696

Bajusz E, Lossnitzer A (1968) A new disease model of chronic congestive heart failure: Studies on its pathogenesis. Trans NY Acad Sci 30:939–948

Bajusz E, Homburger F, Baker JR, Opie LH (1966) The heart muscle in muscular dystrophy with special reference to involvement of the cardiovascular system in the hereditary myopathy of the hamster. Ann NY Acad Sci 138:213–229

Bajusz E, Baker JR, Nixon CW, Homburger F (1969a) Spontaneous hereditary myocardial degeneration and congestive heart failure in a strain of Syrian hamsters. Ann NY Acad Sci 156:105–129

Bajusz E, Homburger F, Baker JR, Bogdonoff P (1969b) Dissociation of factors influencing myocardial degeneration and generalized cardiocirculatory failure. Ann NY Acad Sci 156:396–420

Bilate AMB, Salemi VMC, Ramirez FJA, de Brito T, Silva AM, Umezawa ES, Mady C, Kalil J, Cunha-Neto E (2003) The Syrian hamster as a model for the dilated cardiomyopathy of Chagas' disease: a quantitative echocardiographical and histopathological analysis. Microb Infect 5:1116–1124

Capasso JM, Olivetti G, Anvera P (1989) Mechanical and electrical properties of cardiomyopathic hearts of Syrian hamsters. Am J Physiol, Heart Circ Physiol 257:H1836–H1842

Capasso JM, Sonnenblick EH, Anversa P (1990) Chronic calcium channel blockade prevents the progression of myocardial contractile and electrical dysfunction in the cardiomyopathic Syrian hamster. Circ Res 67:1381–1393

Chemla D, Scalbert E, Desché P, Pourny JC, Lambert F, Lecarpentier Y (1992) Effects of perindopril on myocardial inotropy, lusitropy and economy, and on diaphragm contractility in the cardiomyopathic Syrian hamster. J Pharm Exp Ther 262:515–525

Chemla D, Scalbert E, Desché P, Pourny JC, Lambert F, Lecarpentier Y (1993) Myocardial effects of early therapy with perindopril during experimental cardiomyopathy. Am J Cardiol 71:41E–47E

Desjardins S, Mueller RW, Hubert RS, Cauchy MJ (1989) Effects of milrinone treatment in cardiomyopathic hamsters (CHF 147) with severe congestive heart failure. Cardiovasc Res 23:620–630

Devaux JY. Cabane L, Elser M, Flaouters H, Duboc D (1993) Non-invasive evaluation of the cardiac function in golden retriever dogs by radionuclide angiography. Neuromuscular Disord 3:429–432

Dixon IMC, Ju H, Reidl NL, Scammell-La-Fleur T, Werner JP, Jasmin G (1997) Cardiac collagen remodelling in the cardiomyopathic Syrian hamster and the effect of losartan. J Mol Cell Cardiol 29:1837–1850

Factor SM, Sonnenblick EH (1985) The pathogenesis of clinical and experimental congestive cardiomyopathy: recent concepts. Prog Cardiovasc Dis 27:395–420

Factor SM, Minase T, Cho S, Dominitz R, Sonnenblick EH (1982) Microvascular spasm in the cardiomyopathic Syrian hamster: a preventable cause of focal myocardial necrosis. Circ 66:342–354
Forman R, Parmley WW, Sonnenblick EH (1972) Myocardial contractility in relation to hypertrophy and failure in myopathic Syrian hamsters. J Mol Cell Cardiol 4:203–211
Gardi C, Martorana PA, Calzoni P, Cavarra E, Marcolongo P, de Santi MM, van Even P, Lungarella G (1994) Cardiac collagen changes during the development of right ventricular hypertrophy in tight-skin mice with emphysema. Exp Mol Pathol 60:100–107
Gertz EW (1972) Cardiomyopathic Syrian hamster: A possible model of human disease. Prog Exp Tumor Res 16:242–260
Haleen SJ, Weishaar RE, Overhiser RW, Bousley RF, Keiser JA, Rapundalo SR, Taylor DG (1991) Effects of Quinapril, a new angiotensin converting enzyme inhibitor, on left ventricular failure and survival in the cardiomyopathic hamster. Circ Res 68:1302–1312
Hanton G, Barnes P, Shepperson NB, Walley R (1993) Effects of hydrochlorothiazide and captopril on the survival and heart weight of cardiomyopathic hamsters. Res Commun Chem Pathol Pharmacol 81:159–166
Homburger F (1979) Myopathy of hamster dystrophy: history and morphological aspects. Ann NY Acad Sci 317:2–17
Homburger F, Bajusz E (1970) New models of human disease in Syrian hamsters. J Am Med Ass 212:604–610
Jasmin G, Proscheck L (1982) Hereditary polymyopathy and cardiomyopathy in the Syrian hamster: I. Progression of heart and skeleton muscle lesions in the UM-X7.1 line. Muscle Nerve 5:20–25
Jasmin G, Proschek L (1984) Calcium and myocardial cell injury. An appraisal in the cardiomyopathic hamster. Canad J Physiol Pharmacol 62:891–898
Kato M, Takeda N, Yang J, Nagano M (1992) Effects of angiotensin converting enzyme inhibitors and the role of the renin-angiotensin-aldosterone system in J-2-N cardiomyopathic hamsters. Jpn Circ J 56:46–51
Kuo TH, Tsang W, Wang KK, Carlock L (1992) Simultaneous reduction of the sarcolemmal and SR calcium APTase activities and gene expression in cardiomyopathic hamster. Biochim Biophys Acta 1138:343–349
Malhotra A, Karell M, Scheuer J (1985) Multiple cardiac contractile protein abnormalities in myopathic Syrian hamsters (Bio 53:58). J Mol Cell Cardiol 17:95–107
Martorana PA, Wilkinson M, van Even P, Lungarella G (1990) Tsk mice with genetic emphysema. Right ventricular hypertrophy occurs without hypertrophy of muscular pulmonary arteries or muscularization of arterioles. Am Rev Resp Dis 142:333–337
Nigro V, Okazaki Y, Belsito A, Piluso G, Matsuda Y, Politano L, Nigro G, Ventura C, Abbondanza C, Molinari AM, Acampora D, Nishimura A, Hayashizaki Y, Puca GA (1997) Identification of the Syrian hamster cardiomyopathy gene. Hum Mol Genet 6:601–607
Sen L, O'Neill M, Marsh JD, Smith TW (1990) Inotropic and calcium kinetic effects of calcium channel agonist and antagonist in isolated cardiac myocytes from cardiomyopathic hamsters. Circ Res 67:599–608
Strobeck JE, Factor SM, Bhan A, Sole M, Liew CC, Fein F, Sonnenblick EH (1979) Hereditary and acquired cardiomyopathies in experimental animals: Mechanical, biochemical and structural features. Ann NY Acad Sci 317:59–68
Tanguay M, Jasmin G, Blaise G, Dumant L (1997) Coronary and cardiac sensitivity to the vasoselective benzothiazepine-like calcium antagonist, clentiazem, in experimental heart failure. Cardiovasc Drugs Ther 11:71–79
Tapp WN, Natelson BH, Creighton D, Khazam C, Ottenweller JE (1989) Alprazolam reduces stress-induced mortality in cardiomyopathic hamsters. Pharmacol Biochem Behav 32:331–336
Van Meel JC, Mauz ABM, Wienen W, Diederen W (1989) Pimobendan increases survival of cardiomyopathic hamsters. J Cardiovasc Pharmacol 13:508–509
Ver Donck L, Wouters L, Olbrich HG, Mutschler E, Brogers M (1991) Nebivolol increases survival in cardiomyopathic hamsters with congestive heart failure. J Cardiovasc Pharmacol 18:1–3
Whitmer JT, Kumar P, Solaro RJ (1988) Calcium transport properties of cardiac sarcoplasmic reticulum from cardiomyopathic Syrian hamsters (BIO 53.58 and 14.6): evidence for a quantitative defect in dilated myopathic hearts not evident in hypertrophic hearts. Circ Res 62:81–85
Wiederhold KF, Nilius B (1986) Increased sensitivity of ventricular myocardium to intracellular calcium-overload in Syrian cardiomyopathic hamster. Biomed Biochim Acta 45:1333–1337

A.6.0.5 Cardiac Failure in Rabbits

PURPOSE AND RATIONALE

Rabbit models of heart failure were reviewed by Mudders and Elsner (2000).

Rapid pacing was used by Masaki et al. (1994), Porsa et al. (1994), Eble et al. (1998), Li et al. (2003), and Rose et al. (2005); coronary artery ligation by Pennock et al. (1997), Currie and Smith (1999), Romanic et al. (2001), and Miller et al. (2004); combined pressure and volume overload by Ezzaher et al. (1991), Mohammadi et al. (1997), Dekker et al. (1998), and Baartscheer et al. (2003a, 2003b); aortic insufficiency and aortic constriction by Bouanani et al. (1991) and Pogwizd et al. (1999); regurgitation after damage of the mitral valve by Gunawardena et al. (1999); regurgitation after aortic valve destruction by Magid et al. (1988, 1994), Yoshikawa et al. (1993), King et al. (1997), and Liu et al. (1998); Luchner et al. (2001) used a rabbit model of progressive left ventricular dysfunction to investigate differential expression of cardiac atrial natriuretic peptide and brain natriuretic peptide. Ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system.

PROCEDURE

Male rabbits (chinchilla bastard) underwent implantation of a programmable cardiac pacemaker (Medtronic Minix 8340, Minneapolis, Mn., USA). Under anesthesia (ketamine 60 mg/kg xylazine 5 mg/kg i.m.), the right internal jugular vein was dissected and cannulated with a single-lumen central venous catheter (Braun, Germany). The catheter was then advanced

into the right ventricle under pressure guidance. A transvenous screw-in pacemaker lead (Medtronic) was advanced through the catheter into the ventricular apex and implanted endocardially. The pacemaker was implanted subcutaneously into the right abdominal wall and the pacemaker lead was connected subcutaneously with the pacemaker. Rapid ventricular pacing-induced heart failure could be induced with a transvenously implanted pacemaker system. All rabbits were allowed to recover for at least 10 days after surgery before the pacemaker was started for the induction of heart failure. Proper pacemaker function was checked intraoperatively, at the time of programming, and subsequently all 10 days.

Rabbits (CHF group) underwent pacing with a stepwise increase of stimulation frequencies over 30 days. During the first 10 days, animals were paced at 330 beats/min (bpm). This protocol results in ELVD, as defined by significant LV systolic dysfunction with cardiac enlargement and decreased perfusion pressure but no clinical signs failure The pacing rate was then increased to 360 bpm for 10 days and 380 bpm for another 10 days and ELVD evolved to CHF with further cardiac enlargement and further decreased perfusion pressure together with clinical signs of fluid retention (ascites). At baseline (control), after being paced at 330 bpm for 10 days (ELVD) and at the end of the protocol (CHF), conscious arterial pressure was measured invasively via the medial ear artery and a 2-D guided M-mode echocardiogram was obtained. At the end of the pacing protocol, rabbits were killed by i.v. euthanasia and tissue was rapidly harvested. Hearts were trimmed on ice, snap frozen in liquid nitrogen and stored at –80°C until further processing.

Echocardiography

A long and short-axis echocardiogram (HP Sonos 5500, 12 MHz probe) was performed under light sedation (5 mg midazolam i.m.) in a supine position from the left parasternal window. LV end-diastolic (LVEDd) and end-systolic (LVESd) dimensions and diastolic and systolic thickness of the left ventricular anterior wall (AEDth and AESth) and posterior wall (PEDth and PESth) as well as left atrial diameter (LAd) were determined from three repeated 2-D guided M-mode tracings using the ASE convention. From those measurements, fractional shortening (FS) was calculated as: FS = (LVEDd– LVESd) /LVEDd.

Analytical methods

For analysis of cardiac natriuretic peptide expression, mRNA was extracted from all atrial and left ventricular samples utilizing a commercial kit (Fasttrack, Invitrogen).

As a probe for brain natriuretic peptide (BNP), a 750-bp *Eco*R1/*Hind*III DNA restriction fragment containing the gene for rabbit BNP was used.

EVALUATION

Results of the quantitative studies were expressed as mean ± SEM. Comparisons between the control, ELVD and CHF groups were performed by analysis of variance (ANOVA) followed by Fisher's least significant difference test. Comparison between the atrial and LV tissues as well as between atrial natriuretic peptide (ANP) and BNP were performed by paired Student's *t*-test. Statistical significance was defined as $P < 0.05$.

MODIFICATIONS OF THE METHOD

Arnolda et al. (1985) studied adriamycin cardiomyopathy in the rabbit.

Klimtova et al. (2002) performed a comparative study of chronic toxic effects of daunorubicin and doxorubicin in rabbits.

Alexander et al. (1993) studied electrographic changes following corona-virus-induced myocarditis and dilated cardiomyopathy in rabbits.

Sanbe et al. (2005) described a transgenic model for human troponin I-based hypertrophic cardiomyopathy in the rabbit.

REFERENCES AND FURTHER READING

Alexander IK, Keene BW, Small JD, Yount B Jr, Baric RS (1993) Electrographic changes following rabbit corona-virus-induced myocarditis and dilated cardiomyopathy. Adv Exp Med Biol 342:365–370

Arnolda L, McGrath B, Cocks M, Sumithran E, Johnston C (1985) Adriamycin cardiomyopathy in the rabbit: an animal model of low output cardiac failure with activation of vasoconstrictor mechanisms. Cardiovasc Res 19:378–382

Baartscheer A, Schumacher CA, Belterman CNW, Coronel R, Fiolet JWT (2003a) SR calcium handling and calcium after-transients in a rabbit model of heart failure. Cardiovasc Res 58:99–108

Baartscheer A, Schumacher CA, van Borren MMGJ, Belterman VNW, Coronel R, Fiolet JWT (2003b) Increased Na^+/H^+-exchange activity is the cause of increased $[Na^+]_i$ and underlies disturbed calcium handling in the rabbit pressure and volume overload heart failure model. Cardiovasc Res 57:1015–1024

Bouanani N, Cosin A, Gilson N, Crotazier B (1991) Beta-adrenoceptors and adenylate cyclase activity in hypertrophied and failing rabbit left ventricle. J Mol Cell Cardiol 23:573–581

Currie S, Smith GL (1999) Enhanced phosphorylation of phospholamban and downregulation of sarco/endoplasmatic reticulum Ca^{2+} ATPase type 2 SERCA2) in cardiac sarcoplasmatic reticulum from rabbits with heart failure. Cardiovasc Res 41:135–146

Dekker LR, Rademaker H, Vermeulen JT, Opthof T, Coronel R, Spaan JA, Janse MJ (1988) Cellular uncoupling during ischemia in hypertrophied and failing rabbit ventric-

ular myocardium: effects of preconditioning. Circulation 97:1724–1730
Eble DM, Walker JD, Samarei AM, Spinale FG (1998) Myosin heavy chain synthesis during progression of chronic tachycardia induced heart failure in rabbits. Bas Res Cardiol 93:50–55
Ezzaher A, el Houda-Bouanani N, Su JB, Hittinger L, Crozatier B (1991) Increased negative inotropic effect of calcium-channel blockers in hypertrophied and failing rabbit heart. J Pharmacol Exp Ther 257:466–471
Gunawardena S, Bravo E, Kappagoda CT (1999) Rapidly adapting receptors in a rabbit model of mitral regurgitation. J Physiol (Lond) 521:739–748
King RK, Magid NM, Opio G, Borer JS (1997) Protein turnover in compensated chronic aortic regurgitation. Cardiology 88:518–525
Klimtova I, Simunek T, Mazurova Y, Hrdina R, Gersi V, Adamcova M (2002) Comparative study of chronic toxic effects of daunorubicin and doxorubicin in rabbits. Hum Exp Toxicol 21:649–657
Li YL, Sun YS, Overholt JL, Prabhakar NR, Rozanski GJ, Zucker IH, Schultz HD (2003) Attenuated outward potassium currents in carotid body glomus cells of heart failure rabbit: involvement of nitric oxide. J Physiol (Lond) 555:219–229
Liu SK, Magid NR, Fox PR, Goldfine SM, Borer JS (1998) Fibrosis, myocyte degeneration and heart failure in chronic aortic regurgitation. Cardiology 90:101–109
Luchner A, Muders F, Dietl O, Friedrich E, Blumberg F, Protter AA, Riegger GAJ, Elsner D (2001) Differential expression of cardiac ANP and BNP in a rabbit model of progressive left ventricular dysfunction. Cardiovasc Res 51:601–607
Magid NM, Young MS, Wallerson DC, Goldweit RS, Carter JN, Deveraux RB, Borer JS (1988) Hypertrophic and functional response to experimental chronic aortic regurgitation. J Mol Cell Cardiol 20:239–246
Magid NM, Opio G, Wallerson DC, Young MS, Borer JS (1994) Heart failure due to chronic aortic regurgitation. Am J Physiol 267(2 Pt2): H556–H562
Masaki H, Imaizumi T, Harasawa Y, Takeshita A (1994) Dynamic arterial baroreflex in rabbits with hear failure induced by rapid pacing. Am J Physiol 267(1 Pt2): H92–H99
Miller DJ, MacFarlane NG, Wilson G (2004) Altered oscillatory work by ventricular myofilaments from a rabbit coronary artery ligation model of heart failure. Cardiovasc Res 61:94–104
Mohammadi K, Rouet-Benzineb P, Laplace M, Crozatier B (1997) Protein kinase C activity and expression in rabbit left ventricular hypertrophy. J Mol Cell Cardiol 29:1687–1694
Muders F, Elsner F (2000) Animal models of chronic heart failure. Pharmacol Res 41:605–612
Pennock GD, Yun DD, Agarwal PG, Spooner PH, Goldman S (1997) Echocardiographic changes after myocardial infarction in a model of left ventricular dysfunction. Am J Physiol 273(4 Pt2):H2018–2029
Pogwizd SM, Qi M, Yuan W, Samaral AM, Bers DM (1999) Upregulation of Na^+/Ca^{2+} exchanger expression and function in an arrhythmogenic rabbit model of heart failure. Circ Res 85:1009–1019
Porsa E, Freeman GL, Herlihy JT (1994) Tachycardia heart failure alters rabbit aortic smooth muscle responsiveness to angiotensin II. Am J Physiol 266(3 PT2):H1228–1232
Romanic AM, Burns-Curtis CL, Gout B, Berrebi-Bertrand I, Ohlstein EH (2001) Matrix metalloproteinase expression in cardiac myocytes following myocardial infarction in the rabbit. Life Sci 68:799–814
Rose J, Armoundas AA, Tian Y, DeSilvestre D, Burysek M, Halperin V, O'Rourke B, Kass DA, Marban E, Tomaselli GF (2005) Molecular correlates to altered expression of potassium currents in failing rabbit myocardium. Am J Physiol 288:H2077–H2087
Sanbe DM, James J, Tuzcu V, Nas S, Martin L, Gulick J, Osinka H, Sakthivel S, Klevitsky R, Ginsburg KS (2005) Transgenic model for human troponin I-based hypertrophic cardiomyopathy. Circulation 111:2330–2338
Yoshikawa T, Handa S, Yamada T, Wainai Y, Suzuki M, Nagami K, Tani M, Nakamura Y (1993) Sequential changes in sympatho-neuronal regulation and contractile function following aortic regurgitation in rabbit heart. Eur Heart J 14:1404–1409

A.6.0.6 Congestive Heart Failure in Dogs

PURPOSE AND RATIONALE

Several methods are described, to induce congestive heart failure in dogs, such as rapid ventricular pacing (Armstrong et al. 1986; Freeman et al. 1987; Wilson et al. 1987; Komamura et al. 1992, 1993; Perreault et al. 1992; Travill et al. 1992; Cheng et al. 1993; Redfield et al. 1993; Cory et al. 1994; Kiuchi et al. 1994; Ohno et al. 1994; Vatner et al. 1994; Wang et al. 1994; Williams et al. 1994; Eaton et al. 1995; Spinale et al. 1995; Wolff et al. 1995; Zile et al. 1995; Ravens et al. 1996; Shinbane et al. 1997; O'Rourke et al. 1999; Winslow et al. 1999).

Luchner et al. (1996) assessed circulating, renal, cardiac, and vascular angiotensin II in a canine model of rapid ventricular pacing-induced heart failure that evolves from early left ventricular dysfunction to overt congestive heart failure.

PROCEDURE

Male mongrel dogs underwent implantation of a programmable cardiac pacemaker (Medtronic). Under pentobarbital sodium anesthesia and artificial respiration, the heart was exposed via a small left lateral thoracotomy and pericardiotomy, and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker was implanted subcutaneously into the left chest wall and connected to the pacemaker lead. The dogs were allowed to recover for at least 10 days after surgery before the pacemaker was started. During the first 10 days dogs were paced at 180 beats/min (bpm), resulting in early left ventricular dysfunction as defined by significant systolic dysfunction with decreased cardiac output, cardiac enlargement, and increased filling pressures but maintained systemic perfusion pressure and renal sodium

excretion and no clinical signs of heart failure. The pacing rate was then increased weekly to 200, 210, 220 and 240 bpm, and early left ventricular dysfunction evolved to overt congestive heart failure with avid sodium retention and clinical signs of congestion. At baseline (control), after dogs had been paced at 180 bpm for 10 days and at the end of the protocol (overt CHF), urine was collected for measurement of sodium excretion; conscious mean arterial pressure was measured via a port catheter; a 2-D guided M-mode echogram was obtained; and arterial blood was drawn. Cardiac filling pressures and cardiac output were measured by the thermodilution method at baseline and at the end of the protocol. Arterial blood was collected in EDTA tubes for measurement of ANP, BNP, cGMP, PRA, aldosterone, and Ang II. After euthanasia, hearts were rapidly trimmed and left ventricles weighted for calculation of the index LV weight to body weight.

EVALUATION

Results were expressed as mean ± SE. Comparison between the control, early LV dysfunction, and overt CHF were performed by ANOVA followed by Fisher's least significant difference test.

MODIFICATIONS OF THE METHOD

Kleaveland et al. (1988) and Nagatsu et al. (1994) used the technique of experimental **mitral regurgitation** in dogs to induce left ventricular dysfunction. A 30-cm, 7F sheath was introduced across the aortic valve through the carotid artery. A urologic calculus retrieval forceps was advanced through the sheath to the mitral valve apparatus and was used to sever chordae tendineae. When pulmonary capillary wedge pressure rose to 20 mmHg and forward stroke volume was reduced to 50% of its baseline, a ventriculogram was performed to confirm angiographically that severe mitral regurgitation had been created.

Dell'Italia et al. (1995) and Su et al. (1999) induced mitral regurgitation by percutaneous chordal rupture in dogs.

Kinney et al. (1991) published a method to induce acute, reversible tricuspid insufficiency in anesthetized dogs. A wire spiral is advanced through the atrioventricular canal from the right atrium. The spiral causes regurgitation by preventing complete apposition of the valve leaflets while permitting retrograde flow to occur through the spiral lumen. The degree of regurgitation can be controlled by the use of spirals of different size. Creation of tricuspid insufficiency was demonstrated by onset of right atrial pressure V waves, a ballooning of the right atrium during ventricular systole, palpation of an atrial thrill, or by color Doppler echocardiography. The model is reversible and allows repeated trials of various grades of regurgitation.

Carlyle and Cohn (1983) described a non-chirurgical model of chronic left ventricular dysfunction. The method is accomplished by repetitive DC shock with a guidewire introduced percutaneously and positioned in the left ventricle along the intraventricular septum and an external paddle at the left ventricular apex.

McDonald et al. (1992) produced localized left ventricular necrosis without obstruction of the coronary blood flow in dogs by transmyocardial direct-current shock.

Sabbah et al. (1991, 1993, 1994) and Gengo et al. (1992) produced chronic heart failure in dogs by multiple sequential intracoronary **embolizations with microspheres**. The dogs underwent three to nine intracoronary embolizations with polystyrene latex microspheres (70–102 μm in diameter) performed 1–3 weeks apart. Embolizations were discontinued when left ventricular ejection fraction was less than 35%.

Vanoli et al. (2004) used multiple coronary microembolizations in dogs, whereby three to nine embolizations were performed 1 week apart. The first three embolizations consisted of 2 ml of microsphere suspension injected subselectively into either the left anterior descending or left circumflex coronary artery in an alternating fashion. Subsequent embolizations consisted of 3–6 ml of microspheres divided equally between the left anterior descending or left circumflex coronary artery until LV ejection fraction was < 35%.

Magovern et al. (1992) described a canine model of left ventricular dysfunction caused by five weekly intracoronary infusions of **adriamycin**.

Koide (1997) described premorbid determinants of left ventricular dysfunction in a model of gradually induced pressure overload in dogs. Mongrel dogs were studied through 8 weeks of gradually imposed ascending aortic constriction with the use of a **novel banding technique**. During banding, an initial gradient of 30 mmHg was created. Before banding, at 2, 4, and 6 weeks after banding, hemodynamics and left ventricular mechanics were examined at cardiac catheterization; then the pressure overload was increased by tightening the band.

Valentine et al. (1988) and Devaux et al. (1993) described **X-linked muscular dystrophy in dogs** with cardiac insufficiency similar to Duchenne muscular dystrophy in men and recommended this as an animal model for cardiac insufficiency.

REFERENCES AND FURTHER READING

Armstrong PW, Stopps TP, Ford SE, de Bold AJ (1986) Rapid ventricular pacing in the dog: pathophysiological studies of heart failure. Circulation 74:1075–1084

Carlyle PF, Cohn JN (1983) A nonchirurgical model of chronic left ventricular dysfunction. Am J Physiol 244:H769–H774

Cheng CP, Noda T, Nozawa T, Little WD (1993) Effect of heart failure on the mechanism of exercise-induced augmentation of mitral valve flow. Circ Res 72:795–806

Cory CR, Shen H, O'Brien PJ (1994) Compensatory asymmetry in down-regulation and inhibition of the myocardial Ca^{2+} cycle in congestive heart failure in dogs by idiopathic dilated cardiomyopathy and rapid ventricular pacing. J Mol Cell Cardiol 26:173–184

Dell'Italia LJ, Meng QC, Balcells E, Straeter-Knowlen IM, Hankes GH, Dillon R, Cartee RE, Orr R, Bishop SP, Oparil S (1995) Increased ACE and chymase-like activity in cardiac tissue of dogs with chronic mitral regurgitation. Am J Physiol 269(6 Pt2):H2065–H2073

Eaton GM, Cody RJ, Nunziata E, Binkley PF (1995) Early left ventricular dysfunction elicits activation of sympathetic drive and attenuation of parasympathetic tone in the paced canine model of congestive heart failure. Circulation 92:555–561

Freeman GL, Little WC, O'Rourke RA (1987) Influence of heart rate on left ventricular performance in conscious dogs. Circ Res 61:455–464

Gengo PJ, Sabbah HN, Steffen RP, Sharpe KJ, Kono T, Stein PD, Goldstein S (1992) Myocardial beta receptor and voltage sensitive calcium channel changes in a canine model of chronic heart failure. J Mol Cell Cardiol 24:1361–1369

Kinney TE, Olinger GN, Sgar KB, Boerboom LE (1991) Acute, reversible tricuspid insufficiency: creation of a canine model. Am J Physiol 260:H638–H641

Kiuchi K, Shannon RP, Sato N, Bigaud M, Lajoie C, Morgan KG, Vatner SF (1994) Factors involved in delaying the rise in peripheral resistance in developing heart failure. Am J Physiol 267 (1 Pt2):H211–H216

Kleaveland JP, Kussmaul WG, Vinciguerra T, Diters R, Carabello BA (1988) Volume overload hypertrophy in a closed-chest model of mitral regurgitation. Am J Physiol 254(6 Pt2):H1034–H1041

Koide M, Nagatsu M, Zile MR, Hamakawi M, Swindle MM, Keech G, DeFreyte G, Tagawa H, Cooper G, Carabello BA (1997) Premorbid determinants of left ventricular dysfunction in a novel model of gradually induced pressure overload in the adult canine. Circulation 95:1601–1610

Komamura K, Shannon RP, Pasipoularides A, Ihara T, Lader AS, Patrick TA, Bishop SP, Vatner SF (1992) Alterations in left ventricular diastolic function in conscious dogs with pacing-induced heart failure. J Clin Invest 89:1825–1838

Komamura K, Shannon RP, Ihara T, Shen YT, Mirsky I, Bishop SP, Vatner SF (1993) Exhaustion of Frank-Starling mechanism in conscious dogs with heart failure. Am J Physiol 265 (4 Pt2):H1119–H1131

Luchner A, Stevens TL, Borgeson DD, Redfield MM, Bailey JE, Sandberg SM, Heublein DM, Burnett JC Jr (1996) Angiotensin II in the evolution of experimental heart failure. Hypertension 28:472–477

Magovern JA, Christlieb IY, Badylak SF, Lantz GC, Kao RL (1992) A model of left ventricular dysfunction caused by intracoronary adriamycin. Ann Thoracic Surg 53:861–863

McDonald KM, Francis GS, Carlyle PF, Hauer K, Matthews J, Hunter DW, Cohn JN (1992) Hemodynamic, left ventricular structural and hormonal changes after discrete myocardial damage in the dog. J Am Coll Cardiol 19:460–467

Nagatsu M, Zile MR, Tsutsui H, Schmid PG, DeFreyte G, Cooper IV G, Carabello BA (1994) Native β-adrenergic support for left ventricular dysfunction in experimental mitral regurgitation normalizes indexes of pump and contractile function. Circulation 89:818–826

Ohno M, Cheng CP, Little WC (1994) Mechanism of altered patterns of left ventricular filling during the development of congestive heart failure. Circulation 89:2241–2250

O'Rourke B, Kass DA, Tomaselle GF, Kääb S, Tunin R, Marbán E (1999) Mechanisms of altered excitation-contraction coupling in canine tachycardia-induced heart failure. I: experimental studies. Circ Res 84:562–570

Ravens U, Davia K, Davies CH, O'Gara P, Drake-Holland AJ, Hynd JW, Noble MIM, Harding SE (1996) Tachycardia-induced failure alters contractile properties of canine ventricular myocytes. Cardiovasc Res 32:613–621

Perreault CL, Shannon RP, Komamura K, Vatner SF, Morgan JP (1992) Abnormalities in intracellular calcium regulation and contractile function in myocardium from dogs with pacing-induced heart failure. J Clin Invest 83:932–938

Redfield MM, Aarhus LL, Wright RS, Burnett JC (1993) Cardiorenal and neurohumoral function in a canine model of left ventricular dysfunction. Circulation 87:2016–2022

Sabbah HN, Stein PD, Kono D, Gheorghiade M, Levine TB, Jafri S, Hawkins ET, Goldstein S (1991) A canine model of chronic heart failure produced by multiple sequential coronary microembolizations. Am J Physiol 260(4 Pt2):H1379–H1384

Sabbah HN, Hansen-Smith F, Sharov VG, Kono T, Lesch M, Gengo PJ, Steffen RP, Levine TB, Goldstein S (1993) Decreases proportion of type I myofibers in skeletal muscle of dogs with chronic heart failure. Circulation 87:1729–1737

Sabbah HN, Shimoyama H, Kono T, Gupta RC, Sharov VG, Scicli G, Levine TB, Doldstein S (1994) Effects of long-term monotherapy with enalapril, metoprolol, and digoxin on the progression of left ventricular dysfunction and dilation in dogs with reduced ejection fraction. Circulation 89:2852–2859

Shinbane JS, Wood Ma, Jensen DN, Ellenbogen KA, Fitzpatrick AP, Scheinman MM (1997) Tachycardia-induced cardiomyopathy: a review of animal models and clinical studies. J Am Coll Cardiol 29:709–715

Spinale FG, Holzgrefe HH, Mukherjee R, Hird B, Walker JD, Armin-Baker A, Powell JR, Koster WH (1995) Angiotensin-converting enzyme inhibition and the progression of congestive cardiomyopathy. Circulation 92:562–578

Su X, Wei CC, Machida N, Bishop SP, Hankes GH, Dillon AR, Oparil S, Dell'Italia LJ (1999) Differential expression of angiotensin-converting enzyme and chymase in dogs with chronic mitral regurgitation. J Mol Cell Cardiol 31:1033–1045

Travill CM, Williams TD, Pate P, Song G, Chalmers J, Lightman SL, Sutton R, Noble MI (1992) Hemodynamic and neurohumoral response in heart failure produced by rapid ventricular pacing. Cardiovasc Res 26:783–790

Valentine BA, Cooper BJ, DeLahunta A, O'Quinn R, Blue JT (1988) Canine X-linked muscular dystrophy. An animal model of Duchenne muscular dystrophy: Clinical studies. J Neurol Sci 88:69–81

Vanoli E, Bacchini S, Panigada S, Pentimalli F, Adamson PB (2004) Experimental models of heart failure. Eur Heart J Suppl 6 (Suppl F):F7–F15

Vatner DE, Sato N, Kiucho K, Shannon RP, Vatner SF (1994) Decrease in myocardial ryanodine receptors and altered ex-

citation-contraction coupling early in the development of heart failure. Circulation 90:1432–1430
Wang J, Seyedi N, Xu XB, Wolin MS, Hintze TH (1994) Defective endothelium-mediated control of coronary circulation in conscious dogs after heart failure. Am J Physiol 266(2Pt2):H670–H680
Williams RE, Kass DA, Kawagoe Y, Pak P, Tunin RS, Shah R, Hwang A, Feldman AM (1994) Endomyocardial gene expression during development of pacing tachycardia-induced heart failure in the dog. Circ Res 75:615–623
Wilson JR, Douglas P, Hickey WF, Lanoce V, Ferraro N, Muhammad A, Reicheck N (1987) Experimental congestive heart failure produced by rapid ventricular pacing in the dog: cardiac effects. Circulation 75:857–867
Winslow RL, Rice J, Jafri S, Marbán E, O'Rourke B (1999) Mechanisms of altered excitation-contraction coupling in canine tachycardia-induced heart failure, II: model studies. Circ Res 84:571–586
Wolff MR, Whitesell LF, Moss RL (1995) Calcium sensitivity of isometric tension is increased in canine experimental heart failure. Circ Res 76:781–789
Zile MR, Mukherjee R, Clayton C, Kato S, Spinale FG (1995) Effects of chronic supraventricular pacing tachycardia on relaxation rate in isolated cardiac muscle cells. Am J Physiol 268(5 Pt2):H2104–H2113

A.6.0.7 Cardiac Failure in Pigs

PURPOSE AND RATIONALE

Cardiac failure was studied in pigs using several experimental procedures.

Chow et al. (1990) recommended rapid ventricular pacing in pigs as an experimental model of congestive heart failure.

Farrar et al. (1993) studied pacing-induced dilated cardiomyopathy in pigs. Congestive heart failure was produced by rapid ventricular pacing at 230 bpm for 1 week.

Spinale et al. (1990a, 1990b, 1991, 1992) examined the consequences of chronic supraventricular tachycardia on various parameters of ventricular dysfunction and subendocardial changes in pigs.

Carroll et al. (1995) investigated gene expression in a swine model of ventricular hypertrophy during pressure overload.

Multani et al. (2001) studied long-term angiotensin converting enzyme and angiotensin I-receptor inhibition in pacing-induced heart failure in pigs. Heart failure was induced by rapid atrial pacing (240 bpm for 3 weeks).

Kassab et al. (1993, 2000) investigated remodeling of right ventricular branches after hypertrophy in pigs.

Krombach et al. (1999) studied the effects of amlodipine in congestive heart failure in pigs at rest and after treadmill exercise.

PROCEDURE

Left thoracotomy was performed in Yorkshire pigs in anesthesia. Catheters connected to a vascular access port were placed in the thoracic aorta, the pulmonary artery, and the left atrium. The access ports were then placed in a subcutaneous pocket. A 20-mm flow probe was placed around the pulmonary artery immediately distal to the pulmonary catheter and the electrical connection exteriorized through the thoracolumbar fascia. A shielded stimulating electrode was sutured onto the left atrium, connected to a programmable pacemaker and buried in a subcutaneous pocket. The thoracotomy was closed in layers and the pleural space evacuated of air. After a 14- to 21-day recovery, measurements were performed under normal resting conditions and after exercise. The pacemakers were activated to 240 bpm for a period of 21 days. During the last 3 days, one group was treated with drug, the other served as control. At the day of the study, electrocardiograms were performed, and the pacemakers deactivated. After a 30-min stabilization period, 2-D and M-mode echocardiographic studies were used to image the left ventricle from the parasternal approach. Left ventricular fractional shortening was calculated as (end-diastolic dimension – end-systolic dimension)/diastolic dimension, and was expressed as a percentage. The access ports were entered and pressures obtained using externally calibrated transducers. The flow probe was connected to a digital flowmeter. From the digitized flow signal, stroke volume was computed on a beat-to-beat basis and averaged for a minimum of 25 ejections. Pulmonary and systemic vascular resistances were computed as the mean pressure divided by cardiac output multiplied by the constant 80 to convert to resistance units of dyne$\cdot s \cdot$cm^{-5}. Samples were drawn from the pulmonary artery and atrial catheters for measurement of oxygen saturation and hemoglobin content. The plasma samples were assayed for renin activity, endothelin concentration, and catecholamine levels.

EVALUATION

Results were presented as mean $\pm$ SEM. Pairwise tests of individual group means were compared using Bonferroni probabilities.

MODIFICATIONS OF THE METHOD

Zhang et al. (1996) studied functional and bioenergetic consequences of post-infarction left remodeling in a porcine model. Proximal left coronary artery occlusion was used to generate a myocardial infarction in young pigs. The animals were then followed over sev-

eral months while remodeling of the left ventricle developed. Left ventricular wall thickness, ejection fraction, and wall stress were measured by MRI. Myocardial ATP, creatine phosphate, and inorganic phosphate levels were measured by spatially localized ^{31}P-NMR spectroscopy, and regional myocardial blood flow was measured with radioactive microspheres.

PROCEDURE

MRI Protocols

All MRI studies were performed on the standard Siemens Medical System VISION operating at 1.5 T. The animals were anesthetized with sodium pentobarbital. A catheter was placed into the femoral artery and advanced into the LV chamber for LV pressure recording. Animals then were placed on their left side in a Helmholtz coil with a diameter of 18 cm, which was used to improve signal to noise. To compute LV wall stress, the image acquisition was triggered by the LV pressure through the fluid-filled LV catheter. All of the imaging sequences were synchronized to the LV pressure trace. The electronic LV pressure signal was recorded and fed to a comparator set to a threshold level of 10% of the upslope of the LV pressure curve at the beginning of systole. The signal from the comparator was sent to a pulse former and then fed to the ECG port of the magnetic resonance system, where it was treated like the standard electrographic input to run the pulse sequences. Scout images were taken in the axial plane with a single-shot, ultrafast gradient echo sequence (McDonald et al. 1992; Wilke et al. 1993; Geiger et al. 1995). From the axial image, both horizontal and vertical long-axis images were obtained. By alternating back and forth several times, a true vertical long axis of the left ventricle was obtained. From the long-axis scout image, short-axis segmented cine turboflash slices were prescribed to cover the myocardium from apex to base. The double-oblique, short-axis turboflash images cover the heart from apex to base with a slice thickness of 10 mm, with no interslice gap.

MRI Cine Technique

The parameters of the segmented cine sequence were TR/TE/flip angle = 33 ms/6.1 ms/25 degrees with an FOV = 17.5 cm and a matrix of 87 × 128 (pixel size, 2 mm × 1.4 mm) and slice thickness of 7–10 mm (Atkinson et al. 1991). The sequence used segmented k-space acquisition such that three phase-encoded lines were gathered per cardiac phase per heartbeat. Total image acquisition required approximately 52 heartbeats for each slice location. The temporal image resolution (data acquisition window) of this sequence was 33 ms per cardiac image. Each myocardial level took < 1.5 min to acquire, since two acquisitions were used and the average heart rate of the animals was 120 bpm. The average number of short-axis slices needed to image the entire myocardium from apex to base was six to eight. This 10-min protocol provided high signal-to-noise cine sequences covering the entire heart.

Spin-Echo Images

To obtain high-resolution anatomic heart images, multislice, single-phase spin-echo images trigged in the systolic phase were acquired to cover the entire heart. These images permitted the precise delineation of the extent of the scar region of the heart. Images were taken with a slice thickness of 5 mm and a FOV of 17.5 cm, resulting in a true spatial resolution of 2 mm × 1.4 mm pixel size. The TR for this sequence equals the RR interval (500 ms) and the echo time TE was set to 30 ms. Total measurement time for an average of 10–14 slices was 5 min.

Image Analysis of the MRI Cine Studies

The imaging data were archived to optical disk and copied to a SUN SPARC 10 workstation for evaluation with the use of an automatic segmentation program (ImageView, Siemens Cooperate Research). The program is based on robust deformable models of endocardial and epicardial border segmentation of ventricular boundaries in cardiac magnetic resonance images. This segmentation technique has been combined with a user interface that allows one to load, sort, visualize, and analyze a cardiac study in < 20 min. The segmentation algorithm is based on the steepest descent as well as dynamic programming strategies integrated via multiscale analysis for minimizing the energy function of the resulting contour. The ventricular boundaries are used to construct a three-dimensional model for visualization and to compute hemodynamic parameters. Automatic segmentation of endocardial and epicardial boundaries was performed for calculation of ventricular volumes, EF, LV diastolic and systolic volumes, and absolute myocardial mass from multislice, multiphase magnetic resonance cine images. Starting with a user-specified approximate boundary or an interior point of the ventricle for one starting image in one slice, the algorithm generated automatic contours corresponding to the epicardium and the endocardium and automatically propagated them to other slices in the cardiac phase (spatial propagation) and to other phases for a given slice location (temporal propagation) of

the cardiac study. The observer then could make some manual corrections to the six or seven pairs of contours in the first column of the temporal-spatial matrix. Manual modifications generally were made on the apex and base levels.

EVALUATION

Mean LV wall thickness for each short-axis ring was averaged from three measurements of the remote zone (anterior wall and septum wall). The thickness of the scar was averaged from three measurements of the scar area. LVSA measurement in each slice was computed by subtracting the total area enclosed by the endocardium from that enclosed by the subepicardium; the resultant area was multiplied by the slice thickness to obtain the volume of each slice; the total LV mass volume was calculated by adding up the volumes of all the short-axis slices. The total LVSA was obtained by dividing the total LV wall mass volume by the mean of LV wall thickness of each slice. Similarly, the LVSSA was obtained by dividing the total scar volume, which was the sum of the scar volume of each short axis, by the mean of the scar thickness of each short axis. LV mass was computed by the total LV wall mass volume multiplied by 1.05 (specific gravity of myocardium) to calculate the LV mass. The LV end-diastolic volume (V_d) and end-systolic volume (V_s) of each slice were represented by the area enclosed by the endocardium. The total LV volume was computed by adding the volumes of all slices. LVEF was calculated by $100 \times (V–V_s)/V_d\%$. Interobserver and intraobserver errors for the calculations of LV mass and LV volumes have been shown to be < 3 mg and 3 ml, respectively (McDonald et al. 1994). Meridional wall stress was computed from the LV pressure and simultaneously obtained short-axis view of LV MRI (LV cavity diameter and average thickness the remote LV wall) as described by Grossman et al. (1975).

Spatially Localized ^{31}P-NMR Spectroscopic Technique

Measurements were performed in a 40-cm-bore, 4.7-T magnet interfaced with a SISCO (Spectroscopy Imaging Systems Corporation) console. The LV pressure signal was used to gate NMR data acquisition to the cardiac cycle, while respiratory gating was achieved by triggering the ventilator to the cardiac cycle between data acquisitions (Robitaille et al. 1990). ^{31}P- and ^{1}H-NMR frequencies were 81 and 200.1 MHz, respectively. Spectra were recorded in late diastole with a pulse repetition time of 6–7 s. This repetition time allowed full relaxation for ATP and P_i resonances and ≈90% relaxation for the CP resonance (Zhang and McDonald 1995). CP resonance intensities were corrected for this minor saturation; the correction factor was determined for each heart from two spectra recorded consecutively without transmural differentiation, one with 15-s repetition time to allow full relaxation and the other with the 6- to 7-s repetition time used in all the other measurements.

Radiofrequency transmission and signal detection were performed with a 25-mm-diameter surface coil. The coil was cemented to a sheet of silicone rubber 0.7 mm in thickness and ≈50% larger in diameter than the coil itself. A capillary containing 15 µl of 3 M phosphonoacetic acid was placed at the coil center to serve as a reference. The proton signal from water detected with the surface coil was used to homogenize the magnetic field and to adjust the position of the animal in the magnet so that the coil was at or near the magnet and gradient isocenters. This was accomplished with a spin-echo experiment and a readout gradient. The information gathered in this step also was used to determine the spatial coordinates for spectroscopic localization. Chemical shifts were measured relative to CP, which was assigned a chemical shift of –2.55 ppm relative to 85% phosphoric acid at 0 ppm.

Spatial localization across the LV wall was performed with the RAPP-ISIS/FSW method (Hendrich et al. 1991). Signal origin was restricted with the use of B_0 gradients and adiabatic inversion pulses to a column coaxial with the surface coil perpendicular to the LV wall. The column dimensions were 17 × 17 mm. Within this column, the signal was further localized using the B_1 gradient to five voxels centered about 45°, 60°, 90°, 120°, and 135° spin rotation increments. FSW localization used a nine-term Fourier series expansion. The Fourier coefficients, the number of free induction decays acquired for each term in the Fourier expansion, and the multiplication factors used to construct the voxels have been reported previously. The position of the voxels relative to the coil was set using the B_1 magnitude at the coil center, which was experimentally determined in each case by measurement of the 90° pulse length for the phosphonoacetic acid reference located in the coil center. Each set of spatially localized transmural spectra were acquired in 10 min. A total of 96 scans was accumulated within each 10-min block.

EVALUATION

Resonance intensities were quantified with the use of integration routines provided by the SISCO software. ATPγ resonance was used for ATP determination. Since data were acquired with the transmitter fre-

quency being positioned between the ATPγ and CP resonance, the off-resonance effects on these peaks were negligible. The numeric values for CP and ATP in each voxel were expressed as ratios of CP/ATP. P_i levels were measured as changes from baseline values (ΔP_i) with the use of integrals obtained in the region covering the P_i resonance.

REFERENCES AND FURTHER READING

Atkinson DJ, Edelman RR.(1991) Cineangiography of the heart in a single breath hold with segmented turboflash sequence. Radiology 178:357–360

Carroll SM, Nimmo LE, Knoepfler PS, White FC, Bloor CM (1995) Gene expression in a swine model of ventricular hypertrophy: Intercellular adhesion molecule, vascular endothelial growth factor and plasminogen activators are upregulated during pressure overload. J Mol Cell Cardiol 27:1427–1441

Chow E, Woodard JC, Farrar DJ (1990) Rapid ventricular pacing in pigs: an experimental model of congestive heart failure. Am J Physiol 258:H1603–H1605

Farrar DJ, Woodard JC, Chow E (1993) Pacing-induced dilated cardiomyopathy increases left-to-right ventricular systolic interaction. Circulation 88:720–725

Geiger D, Gupta A, Costa LA, Vlontzos J (1995) Dynamic programming for detecting, tracking and matching deformable contours. IEEE Trans Pattern Analysis Machine Intelligence 17:294–302

Grossman W, Jones D, McLaurin LP.(1975) Wall stress and patterns of hypertrophy in human left ventricle. J Clin Invest 56:56–64

Hendrich K, Merkle H, Weisdorf S, Vine W, Garwood M, Ugurbil K (1991) Phase modulated rotating frame spectroscopic localization using an adiabatic plane rotation pulse and a single surface coil. J Magn Reson 92:258–275

Kassab GS, Imoto K, White FC, Rider CA, Fung YC, Bloor CM (1993) Coronary arterial tree remodelling in right ventricular hypertrophy. Am J Physiol 265(1 Pt2):H366–H375

Kassab GS, Schatz A, Imoto K, Fung YC (2000) Remodeling of the bifurcation asymmetry of right ventricular braches in hypertrophy. Ann Biomed Eng 28:424–430

Krombach RS, Clair MJ, Hendrick JW, Mukherjee R, Houck WV, Hebbar L, Kribbs SB, Dodd MG, Spirale FG (1999) Amlodipine therapy in congestive heart failure: hemodynamic and neurohormonal effects at rest and after treadmill exercise. Am J Cardiol 84:3L–15L

McDonald K, Parrish T, Wennberg P, Stillman AE, Francis GS, Cohn JN.(1992) Rapid, accurate and simultaneous noninvasive assessment of right and left ventricular mass with nuclear magnetic resonance imaging using the snapshot gradient method. J Am Coll Cardiol 19:1601–1607

McDonald KM, Yoshiyama M, Francis GS, Ugurbil K, Cohn JN, Zhang J (1994) Abnormal myocardial bioenergetics in canine asymptomatic left ventricular dysfunction. J Am Coll Cardiol. 23:786–793

Multani MM, Krombach RS, Hednrick JW, Baicu SC, Joffs C, Sample JA, deGasparo M, Spinale FG (2001) Long-term angiotensin-converting enzyme and angiotensin I-receptor inhibition in pacing-induced heart failure. Effects on myocardial interstitial bradykinin levels. J Cardiac Fail 7:348–354

Robitaille PM, Lew B, Merkle H, Path G, Sublett E, Hendrich K, Lindstrom P, From AHL, Garwood M, Bache RJ, Ugurbil K (1990) Transmural high energy phosphate distribution and response to alterations in workload in the normal canine myocardium as studied with spatially localized 31P NMR spectroscopy. Magn Reson Med 16:91–116

Spinale FG, Hendrick DA, Crawford FA, Smith AC, Hamada Y, Carabello BA (1990a) Chronic supraventricular tachycardia causes ventricular dysfunction and subendocardial injury in swine. Am J Physiol 259(1 Pt2):H218–H229

Spinale FG, Hendrick DA, Crawford FA, Carabello BA (1990b) Relationship between bioimpedance, thermodilution, and ventriculographic measurements in experimental congestive heart failure. Cardiovasc Res 24:423–429

Spinale FG, Tomita M, Zellner JL, Cook JC, Crawford FA, Zile MR (1991) Collagen remodelling and changes in LV function during development and recovery from supraventricular tachycardia. Am J Physiol 261(2 Pt2):H308–H318

Spinale FG, Fulbright BM, Mukherjee R, Tanaka R, Hu J, Crawford FA, Zile MR (1992) Relation between ventricular and myocytes function with tachycardia-induced cardiomyopathy. Circ Res 71:174–187

Wilke N, Simm C, Zhang J, Ellermann J, Ya X, Merkle H, Path G, Ludemann H, Bache RJ, Ugurbil K (1993) Contrast-enhanced first pass myocardial perfusion imaging: correlation between myocardial blood flow in dogs at rest and during hyperemia. Magn Reson Med 29:485–497

Zhang J, McDonald K (1995) Bioenergetic consequence of left ventricular remodeling secondary to discrete myocardial infarction. Circulation 92:1011–1019

Zhang J, Wilke N, Wang Y, Zhang Y, Wang C, Eijgelshoven MHJ, Cho YK, Murakami Y, Ugurbil K, Bache RJ, From AHL (1996) Functional and bioenergetic consequences of postinfarction left remodelling in a new porcine model. Circulation 94:1089–1100

A.6.0.8 Cardiac Failure in Sheep

PURPOSE AND RATIONALE

Various methods have been used to induce cardiac failure in sheep: pressure overload after aortic banding (Aoyagi et al. 1993; Charles et al. 1996), volume loading after myocardial infarction (Charles et al. 2003), rapid ventricular pacing (Rademaker 1997, 2002b, 2005; Byrne et al. 2002; Moreno et al. 2005), coronary microembolization (Huang et al. 2004; Monreal et al. 2004).

Rademaker et al. (2002a) studied combined angiotensin converting enzyme inhibition and adrenomedullin in an ovine model of heart failure induced by rapid ventricular pacing.

PROCEDURE

Surgical Preparation

Coopworth ewes (38 ± 47 kg) were instrumented via a left lateral thoracotomy. Under general anesthesia (induced by 17 mg/kg thiopentone; maintained with halothane/nitrous oxide), two polyvinyl chloride catheters were inserted in the left atrium for blood sampling and left atrial pressure (LAP) determination; a Konigsberg pressure-tip transducer was inserted in

the aorta to record mean arterial pressure (MAP); an electromagnetic flow probe was placed around the ascending aorta to measure cardiac output (CO); a 7 French Swan-Ganz catheter was inserted in the pulmonary artery for infusions; and a 7 French His-bundle electrode was stitched subepicardially to the wall of the left ventricle for left ventricular pacing. All leads were externalized through incisions in the back. A bladder catheter was inserted per urethra for urine collections.

The animals were allowed to recover for 14 days before commencing the study protocol. During the experiments, the animals were held in metabolic cages, had free access to water and ate a diet of chaff and sheep pellets (containing 40 mmol/day sodium and 200 mmol/day potassium). A further 40 mmol of sodium was administered orally daily as NaCl tablets using an applicator.

Study Protocol

Heart failure was induced by 7 days of rapid left ventricular pacing (225 bpm) [15] and maintained by continuous pacing for the duration of the study. On four separate days with a rest day between each, the sheep received, in random order, a vehicle control (Haemaccel), human adrenomedullin alone (50 ng/min per kg infusion for 3 h), an ACE inhibitor alone (captopril: 25 mg bolus + 2 mg/h infusion for 3 h), and both agents combined. Infusions were administered in a total volume of 60 ml via the pulmonary artery catheter, commencing at 10:00 hours.

Mean arterial pressure, left atrial pressure, cardiac output and calculated total peripheral resistance

(CTPR = mean arterial pressure/cardiac output) were recorded at 15-min intervals in the 1-h prior to infusion (baseline), and at 15, 30, 45, 60, 90, 120 and 180 min during both the 3-h infusion and post-infusion periods. Hemodynamic measurements were determined by on-line computer-assisted analysis.

Blood samples were drawn from the left atrium at 30 min and immediately pre-infusion (baseline), and at 30, 60, 120 and 180 min during the 3-h infusion and post-infusion periods. Samples were taken into tubes on ice, centrifuged at 3939 *g* for 10 min at 4°C and stored at either –20°C or –80°C before assay for immunoreactive (ir-) adrenomedullin, cAMP, plasma renin activity, angiotensin II, aldosterone, atrial natriuretic peptide, brain natriuretic peptide, endothelin-1, catecholamines and cortisol.

All samples from individual animals were measured in the same assay to avoid inter-assay variability. Plasma electrolytes and hematocrit were measured in every sample taken. Urine volume and samples for the measurement of urine cAMP, sodium, potassium and creatinine excretion were collected every 1 h. Creatinine clearance was calculated as urine creatinine/plasma creatinine.

EVALUATION

Results are expressed as mean ± SEM. Baseline hemodynamic and hormone values represent the means of the four and two measurements respectively made in the 1 h immediately pre-infusion. Statistical analysis was performed by repeated-measures ANOVA. Baseline data from all treatments were compared. Treatment- and time-related differences between all four study limbs were determined using a two-way ANOVA (treatment–time interactions are quoted in the text). Statistical significance was assumed when $P < 0.05$

REFERENCES AND FURTHER READING

Aoyagi T, Fujii AM, Flaganan MF, Arnold LW, Brathwaite KW, Colan SD, Mirsky I (1993) Transition from compensated hypertrophy to intrinsic myocardial dysfunction during development of left ventricular pressure-overload hypertrophy in conscious sheep. Systolic dysfunction precedes diastolic dysfunction. Circulation 88:2415–2425

Byrne MJ, Raman JS, Alferness CA, Elser MD, Kaye DM, Power JM (2002) An ovine model of tachycardia-induced degenerative dilated cardiomyopathy and heart failure with prolonged onset. J Cardiac Fail 8:108–115

Charles CJ, Kaaja RJ, Espiner EA, Nicholls MG, Pemberton CJ, Richards AM, Yandle TG (1996) Natriuretic peptides in sheep with pressure overload left ventricular hypertrophy. Clin Exp Hypertens 18:1051–1071

Charles CJ, Elliott JM, Nicholls MG, Rademaker MT, Richards AM (2003) Natriuretic peptides maintain sodium homoeostasis during chronic volume loading post-myocardial infarction in sheep. Clin Sci 104:429–436

Huang Y, Hunyor SN, Liang L, Kawaguchi O, Shirota K, Ikeda Y, Yuasa T, Gallagher G, Zeng B, Zheng X (2004) Remodeling of chronic severely failing ischemic sheep heart after coronary microembolization: functional, energetic, structural, and cellular responses. Am J Physiol 286:H2141–H2150

Monreal G, Gerhardt MA, Kambara A, Abrishamchian AR, Bauer JA, Goldstein AH (2004) Selective microembolization of the circumflex coronary artery in an ovine model: dilated, ischemic cardiomyopathy and left ventricular dysfunction. J Cardiac Fail 10:174–183

Moreno J, Zaitsev AV, Warren M, Berenfeld O, Kalifa J, Lucca E, Mironov S, Guha P, Jalife J (2005) Effect of remodelling, stretch and ischemia on ventricular fibrillation frequency and dynamics in a heart failure model. Cardiovasc Res 65:158–166

Rademaker MT, Charles CJ, Lewis LK, Yandle TG, Cooper GJS, Coy DH, Richards AM, Nicholls MG (1997) Beneficial hemodynamic and renal effects of adrenomedullin in an ovine model of heart failure. Circulation 96:1983–1990

Rademaker MT, Charles CJ, Cooper JS, Coy DH, Espiner EA, Lewis LK, Nicholls MG, Richards AM (2002b)

Combined angiotensin-converting enzyme inhibition and adrenomedullin in an ovine model of heart failure. Clin Sci 102:653–660

Rademaker MT, Charkles CJ, Espiner EA, Frampton CM, Lainchbury JG, Richards AM (2005) Four-day urocortin-I administration has sustained beneficial hemodynamic, hormonal, and renal effects in experimental heart failure. Eur Heart J 26:2055–2062

A.6.0.9 Cardiac Failure in Monkeys

PURPOSE AND RATIONALE

Several authors used monkeys for studies of cardiac failure. Hollander et al. (1977) investigated the role of hypertension in ischemic heart disease in the **cynomolgus monkey** with coarctation of the aorta. Sieber et al. (1980) studied cardiotoxic effects of adriamycin in **macaques.**

Various studies were performed by the group of Hoit and Walsh in **baboons** (Hoit et al. 1955a, 1955b, 1997a, 1997b; Khoury et al. 1996). Hoit et al. (1997a) studied the effects of thyroid hormone on cardiac β-adrenergic responsiveness in conscious baboons.

PROCEDURE

Animal Instrumentation

Adult male baboons (*Papio anubis*) weighing 21–30 kg were pre-instrumented for physiological monitoring in a lightly anesthetized, sedated state. Animals were pre-instrumented with a Konigsburg micromanometer and a polyvinyl catheter in the LV apex, miniaturized sonomicrometer pairs (3 MHz, 6 mm) across the LV anteroposterior minor axis, a polyvinyl catheter in the right atrium for central venous access, and pacing wires on the right atrial appendage. Wires and tubes were tunneled subcutaneously into the interscapular area for later use. Postoperative pain was reduced by the use of Buprenex (0.01 mg/kg i.m., q 6 h), and postoperative antibiotic (Monocid 25 mg/kg) was administered for 5 days to reduce the risk of infection. Baseline hemodynamic studies were performed after a minimum of 1 week for postoperative recovery.

Hemodynamic data acquisition and analysis The micromanometers and fluid-filled catheters were calibrated with a mercury manometer. Zero drift of the micromanometer was corrected by matching the LV end-diastolic pressure measured simultaneously through the LV catheter. The fluid-filled LV catheter was connected to a pre-calibrated Statham 23 dB transducer with zero pressure at the level of the mid right atrium. The transit time of ultrasound between the ultrasonic dimension crystals was measured with a multichannel sonomicrometer (Triton Technology) and converted to distance assuming a constant velocity of sound in blood of 1.55 mm/ms.

The analog LV dP/dI signal was obtained on-line by electronic differentiation of the high-fidelity LV pressure signal. τ was derived from the high-fidelity LV pressure tracing by the method of Weiss et al. (1976), which assumes a monoexponential decay of LV pressure to a zero asymptote and has been shown to be directionally equivalent to other mathematical approaches for quantification of isovolumic pressure decay. τ is equal to the time in milliseconds for LV pressure to decay to $1/e$; thus, decreases in τ reflect improved isovolumic ventricular relaxation.

Fractional shortening of the LV minor axis was calculated as (EDD–ESD)/EDD, where EDD is LV end-diastolic dimension and ESD is LV end-systolic dimension. LV end-diastole was defined as the time in which LV dP/dt_{max} increased by $\geq$ 150 mmHg/s for 50 ms, and LV end-systole was defined as the time of the maximum ratio of LV pressure to LV minor-axis dimension. LV volumes were derived from minor-axis diameter (D) measurements: LV volume $= \pi/6(D)^3$.

V_{cf} was calculated as LV fractional shortening divided by LV ejection time; LV ejection time was defined as the time from peak positive to peak negative dP/dt.

Analog signals for high-fidelity and fluid-filled LV pressures, LV short-axis dimension, LV dP/dt, and the ECG were recorded on-line on a Gould multichannel recorder at 25 and 100 mm/s paper speed and digitized through an analog-to-digital board (Dual Control Systems) interfaced to an IBM AT computer at 500 Hz and stored on a floppy disk. Data were analyzed using an algorithm and software developed in our laboratory. Steady-state data were acquired over 5–10 s during spontaneous respiration and averaged.

Experimental Protocols

Hemodynamic studies were performed a minimum of 1 week after instrumentation and were repeated after 22–30 (26.8 ± 2.7) days of thyroid T_4 administration. Animals were tranquilized with Valium (1–5 mg) and ketamine (100 mg), and cholinergic blockade was achieved with atropine (0.4–0.8 mg i.v.); additional ketamine was administered as necessary, to a maximum cumulative dose of 40 mg/kg. Animals were atrially paced at a rate 40% to 50% greater than the control heart rate in order to obtain data at matched heart rates after thyrotoxicosis was produced.

Dobutamine Group

After hemodynamic stability was ensured and baseline data were recorded, intravenous dobutamine was infused at 5-min intervals at upwardly titrated rates of 2.5, 5.0, 7.5, and 10.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ to examine the effects of β_1-adrenergic stimulation. The dose range of catecholamine for these studies was chosen to alter inotropic and lusitropic states without causing an untoward increase in heart rate. Steady-state hemodynamic measurements were made during minutes 4 and 5 of each infusion period. At each level, the pacemaker was briefly turned off to determine the effect of dobutamine on the heart rate.

Four of the animals in this group were studied with incremental pacing both before and after β-adrenergic blockade with esmolol (0.3 $mg \cdot kg^{-1} \cdot min^{-1}$ i.v.V). The pacing protocol and the results from a larger group of animals studied before β-adrenergic blockade were detailed in a previous report. Briefly, atrial pacing was instituted at a rate above the intrinsic heart rate to avoid competing rhythms and was increased at 0.2-Hz increments until the critical heart rate was achieved. The critical heart rate was defined as the rate at which dP/dt_{max} and τ reached a maximum and minimum, respectively, during progressive increases in heart rate. We showed previously that hyperthyroidism significantly increases the critical heart rates for both dP/dt_{max} and τ.

The EC_{50} of dobutamine for LV dP/dt_{max} was determined by fitting log(dose)-transformed data to a sigmoidal relation with software from Graph Pad.

Terbutaline Group

Additional animals were chronically instrumented so that we could examine the effects of β_2-adrenergic stimulation. One animal died suddenly after receiving thyroid hormone for 20 days. In the remaining three animals, the β_2-adrenergic agonist terbutaline was infused both before and after production of the hyperthyroid state. Incremental doses of terbutaline (15 min/dose) were infused over a dosing range of 25 to 300 $ng \cdot kg^{-1} \cdot min^{-1}$.

Thyroid Function Tests

Thyroid function tests were performed before the baseline experiment in the euthyroid state and before the terminal experiment (within 24 h of the last dose of T_4) in the hyperthyroid state. T_3 radioimmunoassay, T_4, and free T_4 levels were measured at each state.

EVALUATION

Paired mean data were compared by Student's *t*-test. The effects of thyroid status, catecholamine dose, and β-blockade on hemodynamic and dimension variables were examined with repeated-measures ANOVA (SuperAnova, Abacus Concepts). When significant differences were found, group means were compared with contrasts. A value of $P < 0.05$ was considered significant. Unless specified, data are expressed as mean $\pm$ SD.

REFERENCES AND FURTHER READING

Hoit BD, Shao Y, Gabel M, Walsh RA (1995a) Disparate effects of early pressure overload hypertrophy and force-dependent indices of ventricular performance in the conscious baboon. Circulation 91:1213–1220

Hoit BD, Shao Y, Kinoshita A, Gabel M, Husain A, Walsh RA (1995b) Effects of angiotensin II generated by an angiotensin converting enzyme-independent pathway on left ventricular performance in the conscious baboon. J Clin Invest 95:1519–1527

Hoit BD, Khoury SF, Shao Y, Gabel M, Liggett SB, Walsh RA (1997a) Effects of thyroid hormone on cardiac β-adrenergic responsiveness in conscious baboons. Circulation 96:592–598

Hoit BD, Pawlowski-Dam CM, Shao Y, Gabel M, Walsh RA (1997b) The effects of a thyroid hormone analog on left ventricular performance and contractile and calcium cycling proteins in the baboon. Proc Assoc Am Physicians 109:136–145

Hollander W, Prusty S, Kirkpatrick B, Paddok J, Nagraj S (1977) Role of hypertension in ischemic heart disease and cerebral vascular disease in the cynomolgus monkey with coarctation of the aorta. Circ Res 40 [5 Suppl 1]: I70–I80

Khoury SF, Hoit BD, Dave V, Pawlowski-Dahm CM, Shao Y, Gavel M, Periasamy M, Walsh RA (1996) Effects of thyroid hormone on left ventricular performance and regulation of contractile and Ca^{2+}-cycling proteins in the baboon. Circ Res 79:727–735

Sieber SM, Correa P, Young DM, Dalgard DW, Adamson RH (1980) Cardiotoxic and possible leukemogenic effects of adriamycin in nonhuman primates. Pharmacology 20:9–14

Weiss JL, Frederickson JW, Weisfeldt ML.(1976) Hemodynamic determinants of the time course of fall in canine left ventricular pressure. J Clin Invest 58:751–760

A.6.0.10 Cardiac Failure in Other Species

PURPOSE AND RATIONALE

Various species have been used to study experimental cardiac failure.

Breisch et al. (1984) studied the effects of pressure-overload hypertrophy in the left myocardium of young adult **cats**. Hypertrophy was induced by a 90% constriction of the ascending aorta.

Genao et al. (1996) recommended dilated cardiomyopathy in **turkeys** as an animal model for the study of human heart failure.

Do et al. (1997) studied energy metabolism in normal and hypertrophied right ventricle of the **ferret** heart.

Wang et al. (1994) studied Ca^{2+} handling and myofibrillar Ca^{2+} sensitivity in **ferret** cardiac myocytes with pressure-overload hypertrophy.

Bovine hereditary cardiomyopathy was recommended as an animal model of human dilated cardiomyopathy by Eschenhagen et al. (1995).

REFERENCES AND FURTHER READING

Breisch EA, White FC, Bloor CM (1984) Myocardial characteristics of pressure overload hypertrophy. A structural and functional study. Lab Invest 51:333–342

Do E, Baudet S, Verdys M, Touzeau C, Bailly F, Lucas-Héron B, Sagniez M, Rossi A, Noireaud J (1997) Energy metabolism in normal and hypertrophied right ventricle of the ferret heart. J Mol Cell Cardiol 29:1903–1913

Eschenhagen T, Dieterich M, Kluge SH, Magnussen O, Mene U, Muller F, Schmitz W, Scholz H, Weil J, Sent U (1995) Bovine hereditary cardiomyopathy: an animal model of human dilated cardiomyopathy. J Mol Cell Cardiol 27:357–370

Genao A, Seth K, Schmnidt U, Carles M, Gwathmey JK (1996) Dilated cardiomyopathy in turkeys: an animal model for the study of human heart failure. Lab Animal Sci 46:399–404

Wang J, Flemal K, Qiu Z, Ablin L, Grossman W, Morgan JP (1994) Ca^{2+} handling and myofibrillar Ca^{2+} sensitivity in ferret cardiac myocytes with pressure-overload hypertrophy. Am J Physiol 267(3 Pt2):H918–H924

A.6.0.11 Hypertrophy of Cultured Cardiac Cells

PURPOSE AND RATIONALE

Kojima et al. (1994), Komuro et al. (1990, 1991, 1993), Yamazaki et al. (1993, 1994, 1996) described a method to induce hypertrophy of cardiomyocytes by mechanical stress *in vitro*.

PROCEDURE

Primary cultures from cardiomyocytes are prepared from ventricles of 1-day-old neonatal Wistar Kyoto rats. According to the method of Simpson and Savion (1982), the cultures are treated for 3 days with 0.1 mM bromodeoxyuridine to suppress proliferation of nonmyocardial cells. Elastic culture dishes ($2 \times 4 \times 1$ cm) are made by vulcanizing liquid silicone rubber consisting of methylvinyl polysiloxane and dimethyl hydrogen silicone resin using platinum as a catalyst. The bottom of the disc is 1-mm thick, and it is highly transparent because of no inorganic filler in either component. Cells are plated in a field density of 1×10^5 cells/cm^2 in culture medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Mechanical stress on cardiac cells is applied by gently pulling and hanging the dish on pegs. A 10% change in length of the dish results in an almost identical change in the length of the cell along a single axis (Kimuro et al. 1990). Cardiocytes are stretched by 5%, 10%, or 20%. Drugs, e. g., an angiotensin-II receptor antagonist, are added 30 min before stretch.

For protein analysis, the silicone dishes are stretched for 24 h after 2 days of serum starvation and [^{3}H]phenylalanine (1 µCi/ml) is added for 60 min. At the end of each stress, the cells are rapidly rinsed four times with ice-cold phosphate-buffered saline and incubated for 20 min on ice with 1 ml of 5% trichloroacetic acid. The total trichloroacetic acid-insoluble radioactivity in each dish is determined by liquid scintillation counting.

For determination of mitogen-activated protein kinase, cardiomyocytes are lysed on ice and centrifuged. Aliquots of the supernatants of myocyte extracts are incubated in kinase buffer (25 mM/l Tris-HCl, pH 7.4, 10 mM/l $MgCl_2$, 1 mM/l dithiothreitol, 40 µM/l APT, 2 µCi [γ-^{32}P]ATP, 2 µM/l protein kinase inhibitor peptide, and 0.5 mM/l EGTA) and substrates (25 µg myelin basic protein). The reaction is stopped by adding stopping solution containing 0.6% HCl, 1 mM/l ATP, and 1% bovine serum albumin. Aliquots of the supernatant are spotted on P81 paper (Whatman), washed in 0.5% phosphoric acid, dried and counted.

For determination of*c-fos mRNA*, Northern blot analysis is performed.

EVALUATION

Values are expressed as mean ± SEM. Comparisons between groups are made by one-way ANOVA followed by Dunnett's modified *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The interesting approach to induce hypertrophy of cardiac cells *in vitro* has been used predominantly by one research group. Confirmation by other research groups including modifications of the mechanical procedures seems to be necessary.

REFERENCES AND FURTHER READING

Kojima M, Shiojima I, Yamazaki T, Komuro I, Yunzeng Z, Ying W, Mizuno T, Ueki K, Tobe K, Kadowaki T, Nagai R, Yazaki Y (1994) Angiotensin II receptor antagonist TCV-116 induces regression of hypertensive left ventricular hypertrophy *in vivo* and inhibits the intercellular signaling pathway of stretch-mediated cardiomyocyte hypertrophy *in vitro*. Circulation 89:2204–2211

Komuro I, Yazaki Y (1993) Control of cardiac gene expression by mechanical stress. Annu Rev Physiol 55:55–75

Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, Takaku F, Yazaki Y (1990) Stretching cardiac myocytes stimulates protooncogene expression. J Biol Chem 265:3595–3598

Komuro I, Katoh Y, Kaida T, Shibazaki Y, Kurabayashi M, Hoh E, Takaku F, Yazaki Y (1991) Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. J Biol Chem 266:1265–1268

Simpson P, Savion A (1982) Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Circ Res 50:101–116

Yamazaki T, Tobe K, Hoh E, Maemura K, Kaida T, Komuro I, Tamamoto H, Kadowaki T, Nagai R, Yazaki Y (1993) Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured cardiac myocytes. J Biol Chem 268:12069–12076

Yamazaki T, Komuro I, Shiojima I, Mizuno T, Nagai R, Yazaki Y (1994) *In vitro* methods to study hypertrophy of cardiac cells. J Pharmacol Toxicol Meth 32:19–23

Yamazaki T, Komuro I, Yazaki Y (1996) Molecular aspects of mechanical stress-induced hypertrophy. Mol Cell Biochem 163/164:197–201

A.7 Positive Inotropic Activity (Cardiac Glycosides)

A.7.0.1 General Considerations

Biological standardization of cardiac glycosides was necessary as long as the drugs used in therapy were plant extracts or mixtures of various glycosides. They were standardized in units of an international standard. Some of the pharmacological methods used for these purposes and adopted by many pharmacopoeias have nowadays *historical interest* only. This holds true for the frog method and the pigeon method (Burn et al. 1950).

Particularly, the **frog method** was used for standardization. The method adopted by the U.S Pharmacopoeia X was the 1 h test. Healthy frogs (*Rana pipiens*) weighing 20–30 g were selected from the cold storage room. One hour before assay, their weight was recorded and they were placed in wire cages with a water depth of 1 cm. The doses of digitalis were calculated so that they approximated 0.015 ml/g body weight. Injections were made into the ventral lymph sac. One hour later, the animals were pithed and the heart removed and examined. Systolic arrest of the ventricle and widely dilated atrium indicated the typical result. Calculations of activity in terms of International Units were made from the percentage of dead animals in the test group versus those in the group receiving the international standard.

The **pigeon method** introduced by Hanzlik (1929) and adopted by USP XVII depends on the observation that intravenously injected cardiac glycosides have an emetic action in pigeons. In the original test, adult pigeons weighing 300–400 g are injected with a solution of the cardiac glycoside into a suitable wing vein in the axillary region. Vomiting occurring within 15 min is regarded as positive result. Two doses of test solution and standard are injected and percentage of vomiting pigeons registered. This 4 point assay allows calculation of ED_{50} values and of the potency ratio compared with the standard.

Modifications of other methods, such as the **cat method** introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijngarden (1926), the **guinea pig method** described by Knaffl-Lenz (1926) and the **isolated cat papillary muscle** method introduced by Catell and Gold (1938) still being used for evaluation of synthetic cardioglycosides and other positive inotropic compounds are referenced in detail below.

Surveys on the evaluation of cardiac glycosides have been given by Bahrmann and Greef (1981), for the use of the isolated papillary muscle by Reiter (1981) and for other isolated heart preparations by Greef and Hafner (1981). Moreover, the influence on Na^+/K^+-ATPase, an *in vitro* model specific for cardiac glycosides (Gundert-Remy and Weber 1981), is described.

The mechanisms of action have been reviewed by Scholz (1984) and Grupp (1987).

Analogous to antiarrhythmic agents, Feldmann (1993) proposed a classification system that categorizes inotropic agents according to their supposed mode of action:

Class I: Inotropic agents that increase intracellular cyloAMP, including β-adrenergic agonists and phosphodiesterase inhibitors,

Class II: Inotropic agents affecting sarcolemmal ion pumps and channels, in particular cardiac glycosides inhibiting Na^+/K^+-ATPase,

Class III: Agents that modulate intracellular calcium mechanisms (no therapeutic inotropic agents in this kind yet available),

Class IV: Inotropic agents having multiple mechanisms of action

REFERENCES AND FURTHER READING

Bahrmann H, Greeff K (1981) Evaluation of cardiac glycosides in the intact animal. Handbook of Experimental Pharmacology 56/I:118–152, Springer, Berlin, Heidelberg, New York

Burn JH, Finney DJ, Goodwin LG (1950) Digitalis, strophanthus, and squill. In: Biological Standardization, Chapter XIII, pp 294–310, Oxford University Press, London, New York, Toronto

Cattell M, Gold H (1938) Influence of digitalis glycosides on the force of contraction of mammalian cardiac muscle. J Pharmacol Exp Ther 62:116–125

Di Palma JR (1964) Animal techniques for evaluating digitalis and its derivatives. In: Nodine JH, Siegler PE (eds) Animal and Clinical Pharmacologic Techniques in Drug Evaluation. Chapter 15, pp 154–159, Year Book Medical Publishers, Inc., Chicago
Feldman AM (1993) Classification of positive inotropic agents. J Am Coll Cardiol 22:1223–1227
Greef K (1963) Zur Pharmakologie der herzwirksamen Glykoside. Klin Physiol 1:340–370
Greef K, Hafner D (1981) Evaluation of cardiac glycosides in isolated heart preparations other than papillary muscle. Handbook of Experimental Pharmacology 56/I:161–184. Springer, Berlin, Heidelberg, New York
Grupp G (1987) Selective updates on mechanisms of action of positive inotropic agents. Mol Cell Biochem 76:97–112
Gundert-Remy U, Weber E (1981) ATPase for the determination of cardiac glycosides. Handbook of Experimental Pharmacology 56/I:83–94. Springer, Berlin, Heidelberg, New York
Hanzlik JP (1929) New method of estimating the potency of digitalis in pigeons: *Pigeon emesis*. J Pharmacol Exp Ther 35:363–391
Hatcher RA, Brody JG (1910) The biological standardization of drugs. Am J Pharm 82:360–372
Knaffl-Lenz E (1926) The physiological assay of preparations of digitalis. J Pharmacol Exp Ther 29:407–425
Lind van Wijngaarden C de (1926) Untersuchungen über die Wirkungsstärke von Digitalispräparaten. II. Mitteilung: Über die Genauigkeit der Dosiseichung an der Katze. Arch exp Path Pharmak 113:40–58
Reiter M (1981) The use of the isolated papillary muscle for the evaluation of positive inotropic effects of cardioactive steroids. Handbook of Experimental Pharmacology 56/I:153–159. Springer, Berlin, Heidelberg, New York
Scholz H (1984) Inotropic drugs and their mechanisms of action. J Am Coll Cardiol 4:389–397

A.7.1
In Vitro Tests

A.7.1.1
Ouabain Binding

PURPOSE AND RATIONALE

Cardiac glycosides can be characterized by their binding kinetics (association process, equilibrium binding, and dissociation process) on the ouabain receptor.

PROCEDURE

Heart sarcolemma preparations are obtained from rat or dog heart. From a canine heart or from rat hearts submitted to coronary perfusion myocytes are isolated by collagenase digestion. The isolated membrane fractions consist mainly of myocyte sarcolemma. [^{3}H]ouabain with a specific radioactivity of about 20 Ci/mmol is incubated with ligands to be tested in 10 ml of binding medium consisting of 1 mM $MgCl_2$, 1 mM inorganic phosphate, and 50 mM Tris-HCl, pH 7.4 at 37°C for 10 min.

Association process: After temperature equilibration in the presence of either 10 or 100 nM [^{3}H]ouabain, 200 µg of membrane preparation are added to initiate the reaction. At various times, 4.5 ml are removed and rapidly filtered.

Equilibrium binding: At the end of the temperature equilibration carried out in the presence of increasing concentrations of [^{3}H]ouabain ranging from 10 nM to 3 µM, 40 µg of membranes are added. After 30 min, duplicate aliquots of 4.5 ml are removed and filtered.

Dissociation process: Once equilibrium has been achieved under the experimental conditions used to study association, 10 ml of prewarmed Mg^{2+} plus P_i Tris-HCl solution supplemented with 0.2 mM unlabeled ouabain are added to initiate dissociation of [^{3}H]ouabain. At various times, aliquots of 0.9 ml are removed and rapidly filtered.

All aliquots are filtered under vacuum on HAWP Millipore filters (0.45 µm) and rinsed three times with 4 ml of ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements are determined.

EVALUATION

Kinetic parameters for the association and the dissociation process are calculated. The results of equilibrium binding are analyzed by Scatchard plots.

REFERENCES AND FURTHER READING

Erdmann E, Schoner W (1973) Ouabain-receptor interactions in (Na^++K^+)-ATPase preparations from different tissues and species. Determination of kinetic constants and dissociation constants. Biochim Biophys Acta 307:386–398
Erdmann E, Schoner W (1974) Ouabain-receptor interactions in (Na^++K^+)-ATPase preparations. IV. The molecular structure of different cardioactive steroids and other substances and their affinity to the glycoside receptor. Naunyn Schmiedeberg's Arch Pharmacol 283:335–356
Erdmann E, Philipp G, Scholz H (1980) Cardiac glycoside receptor, (Na^++K^+)-ATPase activity and force of contraction in rat heart. Biochem Pharmacol 29:3219–3229
Lelievre LG, Charlemagne D, Mouas C, Swynghedauw B (1986) Respective involvements of high- and low-affinity digitalis receptors in the inotropic response of isolated rat heart to ouabain. Biochem Pharmacol 35:3449–3455
Maixent JM, Charlemagne D, de la Chapelle B, Lelievre LG (1987) Two Na,K-ATPase isoenzymes in canine cardiac myocytes. Molecular basis of inotropic and toxic effects of digitalis. J Biol Chem 262:6842–6848
Maixent JM, Gerbi A, Berrebi-Bertrand I, Correa PE, Genain G, Baggioni A (1993) Cordil reversibly inhibits the Na,K-ATPase from outside the cell membrane. Role of K-dependent dephosphorylation. J Receptor Res 13:1083–1092

A.7.1.2
Influence on Na^+/K^+ ATPase

PURPOSE AND RATIONALE

The enzyme Na^+/K^+ ATPase is the transport system for Na^+ and K^+ in the cell membranes. The membrane

bound enzyme couples ATP hydrolysis to the translocation of Na^+ and K^+ ions across the plasma membrane through a series of conformational transitions between the E_1 and E_2 states of the enzyme. The enzyme is a heterodimer consisting of a catalytic subunit (110 kDa) associated with a glycosylated β subunit (55 kDa). Three alpha (α_1, α_2, α_3) subunits have been identified by cDNA cloning. In the heart, enzyme Na^+/K^+ ATPase is the target of the positive inotropic glycosides. Therefore, it is of interest for the characterization of positive inotropic compounds. The test is based on the determination of phosphate generated from ATP under special conditions. Inhibition of bovine cerebral Na^+/K^+ ATPase prepared according to Schoner et al. (1967) is measured after addition of various concentrations of the test compound compared with those of the standard (Erdmann et al. 1980).

PROCEDURE

Solutions

1.00 ml 133 mM imidazole pH 7.3
0.04 ml 160 mM $MgCl_2$
0.02 ml DPNH (10 mg/ml)
0.04 ml 310 mM NH_4Cl
0.04 ml 100 mM ATP
0.02 ml 40 mM phosphoenol-pyruvate
0.05 ml pyruvate-kinase (1 mg/ml = 150 U/ml)
0.04 ml lactate-dehydrogenase
(0.5 mg/ml = 180 U/ml)
0.20 ml 1 M NaCl
0.01–0.02 ml bovine cerebral ATPase (depending on activity of the enzyme) up to 2.0 ml distilled water

Test

The enzyme activity is started by addition of the ATP solution at 37°C. After 4 min the inhibitor (various concentrations of the cardiac glycoside) is added. Na^+/K^+ ATPase activity is measured by a coupled optical assay. The reaction is continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of Na^+/K^+ ATPase with 10^{-3} M ouabain.

EVALUATION

Inhibition of ATPase is measured after addition of various concentrations of the test compound. Dose-response curves are established and compared with the standard (k-strophanthin). Potency ratios can be calculated.

MODIFICATIONS OF THE METHOD

Brooker and Jelliffe (1972) and Marcus et al. (1975) described an *in vitro* assay based on displacement of radiolabeled ouabain bound to ATPase by various glycosides. Another method is based on the inhibition of rubidium uptake into erythrocytes (Lowenstein 1965; Belz 1981).

Erdmann et al. (1980) prepared (Na^++K^+)-ATPase-containing cardiac cell membranes from rat hearts.

Maixent et al. (1987, 1991) described two Na,K-ATPase isoenzymes in canine cardiac myocytes as the molecular basis of inotropic and toxic effects of digitalis.

The effect of ouabain on Na^+/K^+ATPase activity in cells of the human rhabdomyosarcoma cell line TE671 was studied by Miller et al. (1993) with a special equipment, the microphysiometer (McConnell et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

The *in vitro* methods being used for determinations of plasma levels of glycosides (Maixent et al. 1995) have been largely substituted by radioimmunoassays specific for individual glycosides. Nevertheless, the inhibition of Na^+/K^+ ATPase can be used as an indicator of activity of new semisynthetic cardiac glycosides.

REFERENCES AND FURTHER READING

Anner B, Moosmayer M (1974) Rapid determination of inorganic phosphate in biological systems by a highly sensitive photometric method. Analyt Biochem 65:305–309

Akera T, Brody T (1978) The role of Na^+, K^+-ATPase in the inotropic action of digitalis. Pharmacol Rev 29:197–201

Belz GG (1981) Rubidium uptake in erythrocytes. Handbook of Experimental Pharmacology 56. Springer, Berlin Heidelberg New York, pp 95–113

Borsch-Galetke E, Dransfeld H, Greef K (1972) Specific activity of Na^++K^+- activated ATPase in rats and guinea pigs with hypoadrenalism. Naunyn-Schmiedeberg's Arch Pharmacol 274:74–80

Brooker G, Jelliffe RW (1972) Serum cardiac glycoside assay based upon displacement of ^{3}H-ouabain from Na-K ATPase. Circulation 45:20–36

Burnett GH, Conklin RL (1968) The enzymatic assay of plasma digitoxin levels. J Lab Clin Med 71:1040–1049

Charlemagne D, Maixent JM, Preteseille M, Lelievre LG (1986) Ouabain binding sites and Na^+,K^+)-ATPase activity in rat cardiac hypertrophy. Expression of the neonatal forms. J Biol Chem 261:185–189

Erdmann E, Philipp G, Scholz H (1980) Cardiac glycoside receptor, (Na^++K^+)-ATPase activity and force of contraction in rat heart. Biochem Pharmacol 29:3219–3229

Gundert-Remy U, Weber E (1981) ATPase for the determination of cardiac glycosides. Handbook of Experimental Pharmacology 56/I:83–94. Springer, Berlin, Heidelberg, New York

Lelievre LG, Maixent G, Lorente P, Mouas C, Charlemagne D, Swynghedauw B (1986) Prolonged responsiveness to ouabain in hypertrophied rat heart: physiological and biochemical evidence. Am J Physiol 250:H923–H931

Lindner E, Schöne HH (1972) Änderungen der Wirkungsdauer und Wirkungsstärke von Herzglykosiden durch Abwandlungen der Zucker. Arzneim Forsch/Drug Res 22:428–435
Lindner E, v. Reitzenstein G, Schöne HH (1979) Das 14,15-β-oxido-Analoge des Proscillaridins (HOE 040) Arzneim Forsch/Drug Res 29:221–226
Lowenstein JM (1965) A method for measuring plasma levels of digitalis glycosides. Circulation 31:228–233
Maixent JM, Charlemagne D, de la Chapelle B, Lelievre LG (1987) Two Na,K-ATPase isoenzymes in canine cardiac myocytes. Molecular basis of inotropic and toxic effects of digitalis. J Biol Chem 262:6842–6848
Maixent JM, Fénard S, Kawamoto RM (1991) Tissue localization of Na,K-ATPase isoenzymes by determination of their profile of inhibition with ouabain, digoxin, digitoxigenin and LND 796, a new aminosteroid cardiotonic. J Receptor Res 11:687–698
Maixent JM, Gerbi A, Berrebi-Bertrand I, Correa PE, Genain G, Baggioni A (1993) Cordil reversibly inhibits the Na,K-ATPase from outside the cell membrane. Role of K-dependent dephosphorylation. J Receptor Res 13:1083–1092
Maixent JM, Gerbi A, Barbey O, Fenard S, Kawamoto RM, Baggioni A (1995) Relation of plasma concentrations to positive inotropic effect of intravenous administration of cordil in dogs. Pharm Pharmacol Lett 1:1–4
Mansier P, Lelievre LG (1982) Ca^{2+}-free perfusion of rat heart reveals a $(Na^+ + K^+)$ATPase form highly sensitive to ouabain. Nature 300:535–537
Marcus FI, Ryan JN, Stafford MG (1975) The reactivity of derivatives of digoxin and digitoxin as measured by the Na-K-ATPase displacement assay and by radioimmunoassay. J Lab Clin Med 85:610–620
McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG, Pitchford S (1992) The Cytosensor microphysiometer. Science 257:1906–1912
Miller DL, Olson JC, Parce JW, Owicki JC (1993) Cholinergic stimulation of the Na^+/K^+ adenosine triphosphatase as revealed by microphysiometry. Biophys J 64:813–823
Noel F, Godfraind T (1984) Heterogeneity of ouabain specific binding sites and $(Na^+ + K^+)$-ATPase inhibition in microsomes from rat heart. Biochem Pharmacol 33:47–53
Schoner W, v. Illberg C, Kramer R, Seubert W (1967) On the mechanism of Na^+- and K^+- stimulated hydrolysis of adenosine triphosphate. Eur J Biochem 1:334–343
Schwarz A, Nagano K, Nakao M, Lindenmayer GE, Allen JC, Matsoi HM (1971) The sodium- and potassium activated adenosine-triphosphatase system. In: Schwartz (ed) Methods in pharmacology Vol 1, Appleton-Century-Crofts, Meredith Corporation, New York, pp 361–388
Skou JC, Esmann M (1992) The Na_2K-ATPase. J Bioenerg Biomembr 24:249–261
Thomas R, Allen J, Pitts BJR, Schwartz A (1974) Cardenolide analogs. An explanation for the unusual properties of AY 22241. Eur J Pharmacol 53:227–237

A.7.2
Tests in Isolated Tissues and Organs

A.7.2.1
Isolated Cat Papillary Muscle

PURPOSE AND RATIONALE

Isolated cardiac tissue has been chosen to study the decrease of performance after prolonged electrical stimulation and during restoration of force under the influence of cardiac glycosides. Cattell and Gold (1938) described a method using cat papillary muscle.

PROCEDURE

Cats of either sex weighing 2.5–3 kg are used. The animal is anesthetized with ether and the thorax is opened rapidly. The heart is removed and a papillary muscle from the right ventricle is isolated and fixed in an organ bath containing oxygenated Ringer's solution at 36°C. One end of the muscle is tied to an tissue holder and the other one to a strain gauge. The muscle is stimulated electrically with 4–6 V, 2 ms duration and a rate of 30/min. The contractions are recorded on a polygraph. After 1 h, the muscle begins to fail and the force of contraction diminishes to a fraction of control. At this point, the cardiac glycoside is added to the bath, restoring the contractile force to levels approaching control. The standard dose is 300 ng/ml ouabain. The potency of natural and semisynthetic glycosides can be determined with this method. Catecholamines, like adrenaline (10 ng/ml) or isoprenaline (10 ng/ml), are active as well.

EVALUATION

The increase of contractile force is calculated as percentage of the predose level. Dose-response curves can be established using various doses.

CRITICAL ASSESSMENT OF THE METHOD

The use of isolated papillary muscle strips can be recommended for evaluation of inotropic compounds of various chemical classes.

MODIFICATIONS OF THE METHOD

Instead of cat papillary muscle the isolated left atrium of guinea pigs can be used (see Sect. A.1.2.6). For testing cardiac glycosides, the calcium content in the Ringer solution is reduced to 50%.

Andersom (1983) compared responses of guinea pig paced left atria to various positive inotropic agents at two different calcium concentrations (1.25 and 2.50 mM). Consistently good results were obtained at the lower calcium concentration with isoproterenol, ouabain, amrinone, and 3-isobutyl-1-methylxanthine.

Böhm et al. (1989) studied positive inotropic substances like isoprenaline and milrinone in isolated cardiac preparations from different sources. They used isolated papillary muscles from Wistar–Kyoto rats and from spontaneously hypertensive rats, but also human papillary muscle strips from patients with moderate

heart failure (NYHA II–III) and compared the effects with papillary muscle strips from patients with severe heart failure (NYHA IV). They recommended that new positive inotropic agents should be screened in human myocardial tissue from patients with heart failure.

Labow et al. (1991) recommended a human atrial trabecular preparation for evaluation of inotropic substances.

Böhm et al. (1989) tested positive inotropic agents in isolated cardiac preparations from different sources, e. g., human papillary muscle strips from patients with severe heart failure (NYHA IV), human papillary muscle strips from patients with moderate heart failure (NYHA II–III), human atrial trabeculae, isolated papillary muscles from Wistar–Kyoto rats, and isolated papillary muscles from spontaneous hypertensive rats. They suggested that positive inotropic effects should be screened in isolated myocardium from patients with heart failure.

REFERENCES AND FURTHER READING

Anderson WG (1983) An improved model for assessment of positive inotropic activity *in vitro*. Drug Dev Res 3:443–451

Böhm M, Diet F, Pieske B, Erdmann E (1989) Screening of positive inotropic agents in isolated cardiac preparations from different sources. J Pharmacol Meth 21:33–44

Brown TG, Lands AM (1964) Cardiovascular activity of sympathomimetic amines. In: Laurence DR, Bacharach AL (eds) Pharmacometrics Vol 1, Academic Press New York, pp 353–368

Cattell M, Gold H (1938) Influence of digitalis glycosides on the force of contraction of mammalian cardiac muscle. J Pharmacol Exp Ther 62:116–125

Di Palma JR (1964) Animal techniques for evaluating digitalis and its derivatives. In: Nodine JH, Siegler PE (eds) Animal and Clinical Pharmacologic Techniques in Drug Evaluation. Chapter 15, Year Book Medical Publishers, Inc., Chicago, pp 154–159

Grupp IL, Grupp G (1984) Isolated heart preparations perfused or superfused with balanced salt solutions. In: Schwartz A (ed) Methods in Pharmacology, Vol 5: Myocardial Biology. pp 111–128. Plenum Press, New York and London

Labow RS, Desjardins S, Keon WJ (1991) Validation of a human atrial trabecular preparation for evaluation of inotropic substances. J Pharm Meth 26:257–268

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydroxy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43:313–319

Reiter M (1981) The use of the isolated papillary muscle for the evaluation of positive inotropic effects of cardioactive steroids. Handbook of Experimental Pharmacology 56/I:153–159. Springer, Berlin, Heidelberg, New York

Turner RA (1965) Cardiotonic agents. In: Screening Methods in Pharmacology. pp 203–209. Academic Press, New York, London

A.7.2.2 Isolated Hamster Cardiomyopathic Heart

PURPOSE AND RATIONALE

Special strains of Syrian hamsters develop cardiomyopathy. These animals can be used for evaluation of cardiotonic drugs (see Sect. A.6.0.4).

PROCEDURE

Hamsters with cardiomyopathy (Bio 14/6) at the age of 50 weeks are used. Controls are normal Syrian hamsters (FIB hybrids) at the same age. The animals are pretreated with heparin (5 mg/kg i.p.) and 20 min later the heart is prepared according the method of Langendorff and perfused with heart Ringer solution under 75 mm H_2O hydrostatic pressure. The preparation is allowed to equilibrate in the isolated state for 60 min at 32°C with a diastolic preload of 1.5 g. The force of contractions is recorded isometrically by a strain gauge transducer on a polygraph, e. g., Heliscriptor He 19 recorder (Hellige GmbH, Freiburg, Germany). From these signals, the heart rate is measured by a chronometer. The coronary flow is measured by an electromagnetic flowmeter. Compounds are injected via the aortic cannula into the inflowing heart-Ringer solution.

EVALUATION

The contractile force and the coronary flow in hearts from diseased and normal animals is registered before and after application of the test drugs. Mean values and standard deviation are calculated before and after drug application and statistically compared using Student's *t*-test.

MODIFICATION OF THE METHOD

Jasmin et al. (1979) showed after prolonged *in vivo* administration beneficial effects of a variety of cardiovascular drugs, including verapamil, prenylamine, dibenamine and propranolol.

After chronic administration (4 or 12 weeks subcutaneously), Weishaar et al. (1987) found beneficial effects of the calcium channel blocker diltiazem, but not by the administration of digitalis.

In contrast, in the experiments of Ottenweller et al. (1987) hamsters treated orally with digoxin survived and showed significant amelioration of the pathological syndrome of heart failure.

REFERENCES AND FURTHER READING

Jasmin G, Solymoss B, Proscheck L (1979) Therapeutic trials in hamster dystrophy. Ann NY Acad Sci 317:338–348

Ottenweller JE, Tapp WN, Natelson BH (1987) The effect of chronic digitalis therapy on the course of heart failure and on endocrine function in cardiomyopathic hamsters. Res Commun Chem Pathol Pharmacol 58:413–416

Rajagopalan R, Ghate AV, Subbarayan P, Linz W, Schoelkens BA (1993) Cardiotonic activity of the water soluble forskoline derivative 8,13-epoxy-6β-(piperidinoacetoxy)-1α,7β,9α-trihydroxy-labd-14-en-11-one. Arzneim Forsch/Drug Res 43(I) 313–319

Weishaar RE, Burrows SD, Kim SN, Kobylarz-Singer DC, Andrews LK, Quade MM, Overhiser R, Kaplan HR (1987) Protection of the failing heart: comparative effects of chronic administration of digitalis and diltiazem on myocardial metabolism in the cardiomyopathic hamster. J Appl Cardiol 2:339–360

A.7.2.3
Potassium Loss from the Isolated Guinea Pig Heart

PURPOSE AND RATIONALE

Cardiac glycosides induce a net loss of potassium from cardiac tissue due to their inhibition of the Na^+/K^+ ATPase. Therefore, potassium is increased in the effluent of the isolated guinea pig heart. This phenomenon can be used as parameter for the activity of digitalis-like compounds (Lindner and Hajdu 1968).

PROCEDURE

The isolated heart of guinea pigs according to LANGENDORFF is prepared as described in Sect. A.3.1.1. The coronary outflow is measured by counting the drops of the effluent by a photocell. The effluent is collected in a funnel with a thin upwards shaped outlet allowing to withdraw small fluid samples for analysis by a flame photometer. A pump attached to a 4-way valve changes the samples to the flame photometer every 15 s in the following sequence: effluent Tyrode-solution from the heart, distilled water, Tyrode-solution used for perfusion, distilled water. The potassium content of affluent and effluent Tyrode-solution is compared and registered on a Varian-recorder. The difference is attributed to the potassium outflow from the heart. The dose-response curve is flat in the therapeutic range, much steeper in the toxic range.

EVALUATION

The following parameters are recorded and calculated:

- coronary flow [ml/min]
- contractile force
- potassium loss [mVal/min]

CRITICAL ASSESSMENT OF THE METHOD

A good correlation was found between the measured potassium loss and the positive inotropic effect of cardiac glycosides. The method is suitable for the quick determination of efficacy of digitalis-like substances and facilitates the discrimination from other positive inotropic compounds like adrenaline.

REFERENCES AND FURTHER READING

Greef K, Hafner D (1981) Evaluation of cardiac glycosides in isolated heart preparations other than papillary muscle. Handbook of Experimental Pharmacology 56/I:161–184. Springer, Berlin, Heidelberg, New York

Lindner E, Hajdu P (1968) Die fortlaufende Messung des Kaliumverlustes des isolierten Herzens zur Bestimmung der Wirkungsstärke digitalisartiger Körper. Arch Int Pharmacodyn 175:365–372

A.7.3
In Vivo Tests

A.7.3.1
Cardiac Toxicity in Cats (Hatcher's Method)

PURPOSE AND RATIONALE

The purpose of the method, originally introduced by Hatcher and Brody (1910) and described in detail by Lind van Wijingaarden (1926), was to establish "cat units" for cardiac glycoside preparations. Hatcher and Brody defined "the cat unit as the amount of crystalline ouabain which is fatal within about ninety minutes to a kilogram of a cat when the drug in injected slowly and almost continuously into the femoral vein". Time to cardiac arrest after intravenous infusion of a solution with defined concentration of the standard was used as reference and the unknown solution of the test preparation compared with the standard. The method can be used for testing natural and semisynthetic glycosides.

PROCEDURE

Cats of either sex weighing 2–3.5 kg are temporarily anesthetized with ether. Anesthesia is maintained with 70 mg/kg chloralose given intravenously. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula is inserted. ECG is recorded from lead II. Then, intravenous infusion of the test solution is started. The endpoint is cardiac arrest which should be reached within 30–60 min by proper adjustment of the concentration of the infused solution.

MODIFICATIONS OF THE METHOD

Hatcher's original method has been modified by many authors. The method using **guinea pigs**, introduced by Knaffl-Lenz (1926) is in its essentials similar to the cat method.

Guinea pigs weighing 400–600 g are anesthetized with urethane (1.75 g/kg i.m.) The animal is secured

on a operating table and the trachea is cannulated. The jugular vein is cannulated for infusion of the test preparation. Cardiac arrest is recorded from ECG lead II.

Dogs and guinea pigs were used by Dörner (1955).

REFERENCES AND FURTHER READING

Bahrmann H, Greeff K (1981) Evaluation of cardiac glycosides in the intact animal. Handbook of Experimental Pharmacology 56/I: 118–152, Springer, Berlin, Heidelberg, New York

Burn JH, Finney DJ, Goodwin LG (1950) Digitalis, strophanthus, and squill. In: Biological Standardization, Chapter XIII, pp 294–310, Oxford University Press, London, New York, Toronto

Dörner J (1955) Zur Frage der Beziehungen zwischen Strophanthintoxicität und Größe der Coronardurchblutung. Arch exper Path Pharmakol 226:152–162

Hatcher RA, Brody JG (1910) The biological standardization of drugs. Am J Pharm 82:360–372

Knaffl-Lenz E (1926) The physiological assay of preparations of digitalis. J Pharmacol Exp Ther 29:407–425

Lind van Wijngaarden C de (1926) Untersuchungen über die Wirkungsstärke von Digitalispräparaten. II. Mitteilung: Über die Genauigkeit der Dosiseichung an der Katze. Arch exp Path Pharmak 113:40–58

A.7.3.2 Decay Rate and Enteral Absorption Rate of Cardiac Glycosides

PURPOSE AND RATIONALE

The basic principle of Hatcher's or Knaffl-Lenz's method is suitable to determine decay rates of cardiac glycosides. The decay of efficacy can by due to excretion or metabolic degradation of the glycoside.

PROCEDURE

Beagle dogs of either sex weighing 8–20 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The animal is fixed on its legs on a heated operating table. Tracheostomy is performed and a tracheal cannula inserted. The vena femoralis is cannulated for continuous infusion of a defined concentration (µg/kg/min) of the test compound. ECG is recorded from lead II. The signs of first toxic effects, e. g., extra systoles, AV-block) are recorded. At this time, the infusion is terminated and the total dose/kg of the applied glycoside registered. After 4, 8, 12, or 24 h the infusion procedure is repeated. Within this period of time the glycoside administered with the first dose is only partially metabolized or excreted. Therefore, the dose needed for observation of ECG changes during the second infusion will be lower than in the first experiment.

EVALUATION

The dose required in the second experiment for induction of ECG changes is equal to the amount of metabolized or excreted glycoside. This value is expressed as percentage of the amount required in the first experiment and indicates the decay rate of the glycoside. Testing after various time intervals, the decay rate can be visualized graphically and half life times be calculated.

MODIFICATIONS OF THE METHOD

Rhesus monkeys have been used since their response to cardiac glycosides is more similar to that of man than that of dogs (Lindner et al. 1979).

The basic principle of Hatcher's or Knaffl-Lenz's method is also suitable to determine *enteral absorption of cardiac glycosides*. Again, for this purpose dogs are preferred instead of cats or guinea pigs. The dose to induce cardiac arrest is determined in 3–6 dogs. To other dogs, the same test compound is given intraduodenally at a dose below the intravenous lethal dose. Ninety or 180 min afterwards, the intravenous infusion with the same infusion speed and the same concentration of the test compound as in the previous experiments is started and time until cardiac arrest determined. The higher the duodenal resorption of the compound, the lower the dose of the intravenous infusion will be. For evaluation, the intravenous dose needed in the second experimental series (with enteral predosing) is subtracted from the dose of the first series (without enteral predosing) and indicates the amount of absorbed compound. This value is expressed as percentage of the value of the first series and indicates the absorption rate.

The efficacy and safety of a novel Na^+,K^+-ATPase inhibitor has been tested in dogs with propranolol-induced heart failure by Maixent et al. (1992).

REFERENCES AND FURTHER READING

Bahrmann H, Greeff K (1981) Evaluation of cardiac glycosides in the intact animal. Handbook of Experimental Pharmacology 56/I:118–152, Springer, Berlin

Kleemann A, Lindner E, Engel J (1985) Herzglykoside und deren Aglykone. In: Arzneimittel, Fortschritte 1972–1985. pp 213–226. Verlag Chemie, Weinheim, Germany

Lindner E, Schöne HH (1972) Änderungen der Wirkungsdauer und Wirkungsstärke von Herzglykosiden durch Abwandlungen der Zucker. Arzneim Forsch/Drug Res 22:428–435

Lindner E, v. Reitzenstein G, Schöne HH (1979) Das 14,15-β-oxido-Analoge des Proscillaridins (HOE 040) Arzneim Forsch/Drug Res 29:221–226

Maixent JM, Bertrand IB, Lelièvre LG, Fénard S (1992) Efficacy and safety of the novel Na^+,K^+-ATPase inhibitor 20R 14β-amino 3β-rhamnosyl 5β-pregnan 20β-ol in a dog model of heart failure. Arzneim Forsch/Drug Res 42:1301–1305

A.8
Effects on Blood Supply and on Arterial and Venous Tonus

A.8.1
Models for Stroke and Multi-Infarct Cerebral Dysfunction

A.8.1.1
Cerebral Ischemia by Carotid Artery Occlusion in Mongolian Gerbils

PURPOSE AND RATIONALE

The Mongolian gerbil (*Meriones unguiculatus*) is extremely susceptible to carotid occlusion because of the peculiar anatomical occurrence of an incomplete circle of Willis without posterior communicating artery and a frequently rudimentary anterior communicating artery. Clamping of both carotid arteries induces a bilateral temporary brain ischemia (Levine and Sohn 1969; Bosma et al. 1981; Mršulja et al. 1983; Hossmann et al. 1983; Chandler et al. 1985). This pathological animal model allows the simulation of circulatory disturbances in the human brain. The hippocampus is one of the most vulnerable regions of the brain to ischemia and anoxia. The gerbil is known to develop selective neuronal damage in the CA1 sector of the hippocampus following brief periods of forebrain ischemia. This damage differs from conventionally described ischemic neuronal injury because of its slow development (Ito et al. 1975; Kirino 1982; Hossmann et al. 1983). The occlusion time can be varied allowing determination of various parameters, e. g. ischemia induced amnesia (see Sect. F.3.1.8).

PROCEDURE

Male Mongolian gerbils (strain: Hoe GerK jirds) weighing 48–88 g are randomly divided into groups (10–15 animals for each test and control group). Prior to testing, the animals are housed in a climate-controlled environment (21°C) with food and water available ad libitum. Fifteen minutes before surgery, the gerbils receive the test compound by oral or intraperitoneal administration. The control group is treated with vehicle alone.

The exposure of the common carotid arteries is performed under anesthesia with sodium pentobarbital (32 mg/kg i.p.), chloralhydrate (100 mg/kg i.p.) and atropine sulfate (0.8 mg/kg i.p.). The carotid arteries are isolated from surrounding tissue and a loop of unwaxed dental floss is placed around each artery. A 2 cm length of double lumen catheter is passed from the level of carotid artery through the muscle layers of the dorsal surface of the neck. Each end of dental floss is threaded through a separate lumen, leaving a loose loop around the artery. Two days later, occlusion of each artery is produced by gently pulling the dental floss until the artery is completely occluded between the floss and the center wall of the catheter. Heifitz clips are placed on the floss against the exterior end of the tubing to maintain occlusion. After various intervals (5 to 30 min), the clips are removed and circulation is restored.

Complete bilateral occlusion of the arteries is confirmed by behavioral symptoms, i. e., depression of spontaneous motor activity, shallow and rapid respiration, and ptosis. Care is taken to avoid a drop of body temperature during any stage of the experiment. After experimental manipulations, animals are placed on a heating pad until complete recovery of motor activity.

Subjects are placed in individual observation glasses which are kept at a temperature of 29°C. They are observed for neurological symptoms (such as circling behavior, jumping and rolling seizures, opisthotonus, tonic convulsions, etc.) for 90 min.

After various intervals, the gerbils are sacrificed and their brains are removed.

EVALUATION

The following parameters are measured two hours after occlusion:

Degree of brain edema: water content (difference in weight of wet and dry brain)

Content of sodium and potassium. The hemispheres are separated and put on pre-weighted watch glasses to determine the wet weight. Then the hemispheres are dried in an open Petri dish for 2 days at a temperature of 95°C. After cooling off, the dry weight is noted. Sodium and potassium concentrations in the dried brain hemispheres are determined by flame photometry. The Na^+/K^+-ratio is calculated.

For *histological examinations*, the animals are sacrificed at 2 or 4 days after ischemia under ether anesthesia by decapitation. The brains are then removed and frozen in CO_2. Hippocampi are sectioned coronally with a cryostat at –14°C. The section thickness for Nissl and glial fibrillary acid protein (GFAP) staining is 20 µm and for the histochemical localization of calcium 30 µm.

Nissl staining and its quantitative evaluation. The sections are mounted by thawing on glass slides, Nissl

stained, and cover slipped with Permount. In order to standardize the histological procedure and to rule out the possibility that differences in the staining intensity were due to technical inconsistency, slides from control and experimental groups are processed together, stained for 5 min, and differentiated in a series of alcohols for 3 min each. The extent of hippocampal nerve cell damage (as reflected by cell loss and decreased stainability) is assessed by measuring the amount of Nissl-stained material in a predetermined representative region of the CA1 area with the aid of a guided densitometer (Leitz Texture Analysis System). The measuring field is 50 × 500 µm, fitting to the width of the CA1 soma layer (about 40 µm).

Calcium localization. A modification of technique described by Kashiwa and Atkinson (1963) is used for the cytochemical localization of ionic calcium. The principle of the technique is that calcium complexes with a chelating agent producing an insoluble chromophore.

Stock solutions: Two solutions were used: a) glyoxalbis-(2-hydroxyanil) (GBHA), 0.4 g/100 ml absolute ethanol (2 ml 0.4% GBHA); and b) NaOH, 5 g/100 ml distilled water (0.3 ml 5% NaOH).

Staining procedure: First, cryostat sections are placed immediately in cold absolute acetone for rapid fixation for 5 min. Next, floating sections are transferred into 96% alcohol for 5 min and then transferred to staining solution for 3–4 min. Sections are then placed in 96% alcohol and mounted on glass slides. Because of quenching, it is necessary to view and photograph immediately.

GFAP (glial fibrillary acid proteinglial fibrillary acid protein) fluorescencemicroscopy. Following slide mounting, cryostat sections are fixed for 15 min in 3.5% formaldehyde solution in 0.01 M phosphate-buffered saline (PBS). The sections are incubated with mouse primary antibody against GFAP (Boehringer, Mannheim, FRG) for 30 min diluted 1:50 PBS. This antibody shows cross reactions also with GFAP from pigs and rats, indicating low species selectivity (Graber and Kreutzberg, 1986). The sections are rinsed in PBS and incubated to tetramethylrhodamine isothiocyanate (TRITC) specific for mouse immunoglobulin G (T-5393 from Sigma) diluted 1:50 in PBS. Control sections are incubated with PBS instead of primary antibody.

Measurements, expressed as extinction units per measuring field, are taken from three slides of each animal and averaged. Statistical analysis is done by Student's *t*-test.

MODIFICATIONS OF THE METHOD

"Sensitive" gerbils can be selected according to the method of Delbarre et al. (1988). In this method, pupil dilatation is obtained with atropine sulfate (1%) 20 min before anesthesia. Ocular fundus is examined with direct ophthalmoscope (Heine) before ligation and 5 min later. Only animals with an absence of retinal blood flow after ligation are considered as positive.

Using ^{31}P nuclear magnetic resonance spectroscopy, Sasaki et al. (1989) studied energy metabolism of the ischemic brain of gerbils *in vivo*.

An unanesthetized-gerbil model of cerebral ischemia was described by Chandler et al. (1985).

Using microdialysis, adenosine and its metabolites were measured directly in the brain of male gerbils by Dux et al. (1990). Two microdialysis probes (CMA/10, Carnegie Medicine, Sweden) were implanted stereotactically in the brain of the animals, one in the left dorsal hippocampus and one in the right striatum. The fibres were fixed to the cranium using dental wax. The dialysate was collected in 5 min intervals, and the concentrations of adenosine, hypoxanthine and inosine were determined by HPLC (Zetterström et al. 1982; Fredholm et al. 1984).

Kindy et al. (1992) measured glial fibrillary acid protein and vimentine on mRNA level by Northern blot analysis and protein content by immunoblot analysis in the gerbil neocortex, striatum and hippocampus after transient ischemia.

McRae et al. (1994) studied the effect of drug treatment on activated microglial antigens in hippocampal sections after ischemia in gerbils with cerebral fluid from patients with Alzheimer's disease and the amyloid precursor protein.

Nurse and Corbett (1996) found neuroprotection in gerbils with global cerebral ischemia after several days of mild, drug-induced hypothermia. The protection by the AMPA-antagonist NBQX may be due to a decrease in body temperature. A protracted period of subnormal temperature during the postischemic period can obscure the interpretation of preclinical studies.

REFERENCES AND FURTHER READING

Bosma HJ, Paschen W, Hossman KA (1981) Cerebral ischemia in gerbils using a modified vascular occlusion model. In: Meyer JS, Lechner H, Reivich M, Ott EO, Aranibar A (1981) Cerebral Vascular Disease 3, Excerpta Medica, Amsterdam, pp 280–285

Chandler MJ, DeLeo J, Carney JM (1985) An unanesthetized-gerbil model of cerebral ischemia-induced behavioral changes. J Pharmacol Meth 14:137–146

Delbarre G, Delbarre B, Barrau Y (1988) A suitable method to select gerbils with incomplete circle of Willis. Stroke 19:126

DeLeo J, Toth L, Schubert P, Rudolphi K, Kreutzberg GW (1987) Ischemia-induced neuronal cell death, calcium accumulation, and glial response in the hippocampus of the Mongolian gerbil and protection by propentofylline (HWA 285). J Cerebr Blood Flow Metab 7:745–751

Dux E, Fastbo J, Ungerstedt U, Rudolphi K, Fredholm BB (1990) Protective effect of adenosine and a novel xanthine derivative propentofylline on the cell damage after bilateral carotid occlusion in the gerbil hippocampus. Brain Res 516:248–256

Fredholm BB, Dunwiddie TV, Bergman B, Lindström K (1984) Levels of adenosine and adenine nucleotides in slices of rat hippocampus. Brain Res 295:127–136

Graeber MB, Kreutzberg GW (1986) Astrocytes increase in glial fibrillary acidic protein during retrograde changes of facial motor neurons. J Neurocytol 15:363–373

Hossman KA, Mies G, Paschen W, Matsuoka Y, Schuier FJ, Bosma HJ (1983) Experimental infarcts in cats, gerbils and rats. In: Stefanovich V (ed) Stroke: Animal Models, Pergamon Press; Oxford, New York, pp 123–137

Ito M, Spatz M, Walker JT, Klatzo I (1975) Experimental cerebral ischemia in Mongolian gerbils. 1. Light microscopic observations. Acta neuropathol (Berl) 32:209–223

Kashiwa HK, Atkinson WG (1963) The applicability of a new Schiff base glyoxal bis (2-hydroxy-anil), for the cytochemical localization of ionic calcium. J Histochem Cytochem 11:258–264

Kindy MA, Bhat AN, Bhat NR (1992) Transient ischemia stimulates glial fibrillary acid protein and vimentine gene expression in the gerbil neocortex, striatum and hippocampus. Mol Brain Res 13:199–206

Kirono T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57–69

Levine S, Sohn D (1969) Cerebral ischemia in infant and adult gerbils. Arch Pathol 87:315–317

Lundy EF, Solik BS, Frank RS, Lacy PS, Combs DJ, Zelenok GB, D'Alecy LG (1986) Morphometric evaluation of brain infarcts in rats and gerbils. J Pharmacol Meth 16:201–214

McRae A, Rudolphi KA, Schubert P (1994) Propentofylline depresses amyloid and Alzheimer's CSF antigens after ischemia. Neuro Report 5:1193–1196

Mršulja BB, Micíc DV, Djuricic BM (1983) Gerbil stroke model: an approach to the study of therapeutic aspects of postischemic brain edema. In: Stefanovich V (ed) Stroke: Animal Models, Pergamon Press; Oxford, New York, pp 45–62

Nurse S, Corbett D (1996) Neuroprotection after several days of mild, drug-induced hypothermia. J Cerebr Blood Flow Metab 16:474–480

Rudolphi KA, Keil M, Hinze HJ (1987) Effect of theophylline on ischemically induced hippocampal damage in Mongolian gerbils: A behavioral and histopathological study. J Cerebr Blood Flow Metab 7:74–81

Sasaki M, Naritomi H, Kanashiro M, Nishimura H, Sawada T (1989) Effects of propentofylline on energy metabolism of the ischemic brain studied by *in vivo* ^{31}P nuclear magnetic resonance spectroscopy. Arzneim Forsch/Drug Res 39:886–889

Zetterström T, Vernet L, Ungerstedt U, Tossman U, Jonzon B, Fredholm BB (1982) Purine levels in the intact rat brain. Studies with an implanted hollow fibre. Neurosci Lett 29:111–115

A.8.1.2 Forebrain Ischemia in Rats

PURPOSE AND RATIONALE

Smith et al. (1984) described a model of forebrain ischemia in rats induced by transient occlusion of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. This method has been used extensively (Nuglisch et al. 1990; Oberpichler et al. 1990; Rischke and Krieglstein 1990; Krieglstein and Peruche 1991; Nuglisch et al. 1991; Prehn et al. 1991; Rischke and Krieglstein 1991; Seif el Nasr et al. 1992; Peruche et al. 1995).

PROCEDURE

Male Wistar rats weighing 250–300 g are anesthetized with 3.5% halothane and then connected to a Starling type respirator delivering 0.8% halothane and 30% O_2 in N_2O. The jugular vein and the tail artery are catheterized for withdrawal of blood and for monitoring blood pressure. Anticoagulation is achieved by intravenous heparin (200 IU/kg) application. Blood gases, blood pH, blood pressure, and blood glucose are measured 5 min prior to ischemia and 10 min after ischemia.

Halothane, but not N_2O, is discontinued and the rats are allowed to recover for 30 min. During this period, muscle paralysis is maintained with 5 mg/kg suxamethonium chloride, repeated every 15 min. After injection of trimethaphan camphor sulfonate (5 mg/kg), forebrain ischemia is induced by clamping of both carotid arteries and exsanguination to a blood pressure of 40 mm Hg. To prevent decay of intra-ischemic brain temperature, the environmental temperature is adjusted to 30°C by means of a infrared heating lamp. After 10 min of ischemia, the carotid clamps are removed and blood pressure is restored by re-infusing the shed blood. To minimize systemic acidosis, the rats receive intravenously 50 mg/kg $NaHCO_3$. The animals are removed from the respirator when they regain spontaneous respiration.

EVALUATION

Various parameters are used to evaluate the consequences of transient forebrain ischemia and the effectiveness of drug treatment:

- Local cerebral blood flow determination with the [^{14}C]iodoantipyrine method (Sakurada et al. 1978),
- Histological assessment of ischemic cell damage in the hippocampus on day 7 after ischemia (Seif el Nasr et al. 1990; Nuglisch et al. 1990),

- Local cerebral glucose utilization using the [^{14}C]deoxyglucose method described by Sokoloff et al. (1977),
- Quantitative analysis of the electrocorticogram (Peruche et al. 1995).

MODIFICATIONS OF THE METHOD

Kochhar et al. (1988) used two focal cerebral ischemia models in **rabbits**: a multiple cerebral embolic model by injection of microspheres into the internal carotid circulation and a spinal chord ischemia model by occluding the aorta for predetermined periods.

Gilboe et al. (1965) described the isolation and mechanical maintenance of the **dog** brain.

Andjus et al. (1967) and Krieglstein et al. (1972) described the preparation of the isolated perfused rat brain for studying effects on cerebral metabolism.

A cerebral ischemia model with conscious **mice** was described by Himori et al. (1990).

REFERENCES AND FURTHER READING

Andjus RK, Suhara K, Sloviter HA (1967) An isolated, perfused rat brain preparation, its spontaneous and stimulated activity. J Appl Physiol 22:1033–1039

Gilboe DD, Cotanch WW, Glover MB (1965) Isolation and mechanical maintenance of the dog brain. Nature 206:94–96

Himori N, Watanabe H, Akaike N, Kurasawa M, Itoh J, Tanaka Y (1990) Cerebral ischemia model with conscious mice. Involvement of NMDA receptor activation and derangement of learning and memory ability. J Pharmacol Meth 23:311–327

Kochhar A, Zivin JA, Lyden PD, Mazzarella V (1988) Glutamate antagonist therapy reduces neurologic deficits produced by focal central nervous system ischemia. Arch Neurol 45:148–153

Krieglstein G, Krieglstein J, Stock R (1972) Suitability of the isolated perfused rat brain for studying effects on cerebral metabolism. Naunyn-Schmiedeberg's Arch Pharmacol 275:124–134

Krieglstein J, Peruche B (1991) Pharmakologische Grundlagen der Therapie der zerebralen Ischämie. Arzneim Forsch/Drug Res 31:303–309

Nuglisch J, Karkoutly C, Mennel HD, Roßberg C, Krieglstein J (1990) Protective effect of nimodipine against neuronal damage in rat hippocampus without changing postischemic cerebral blood flow. J Cerebr Blood Flow Metab 10:654–659

Nuglisch J, Rischke R, Krieglstein J (1991) Preischemic administration of flunarizine or phencyclidine reduces local cerebral glucose utilization in rat hippocampus seven days after ischemia. Pharmacology 42:333–339

Oberpichler H, Sauer D, Roßberg C, Mennel HD, Krieglstein J (1990) PAF antagonist ginkgolide B reduces postischemic neuronal damage in rat brain hippocampus. J Cerebr Blood Flow Metab 10:133–135

Peruche B, Klaassens H, Krieglstein J (1995) Quantitative analysis of the electrocorticogram after forebrain ischemia in the rat. Pharmacology 50:229–237

Prehn JHM, Backhauß C, Karkoutly C, Nuglisch J, Peruche B, Roßberg C, Krieglstein J (1991) Neuroprotective properties of 5-HT$_{1A}$ receptor agonists in rodent models of focal and global cerebral ischemia. Eur J Pharmacol 203:213–222

Rischke R, Krieglstein J (1990) Effects of Vinpocetin on local cerebral blood flow and glucose utilization seven days after forebrain ischemia in the rat. Pharmacology 41:153–160

Rischke R, Krieglstein J (1991) Postischemic neuronal damage causes astroglial activation and increase in local cerebral glucose utilization in rat hippocampus. J Cerebr Blood Flow Metab 11:106–113

Sakurada O, Kennedy C, Jehle J, Brown JD, Carbin O, Sokoloff L (1978) Measurement of local cerebral blood flow with iodo[^{14}C]-antipyrine. Am J Physiol 234:H59–H66

Seif el Nasr M, Peruche B, Roßberg C, Mennel HD, Krieglstein J (1990) Neuroprotective effect of memantine demonstrated *in vivo* and *in vitro*. Eur J Pharmacol 185:19–24

Seif el Nasr M, Nuglisch J, Krieglstein J (1992) Prevention of ischemia-induced cerebral hypothermia by controlling the environmental temperature. J Pharmacol Meth 27:23–26

Smith ML, Auer RN, Siesjö BK (1984a) The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. Acta Neuropathol (Berl) 64:319–332

Smith ML, Bendek G, Dahlgren N, Rosén I, Wieloch T, Siesjö BK (1984b) Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. Acta Neurol Scand 69:385–401

Sokoloff L, Reivich M, Kennedy C, DesRosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M (1977) The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 28:897–916

A.8.1.3 Hypoxia Tolerance Test in Rats

PURPOSE AND RATIONALE

The electrical activity of the brain is dependent on a continuous energy supply. Hypoxia is induced in test animals by inhalation of nitrogen. Marked hypoxia depresses cerebral metabolism resulting in an electrical failure of the brain. The procedure is used to investigate the ability of test compounds to antagonize the hypoxia-induced electrical failure of the brain by measuring the hypoxia-tolerance and the EEG recovery.

PROCEDURE

Male Sprague Dawley rats or stroke-prone rats weighing 250–300 g are used. They are anesthetized with hexobarbital sodium (100–120 mg/kg, i.p) and surgically implanted with 2 epidural EEG-electrodes and a reference electrode to the parietal frontal cortex. After a minimum of a one week recovery period, testing can be started. The rats receive the test compound by intravenous or intraperitoneal administration. The control group is treated with vehicle alone. Thirty to 60 min after i.p. administration (immediately after i.v. administration) the animals are anaesthetized by

hexobarbital sodium at 100–120 mg/kg, i.p. When the stage of surgical anesthesia is reached, the animals are placed in a hypoxia chamber. EEG and ECG are recorded using a Hellige recorder.

Hypoxia is induced by inhalation of nitrogen (1200 l/h). On reaching an isoelectric EEG, the nitrogen inhalation is terminated and the animals are allowed to breath room air. The recording is continued until EEG potentials can again be registered.

EVALUATION

The following parameters are measured in the test and control groups

- Hypoxia tolerance (HT): The time from the start of nitrogen inhalation until the onset of isoelectric EEG.
- Latency of EEG recovery: The time from the end of nitrogen inhalation until the onset of EEG potentials.
- The values of test and control groups are compared.

The percent change is calculated.
Statistics: Student's *t*-test by unpaired comparison.

REFERENCES AND FURTHER READING

Hossman KA, Mies G, Paschen W, Matsuoka Y, Schuier FJ, Bosma HJ (1983) Experimental infarcts in cats, gerbils and rats. In: Stefanovich V (ed) Stroke: Animal Models, Pergamon Press; Oxford, New York, pp 123–137

A.8.1.4 Middle Cerebral Artery Occlusion in Rats

PURPOSE AND RATIONALE

The permanent middle cerebral artery (MCA) occlusion technique in rats has been widely employed to evaluate various kinds of neuroprotective agents in cerebral ischemia (Ginsberg and Busto 1989). Focal cerebral infarction is achieved using a modification of the MCA occlusion model described by Tamura et al. (1981) and Pak et al. (1992).

PROCEDURE

Adult male Sprague-Dawley rats weighing 300–400 g are used in the experiments. The animals are anesthetized with a nitrous oxide-oxygen mixture (70:30%) containing 1.0 to 1.2% halothane. A mask is put on the nose and self-respiration is maintained during surgery, which takes around 20 to 30 min. The right femoral artery and vein are catheterized for monitoring blood pressure, blood sampling and administration of the drug. Left MCA occlusion is performed via a subtemporal approach without removal of zygomatic arch or temporal muscle. Under high magnification of a surgical microscope, the left MCA is coagulated with a microbipolar coagulator from the olfactory tract to the most proximal portion of the MCA through a cranial window, about 3 to 4 mm in diameter, and cut afterward. Anesthesia is stopped just after MCA occlusion. The arterial catheter is removed soon after MCA occlusion, but the venous catheter is maintained for constant infusion of the drug by a swivel system (Harvard Apparatus, UK.). The animals are maintained normothermic (37°C) by a homeothermic system (Homeothermic Blanket System, Harvard Apparatus, UK.). During the surgery, mean arterial blood pressure (MABP) is recorded continuously (Model 7E polygraph, Grass, USA). Arterial gases and pH (178 pH/blood gas analyzer, Corning, USA) as well as hematocrit and blood glucose are measured twice, once about 15 min before MCA occlusion and the other just following MCA occlusion. Bilateral temporal muscle temperature (Therm 2250–1, Ahlborn Mess- und Regelungstechnik, Germany) is monitored during the surgery and several minutes before sacrifice.

Two, six, twelve and twenty-four hours after discontinuing anesthesia, each rat's level of consciousness and motor activity are evaluated using a grading scale of 0 to 3 (0: normal activity; 1: spontaneous activity+/–; 2: not arousable by tactile stimulation; 3: not arousable by painful stimulation, no spontaneous activity). Immediately before sacrifice, neurological status is examined using a grading scale of 0 to 3 (0: no observable deficit; 1: forelimb flexion; 2: decreased resistance to lateral push without circling; 3: the same status as grade 2, with circling). Four groups of rats are studied: vehicle-treated controls, and drug-treated animals at 3 different doses.

All the rats are re-anesthetized 24 h after MCA occlusion and sacrificed by intravenous injection of KCl. Immediately after sacrifice the brain is removed and frozen at –10°C for 10 min. The forebrain is cut into eight coronal slices by a rat brain slicer which are processed with the tetrazolium chloride (TTC) emulsion technique. Areas of brain not stained by TTC are drawn on a diagram at 8 preselected coronal levels of forebrain without knowledge of the experimental treatment. The areas of ischemic damage in the cerebral hemisphere, cerebral cortex, and caudate nucleus, drawn in the diagram are measured with a planimeter (KP-21, Koizumi, Japan) and integrated to determine the volumes.

REFERENCES AND FURTHER READING

Significance of the differences between the control and the treated groups is assessed by analysis of variance with subsequent inter group comparison by Student's *t*-test with Bonferroni correction. $P < 0.05$ is required for significance.

MODIFICATIONS OF THE METHOD

Hossmann (1982) reviewed the experimental models of cerebral ischemia.

Yamamoto et al. (1992) studied the inhibition of NO biosynthesis on the volume of focal ischemic infarction produced by occlusion of the middle cerebral artery in spontaneously hypertensive rats.

Shigeno et al. (1985) described a recirculation model following middle cerebral artery occlusion in rats. The trunk of the middle cerebral artery was isolated between the rhinocortical branch and the lenticulostriate artery and encircled with a loose-fitting suture of nylon thread. The thread was exteriorized through a small polyethylene catheter, which was previously introduced into the craniectomy site through a burr hole in the zygoma. The artery was occluded by retraction of the thread, which was then fixed with biological glue. Recirculation was achieved by cutting and removing the thread.

Bederson et al. (1986a) occluded the middle cerebral artery at different sites in six groups of normal rats and characterized the anatomical sites that produce uniform cerebral infarction. A neurological system was developed that can be used to evaluate the effects of cerebral ischemia.

Bederson et al. (1986b) evaluated the use of 2,3,5-triphenyltetrazolium chloride as a histopathological stain for identification and quantification of infarcted brain tissue after middle cerebral artery occlusion in rats.

Yang et al. (1992) found a reduction of Na,K-ATPase activity in the ischemic hemisphere shortly after middle cerebral artery occlusion in rats.

Du et al. (1996) induced transient focal cerebral ischemia in rats by a 90 min period of ligation of the right middle cerebral artery and both common carotid arteries.

Germano et al. (1987) found a decrease of stroke size and deficits in rats with middle cerebral artery occlusion after treatment with kynurenic acid, a broad spectrum antagonist of excitatory amino acid receptors.

Wu et al. (1999) reported that propentofylline attenuates microglial reaction in the rat spinal cord induced by middle cerebral artery occlusion.

Gotti et al. (1988) found a reduction of the volume of infarcted tissue due to occlusion of the middle cerebral artery in **rats** and **cats** after administration of NMDA receptor antagonists.

Hossmann and Schuier (1980) studied experimental brain infarcts in **cats** after occlusion of the left middle cerebral artery.

Retro-orbital occlusion of the middle cerebral artery in cats was performed by Sundt and Waltz (1966) and by Gotti et al. (1988).

Welsh et al. (1987), Backhauß et al. (1992) described focal cerebral ischemia in **mice** after permanent occlusion of the middle cerebral artery.

Hara et al. (1997) found a reduction of ischemic and excitotoxic neuronal damage by inhibition of interleukin 1β-converting enzyme family proteases after occlusion of the middle cerebral artery in **mice** and **rats**. Nylon filaments were introduced from the carotid artery which were withdrawn after 2 h. One day later, the animals were tested for neurological deficits and the brains analyzed for infarct size and interleukin 1β levels.

Huang et al. (1994) produced **knock-out mice** deficient in the neuronal isoform of NO synthase by targeted disruption of the neuronal nitric oxide synthase gene. In these mice, Hara et al. (1996) found reduced brain edema and infarction volume after transient middle coronary artery occlusion.

Nishimura et al. (1998) described an experimental model of thromboembolic stroke without craniotomy in **cynomolgus monkeys** by delivering an autologous blood clot to the middle cerebral artery. A chronic catheter was implanted in the left carotid artery in male cynomolgus monkeys. A 5 cm long piece of an autologous blood clot was flushed into the internal carotid artery with physiological saline. A neurological score was assigned at 0.167, 0.5, 1, 2, 4, 6, and 24 h after embolization. In the acute phase after embolization, typical behavior consisted of circling gait and moderate deviation towards the side of embolization, long-lasting and strong extensor hypotonia of the contralateral lower and upper limbs, and mild to severe incoordination. Contralateral hemiplegia was observed over the following 24 h. At 24 h the animals were sacrificed immediately after the last neurological scoring, and the cerebral vasculature was inspected for the location of the clot. The brain was then cut into 2 mm thick coronal sections. Cerebral infarction size and location were ascertained by the triphenyl-2H-tetrazolium chloride staining method. The lesions involved mostly the caudate nucleus, internal capsule, putamen and thalamus.

Salom et al. (1999) subjected female **goats** to 20 min global cerebral ischemia under halothane/N_2O anesthesia. An episode of transient global ischemia was achieved by occlusion of the two external carotid arteries and simultaneous external compression of the jugular veins by a neck tourniquet. A reperfusion period started when the occlusions were released, and it was monitored for 2 h.

De Ley et al. (1988) studied experimental thromboembolic stroke induced by injection of a single autologous blood clot into the internal cerebral artery in dogs by**positron emission tomography**.

REFERENCES AND FURTHER READING

Backhauß C, Karkoutly C, Welsch M, Krieglstein J (1992) A mouse model of focal cerebral ischemia for screening neuroprotective drug effects. J Pharmacol Meth 27:27–32

Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H (1986a) Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. Stroke 17:472–476

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986b) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats.Stroke 17:1304–1308

De Ley G, Weyne J, Demeester G, Stryckman K, Goethals P, Van de Velde E, Leusen I (1988) Experimental thromboembolic stroke studied by positron emission tomography: immediate versus delayed reperfusion by fibrinolysis. J Cerebr Blood Flow Metab 8:539–545

Du C, Hu R, Csernansky CA, Liu XZ, Hsu CY, Choi DW (1996) Additive neuroprotective effects of dextrorphan and cycloheximide in rats subjected to transient focal cerebral ischemia. Brain Res 718:233–236

Germano IM, Pitts LH, Meldrum BS, Bartkowski HM, Simon RP (1987) Kynurenate inhibition of cell excitation decreases stroke size and deficits. Ann Neurol 22:730–734

Ginsberg MD, Busto R (1989) Rodent models of cerebral ischemia. Stroke 20:1627–1642

Gotti B, Duverger G, Bertin J, Carter C, Dupont R, Frost J, Gaudilliere B, MacKenzie ET, Rousseau J, Scatton B, Wick A (1988) Ifenprodil and SL 82.0715 as cerebral antiischemic agents. I. Evidence for efficacy in models of focal cerebral ischemia. J Pharm Exp Ther 247:1211–1221

Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz MA (1996) Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. J Cerebr Blood Flow Metab 16:605–611

Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA (1997) Inhibition of interleukin 1β converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci USA 94:2007–2012

Hossmann KA (1982) Treatment of experimental cerebral ischemia. J Cerebr Blood Flow Metab 2:275–297

Hossmann KA, Schuier FJ (1980) Experimental brain infarcts in cats. I. Pathophysiological observations. Stroke 11:583–592

Huang PL, Dawson PM, Bredt DS, Snyder SH, Fishman MC (1994) Targeted disruption of the neuronal nitric oxide synthase gene. Cell 75:1273–1286

Nsihimura A, Hamada T, Fukuzaki K, Miyajima H, Nagata R, Kito G (1998) A new model of experimental thromoboembolic stroke in cynomolgus monkey. Naunyn-Schmiedeberg's Arch Pharmacol 358, Suppl 1:R70

Park CK, Rudolphi KA (1994) Antiischemic effects of propentofylline (HWA 285) against focal cerebral infarction in rats. Neurosci Lett 178:235–238

Park CK, McCulloch J, Kang JK, Choi CR (1992) Efficacy of D-CPPene, a competitive *N*-methyl-D-aspartate antagonist in focal cerebral ischemia in the rat. Neurosci Lett 147:41–44

Salom JB, Barberá MD, Centeno JM, Ortí M, Torregrosa G, Alborch E (1999) Relaxant effects of sodium nitroprusside and NONOates in goat middle cerebral artery: Delayed impairment by global ischemia-reperfusion. Nitric Oxide: Biol Chem 3:85–93

Shigeno T, Teasdale GM, McCulloch J, Graham DI (1985) Recirculation model following MCA occlusion in rats. Cerebral blood flow, cerebrovascular permeability, and brain edema. J Neurosurg 63:272–277

Smith SE, Meldrum BS (1992) Cerebroprotective effect of a non-N-methyl-D-aspartate antagonist, GYKI 52466, after focal ischemia in the rat. Stroke 23:861–864

Sundt TM, Waltz AG (1966) Experimental cerebral infarction: Retro-orbital, extradural approach for occluding the middle cerebral artery. Mayo Clin Proc 41:159–168

Tamura A, Graham DI, McCulloch J, Teasdale GM (1981) Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J Cerebr Blood Flow Metab 1:53–60

Welsh FA, Sakamoto T, McKee AE, Sims RE (1987) Effect of lactacidosis on pyridine nucleotide stability during ischemia in mouse brain. J Neurochem 49:846–851

Wu YP, McRae A, Rudolphi K, Ling EA (1999) Propentofylline attenuates microglial reaction in the rat spinal cord induced by middle cerebral artery occlusion. Neurosci Lett 280:17–20

Yamamoto S, Golanov EV, Berger SB, Reis DJ (1992) Inhibition of nitric oxide synthesis increases focal ischemic infarction in rat. J Cerebr Blood Flow Metab 12:717–726

Yang GY, Chen SF, Kinouchi H, Chan PH, Weinstein PR (1992) Edema, cation content and ATPase activity after middle cerebral artery occlusion in rats. Stroke 23:1331–1336

A.8.1.5 Photochemically Induced Focal Cerebral Ischemia in Rats

PURPOSE AND RATIONALE

Focal cerebral ischemia in rats can be induced by irradiation with intensive green light via a fibre optic through the skull after injection of the dye Rose Bengal. The resulting cerebral infarct can be studied for various parameters, such as infarct volume, water content, local cerebral blood flow and glucose utilization.

PROCEDURE

Male Sprague-Dawley rats (300–350 g) are used. Anesthesia is induced with 3% halothane in oxygen and is maintained with 1% halothane in oxygen applied via a face mask. A small incision is made in the skin over the right femoral vein and a thin catheter is

inserted. One ml of the dye Rose Bengal (in a concentration of 5 mg/ml of saline) is injected. A midline head incision is made and the right side of the skull is exposed. An intense green light, produced by a xenon lamp (75W, Zeiss, FRG) and then passed through a filter (wavelength 570 nm, Schott, Mainz, FRG) and a heat filter (Schott, Mainz, FRG), is directed on the skull at the level of the bregma for 15 min. The 3 mm diameter of the illuminated circle is determined by passing the light through a fibre optic (Schott, Mainz, FRG) while in close contact to the skull. The temperature of the skull underneath the fibre optic does not change during the time of illumination. At the end of the induction period, the temporary catheter is removed from the femoral vein which remains patent following the closure of the catheterization site with liquid suture (Histoacryl, Braun, Melsungen, FRG), and the incisions in the leg and head are likewise sutured after liberal application of local anesthetic. The anesthetic gas mixture is discontinued and the rats are allowed to recover consciousness in a warm environment until such times as the appropriate experiment is to be performed. Due to the non-invasive nature of this technique it is not possible to measure blood pressure or blood gases during the ischemic period. However, rectal temperature and plasma glucose concentrations are controlled.

Measurement of Infarct Volume

Osmotically controlled mini-pumps (Model 2ML1 Alzet, USA) are placed into the peritoneum of two groups of six male Sprague Dawley rats (body weight 300–350 g). The mini-pumps are fitted via thin polyethylene catheters to the femoral vein of the rats. Each pump contains either 2 ml of physiological saline or 2 ml of a solution of the drug to be studied. The animals are then given an ischemic insult as described in the previous section. Seventy-two hours after the induction of ischemia, the rats are sacrificed by decapitation and the brains removed and frozen at –50°C. Coronal sections (20 µ) are cut in a cryotome at –20°C, fixed in Haidenhain's Susa and stained with Cresyl Violet. The ischemic area on 90 sections is measured and the volume of ischemic change is then calculated using a linear trapezoidal extrapolation of the areas measured.

Measurement of Brain Water Content

Three groups each of six rats are used. One group receives no ischemic lesion (the illumination with green light is omitted from the experimental protocol) and two groups are lesioned as described above. One lesioned group receives the test drug orally at 15 min, 30 min, 1, 3 and 5 h after the induction of the ischemia or sham operation. The other group is treated with saline at the same time-points. Twenty-four hours later the rats are sacrificed by decapitation. The brains are rapidly removed and placed on a cutting block with 1 mm gradations. Two cuts are made 1 mm or less anterior and posterior to the lesion. The thick section so produced is then divided into left (non-lesioned) and right (lesioned) halves and placed in pre-weighed vials and the wet weights of the tissue samples are carefully measured. The tissue is then frozen in liquid nitrogen and then left under vacuum (less than 0.1 Torr) for 24 h. On removal the vials are sealed to prevent rehydration and reweighed to obtain the dry weight of tissue from which the water content (expressed as percentage of wet weight of tissue) is calculated.

EVALUATION

All data are presented as mean ± SD of the mean. For left (contralateral to the lesion) against right (ipsilateral to the lesion) comparisons, a *t*-test with paired comparison was used ($P < 0.05$). Statistical differences between groups were calculated using the unpaired *t*-test.

MODIFICATIONS OF THE METHOD

Boquillon et al. (1992) produced cerebral infarction in mice by intravenous injection of 10 mg/kg rose bengal, and by focal illumination of the intact skull surface for 3 min with a laser source, operating at 570 nm with power levels of 2, 5, 10, and 20 mW.

Matsuno et al. (1993) used a similar model to induce cerebral ischemia in rats based on middle cerebral artery occlusion by the photochemical reaction of rose bengal after irradiation with high intensity green light.

Stieg et al. (1999) studied neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. An excellent correlation between infarct size determined by magnetic resonance imaging (MRI) and histopathological analysis in the same animals was found.

REFERENCES AND FURTHER READING

Boquillon M, Boquillon JP, Bralet J (1992) Photochemically induced, graded cerebral infarction in the mouse by laser irradiation. Evolution of brain edema. J Pharm Meth 27:1–6

Grome JJ, Gojowczyk G, Hofmann W (1990) Effect of chronic intravenous administration of propentofylline (HWA 285) on the volume of focal ischemic damage in the rat. Stroke 21, Suppl I:I-134–I-135, PS-12–11

Matsuno H, Uematsu T, Umemura K, Tagiguchi Y, Asai Y, Murakana Y, Nakashima M (1993) A simple and reproducible cerebral thrombosis model in rats induced by a photochemical reaction and the effect of a plasmin-plasminogen activator chimera in this model. J Pharm Toxicol Meth 29:165–173

Stieg PE, Sathi S, Warach S, Le DA, Lipton SA (1999) Neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. Eur J Pharmacol 375:115–120

A.8.1.6 Microdialysis and Neuroprotection Experiments After Global Ischemia in Rats

PURPOSE AND RATIONALE

Transient global ischemia can be induced in rats by electrocauterization of the vertebral arteries followed by clamping of the carotid arteries.

PROCEDURE

Male Wistar rats weighing 280–340 g are used. The rats are anesthetized with methohexital sodium (60 mg/kg i.v.) and the vertebral arteries are electrocauterized (Pulsinelli and Brierley 1979). The rats are fasted overnight and re-anesthetized on the following day with halothane and intubated. The femoral artery and vein are cannulated to allow blood sampling, blood withdrawal and recording of mean arterial pressure (MAP). Samples of blood are taken at regular time intervals and blood gas/acid base status is analyzed (Instrumentation Laboratory, 1306). Rectal temperature is measured with a thermistor and controlled at 37°C by means of a heating lamp. Four-vessel occlusion ischemia is induced for 20 min by bilateral carotid clamping followed by a period of reflow.

Microdialysis Experiments

The head of the rat is fixed in a stereotactic frame. The skin is incised over the head, pulled apart and a 3 × 3 mm hole is drilled through the cranium. A microdialysis probe (Sandberg et al. 1986) is implanted into CA1 region of the hippocampus (2.2 mm lateral and 3.8 mm dorsal to bregma, and the window of the dialysis membrane 1.4–2.9 mm below the cortical surface). The electroencephalogram (EEG) is measured continuously with a tungsten electrode attached to the dialysis probe. The probe is perfused at a rate of 2.5 µl/min with a modified Ringer solution. Dialysates are sampled every 10 min and analyzed for purines (Hagberg et al. 1987) and amino acids (Lindroth et al. 1985). One group receives the test drug i.p. 15 min prior to ischemia whereas the control group obtains saline. The animals are followed during 20 min of ischemia and 2 h of reflow.

HPLC analyses are carried out using a reverse-phase C_{18} column (Waters 10 µm µBondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM $NH_4H_2PO_4$, pH 6.0, 13% methanol.

Neuroprotection Experiments

During ischemic insult and for 20 min of reflow, the temperature of the temporalis muscle is controlled at 37°C. Immediately following ischemia the rats are divided into two groups. One group is treated with the test drug and the other with saline. A bolus injection is administered i.p. 15 min after ischemia and a mini-osmotic pump is implanted into the abdominal cavity which delivers the test drug for 7 days. Control animals receive a bolus of saline and mini-osmotic pumps filled with saline. Seven days later the rats are anesthetized with pentobarbital and perfusion-fixed with formol saline. The histological evaluation is done "blind". The hippocampal damage is semiquantified according to the following scoring system:

0 = no damage
1 = scanty damage,
2 = moderate damage
3 = severe damage
4 = complete loss of pyramidal cells in the hippocampus.

EVALUATION

The purine and amino acid data are expressed as means ± SEM and differences are evaluated with the non-parametric Mann-Whitney test. The neuroprotective efficacy of the test drug is evaluated with two-tailed Student's *t*-test.

MODIFICATIONS OF THE TEST

In addition to the assessment of neuronal damage, Block et al. (1996) tested spatial learning of treated rats one week after 4-vessel occlusion in a Morris water maze.

REFERENCES AND FURTHER READING

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43:1396–1374

Block F, Schmitt W, Schwarz M (1996) Pretreatment but not post-treatment with GYKI 52466 reduces functional

deficits and neuronal damage after global ischemia in rats. J Neurol Sci 139:167–172
Choi DW (1990) Methods for antagonizing glutamate neurotoxicity. Cerebrovasc Brain Metab 2:105–147
Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M (1987) Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. J Neurochem 49:227–231
Hagberg H, Andiné P, Fredholm B, Rudolphi K (1990) Effect of the adenosine uptake inhibitor propentofylline on the extracellular adenosine and glutamate and evaluation of its neuroprotective efficacy after ischemia in neonatal and adult rats. In: Krieglstein J, Oberpichler (eds) Pharmacology of Cerebral Ischemia. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, pp 427–437
Lindroth P, Hamberger A, Sandberg M (1985) Liquid chromatographic determination of amino acids after precolumn fluorescence derivatization. In: Boulton AA, Baker GB, Wood JD (eds) Neuromethods, Vol 3, Amino acids. HUMANA Press Inc., pp 97–116
Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10:267–272
Sandberg M, Butcher SP, Hagberg H (1986) Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: *In vivo* dialysis of the rat hippocampus. J Neurochem 47:178–184

A.8.1.7 Hypoxia/Hypoglycemia in Hippocampal Slices

PURPOSE AND RATIONALE

The *in vitro* release of adenosine, inosine, and hypoxanthine from rat hippocampal slices can be determined with and without drug.

PROCEDURE

Male Sprague-Dawley rats weighing 150–275 g are decapitated, the hippocampi isolated and cut into 400 µm thick slices which are placed into KRB (4°C) containing (mM) NaCl (118), KCl (4.85), $MgSO_4$ (1.15), KH_2PO_4 (1.15), $NaHCO_3$ (25), glucose (5.5), $CaCl_2$ (1.3) equilibrated with 95% O_2/5% CO_2. The incubation medium is brought up to room temperature over a period of 30 min. The KRB is then replaced with fresh medium and the slices are incubated for a further 30 min at room temperature, followed by 30 min at 37°C in fresh KRB. Following the initial incubations, slices are labelled for 45 min with ^{3}H-adenine (5 µCi/ml) at 37°C. Two labelled slices are transferred into plastic cylinders which have nylon net bases and these, together with the slices, are placed into glass superfusion chambers. Slices are superfused at a flow rate of 0.5 ml/min with KRB at 37°C. After a 60 min wash, collection of 5 min fractions begins, which continues throughout the remainder of the experiment. A 1.25 ml aliquot of the fractions is taken for determination of radioactivity using scintillation spectrometry (scintillation fluid: Picofluor 15). The remaining 1.25 ml is taken for HPLC analysis of purines and amino acids.

Hypoxia/hypoglycemia is induced by superfusion with KRB containing no glucose and 95% N_2 and 5% CO_2 for 35 min followed by recovery. All other procedures are as described above. The test drug is added to the perfusion fluid at an appropriate concentration. The 1.25 ml aliquots taken for HPLC analysis are pooled with two other aliquots (total 3.75 ml), lyophilized and concentrated 10 fold before analysis. Samples are analyzed for adenosine, inosine and hypoxanthine with HPLC. The radioactivity associated with each of these fractions is also determined by collecting the eluent from the column at the appropriate times. HPLC analyses are carried out using a reverse-phase C_{18} column (Waters 10 µm µBondapak) with isocratic elution, a flow rate of 1.0 ml/min and at ambient temperature. For adenosine, inosine and hypoxanthine the mobile phase is 10 mM $NH_4H_2PO_4$, pH 6.0, 13% methanol.

EVALUATION

Values for adenosine, inosine and hypoxanthine can be expressed in two ways: (1) release rate per slice (pmol/min × slice); (2) percentage of the total amount of released radioactivity (% total $_3$H-label released). The purine data are expressed as means ± SEM and differences are evaluated with the non-parametric Mann-Whitney test.

REFERENCES AND FURTHER READING

Dunwiddie TV (1986) The use of *in vitro* brain slices in neuropharmacology. In: Electrophysiological Techniques in Pharmacology. Alan R Liss, Inc., pp 65–90
Hagberg H, Andiné P, Fredholm B, Rudolphi K (1990) Effect of the adenosine uptake inhibitor propentofylline on the extracellular adenosine and glutamate and evaluation of its neuroprotective efficacy after ischemia in neonatal and adult rats. In: Krieglstein J, Oberpichler (eds) Pharmacology of Cerebral Ischemia. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, pp 427–437

A.8.1.8 Measurement of Local Cerebral Blood Flow and Glucose Utilization in Rats

PURPOSE AND RATIONALE

Cerebral glucose utilization can be determined using [^{14}C]-2-deoxyglucose according to Sokoloff et al. (1977). Local cerebral blood flow using [^{14}C]-iodoantipyrine is measured as described by Sakurada et al. (1978).

PROCEDURE

Animal Preparation

The experiments are performed on male Sprague-Dawley rats (260–300 g). Catheters are placed, under light halothane (1%) anesthesia, into the femoral vein and artery of each hind limb (for the measurement of mean arterial pressure, the sampling of arterial blood and the intravenous administration of drugs and radioisotopic tracer). The wound sites are then infused with local anesthetic, sutured, and protected by pads. The lower abdomen is covered by an elastic stocking, followed by a loose-fitting plaster coat. Anesthesia is discontinued and the rats are allowed at least two hours to recover before any further manipulations are performed.

Experimental Protocol

The rats are given an intravenous infusion (50 µl/min) of either saline or the test drug dissolved in saline. This infusion is maintained throughout the measurement of local cerebral blood flow or local cerebral glucose utilization.

Local Cerebral Glucose Utilization

A full description of the method for measuring local cerebral glucose utilization using [^{14}C]-2-deoxyglucose has been published (Sokoloff et al. 1977). Five minutes after the administration of the test drug, the experiment is started with the intravenous administration of [^{14}C]-2-deoxyglucose (125 µCi/kg). Fourteen timed arterial blood samples are taken during the following forty-five minutes. These samples are centrifuged and the plasma is measured for glucose concentration (using an automated glucose analyzer) and [^{14}C] levels (by liquid scintillation counting). At the end of this period the rats are sacrificed by decapitation, the brain rapidly removed and frozen at –45°C. Twenty micron thick coronal sections are cut in a cryostat (–22°C) and autoradiograms are prepared by placing these sections in an array against Kodak SB-5 X-ray film along with pre-calibrated plastic standards range (55–851 nCi/g) for seven days in light-tight cassettes.

Local Cerebral Blood Flow

The autoradiographic measurement of local cerebral blood flow using [^{14}C]-iodoantipyrine is carried out as described by Sakurada et al. (1978). [^{14}C]-iodoantipyrine (125 µCi/kg) is administered fifteen minutes after the infusion of the test drug has commenced. In a period of sixty seconds, eighteen timed arterial samples are collected in pre-weighed filter-paper discs from a free flowing arterial catheter. The discs are reweighed and the [^{14}C] concentration of each is measured by liquid scintillation counting. At the end of one minute, the rat is decapitated and autoradiograms are prepared in the same manner as for the measurement of local cerebral glucose utilization.

Densitometric Analysis of Autoradiograms

Tissue [^{14}C] concentrations were determined using a densitometer system (Zeiss, FRG) by reference to the images of the precalibrated standards. For each structure of interest, bilateral determination of optical densities are made on six different autoradiographic images in which the structure is best defined.

The mean optical density is used to calculate [^{14}C] concentrations. From this value, and the history of [^{14}C] in the blood, values of local cerebral blood flow and glucose utilization are obtained using the respective operational equations of these methods (Sakurada et al. 1978; Sokoloff et al. 1977).

EVALUATION

Groups of data are statistically compared by *t*-test with unpaired comparison using the BONFERRONI correction factor for multiple group analyses. Linear regression data for comparing cerebral blood flow (CBF) and glucose utilization (GU) undergo log transformation as recommended by McCulloch et al. (1982).

MODIFICATIONS OF THE METHOD

Ito et al. (1990) measured glucose utilization in the mouse brain by the simultaneous use of [^{14}C]2-deoxyglucose and [^{3}H]3-O-methylglucose.

High-resolution animal positron emission tomography was recommended by Magata et al. (1995) for noninvasive measurement of cerebral blood flow with ^{15}O-water and glucose metabolic rate with ^{18}F-2-fluoro-2-deoxyglucose.

The effect of ginseng pretreatment on cerebral glucose metabolism in ischemic rats using animal positron emission tomography (PET) with [^{18}F]-2 fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) was described by Choi et al. (1997).

Hawkins et al. (1993) developed a method for evaluating tumor glycolytic rates *in vivo* with nude mice injected with 2-[^{18}F]fluoro-2-deoxy-D-glucose and a dedicated animal positron emission tomography scanner.

Positron emission tomography has been used with specific ligands for CNS imaging (de la Sayette 1991; Jones et al. 1991; Kung 1993).

Rogers et al. (1994) synthesized ^{18}F-labelled vesamicol derivates to be evaluated in small animal positron emission tomography.

Hume et al. (1997) measured *in vivo* saturation kinetics of two dopamine transporter probes using a small animal positron emission tomography scanner.

REFERENCES AND FURTHER READING

Choi SR, Magata Y, Saji H, Tajima K, Kitano H, Konishi J, Yokoyama A (1997) Effect of ginseng pretreatment on cerebral glucose metabolism in ischemic rats using animal positron emission tomography (PET) and ([^{18}F]-FDG). Phytother Res 11:437–440

De la Sayette T, Chavoix C, Brouillet A, Hantraye P, Kunimoto M, Khalili-Varasteh M, Guibert B, Prenant C, Mazière M (1991) *In vivo* benzodiazepine receptor occupancy by CL 218,872 visualized by positron emission tomography in the brain of the living baboon: Modulation by GABAergic transmission and relation with anticonvulsant activity. Exp Brain Res 83:397–402

Grome J, Stefanovich V (1985) Differential effects of xanthine derivatives on local cerebral blood flow and glucose utilization in the conscious rat. In: Stefanovich V, Rudolphi K, Schubert P (eds) Adenosine: Receptors and Modulation of Cell Function. IRL Press Ltd., Oxford, England, pp 453–458

Hawkins RA, Choi Y, Scates S, Rege S, Hoh CK, Glaspy J, Phelps ME (1993) An animal model for *in vivo* evaluation of tumor glycolytic rates with positron emission tomography. J Surg Oncol 53:104–109

Hume SP, Brown DJ, Ashwoth S, Hirani E, Luthra SK, Lammertsma AA (1997) *In vivo* saturation kinetics of two dopamine transporter probes measured using a small animal positron emission tomography scanner. J Neurosci Meth 76:45–51

Ito K, Sawada Y, Ishizuka H, Sugiyama Y, Suzuki H, Iga T, Hanano M (1990) Measurement of cerebral glucose utilization from brain uptake of [^{14}C]2-deoxyglucose and [^{3}H]3-O-methylglucose in the mouse. J Pharmacol Meth 23:129–140

Jones HA, Rhodes CR, Law MP, Becket JM, Clark JC, Boobis AR, Taylor GW (1991) Rapid analysis of ^{11}C-labelled drugs: fate of [^{11}C]-*S*-4-(*tert*.-butylamino-2-hydroxypropoxy)-benzimidazol-2-one in the dog. J Chromatogr Biomed Appl 570:361–370

Kung HF (1993) SPECT and PET ligands for CNS imaging. Neurotransmissions 9/4:1–6

Lacombe B, Meric P, Seylaz J (1980) Validity of cerebral blood flow measurements obtained with quantitative tracer techniques. Brain Res Rev 2:105–169

Magata Y, Saji H, Choi SR, Tajima K, Takagaki T, Sasayama S, Yonekura Y, Kitano H, Watanabe M, Okada H, Yoshikawa E, Yamashita T, Yokoyama A, Konishi J (1995) Noninvasive measurement of cerebral blood flow and glucose metabolic rate in the rat with high-resolution animal positron emission tomography (PET): A novel *in vivo* approach for assessing drug action in the brains of small animals. Biol Pharm Bull 18:753–756

McCulloch J, Kelly PAT, Ford I (1982) Effect of apomorphine on the relationship between local cerebral glucose utilization and local cerebral blood flow. (with an appendix on its statistical analysis). J Cerebr Blood Flow Metab 2:487–499

Rogers GA, Stone-Elander S, Ingvar M, Eriksson L, Parsons SM, Widen L (1994) ^{18}F-labelled vesamicol derivates: Syntheses and preliminary *in vivo* small animal positron emission tomography evaluation. Nucl Med Biol 21:219–230

Sakurada O, Kennedy C, Jehle J, Brown JD, Carbin O, Sokoloff L (1978) Measurement of local cerebral blood flow with iodo(^{14}C)-antipyrine. Am J Physiol 234:H59–H66

Smith CB (1981) Age-related changes in local rates of cerebral glucose utilization in the rat. In: Enna SJ et al (eds) Brain. Neurotransmitters and Receptors in Aging and Age-Related Disorders (Aging, Vol 17) Raven Press, New York, pp 195–201

Sokoloff L, Reivich M, Kennedy C, DesRosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M (1977) The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 28:897–916

Winn HR, Rubio GR, Berne RM (1981) The role of adenosine in the regulation of cerebral blood flow. J Cerebr Blood Flow Metab 1:239–244

Wolfson LI, Sakurada O, Sokoloff L (1977) Effects of γ-butyrolactone on local cerebral glucose utilization in the rat. J Neurochem 29:777–783

A.8.1.9 Cerebrovascular Resistance in Anesthetized Baboons

PURPOSE AND RATIONALE

Cerebral blood flow and cerebrovascular resistance can be measured by injection of inert radioactive gas (^{133}Xenon) and evaluation of the ^{133}Xe clearance curve in anesthetized baboons.

PROCEDURE

Animal Preparation

The experiments are performed on baboons (*Papio* or *cynocephalus*) weighing 9–13 kg. Initial sedation with phencyclidine (10 mg i.m.) is followed by an intravenous injection of sodium thiopental (75 mg/kg). The animals are intubated to a positive pressure ventilator delivering 70:30% nitrous oxide and oxygen in open circuit. A continuous intravenous infusion of phencyclidine (0.01 mg/kg/min) is given throughout the course of the experiment. Suxamethonium (50 mg i.m.) is administered every 30 min in order to assist control of ventilation with the respiratory pump.

During the experiments, the end tidal concentration of CO_2 is continuously measured and the ventilating pump adjusted to maintain an arterial CO_2 tension ($PaCO_2$) of between 37 and 40 mm Hg for the control measurements. Arterial blood samples are taken during every measurement of cerebral blood flow (CBF) to measure $PaCO_2$, PaO_2, and pH by direct reading electrodes (Corning). Body temperature is maintained around 37°C by means of an electrically heated blanket and infrared heating lamps.

Catheters are inserted into the aorta via femoral arteries for the continuous measurement of arterial blood pressure and for the withdrawal of arterial blood samples. Both femoral veins are cannulated, one for the continuous infusion of phencyclidine and the other for the infusion of the test drug. A catheter is inserted into the right lingual artery so that its tip lays just distal to the carotid bifurcation. This catheter is flushed at regular intervals with heparinized saline to prevent platelet aggregation at the tip. In the studies with required intravenous administration of test drug, the other branches of the external carotid artery are ligated. Where the requirement is for intracarotid administration of test drug, another catheter is retrogradely advanced into the external carotid artery so that its tip lays next to the tip of the lingual catheter. This catheter is then attached to a constant-rate infusion pump (Sage Instruments). Heparinized saline is infused at a rate of 0.2 ml/min to act as a control for drug infusion.

A burr hole is made over the midline fissure and a catheter inserted into the sagittal sinus for the withdrawal of cerebral venous blood samples. The hole is sealed with plaster of Paris. The scalp and temporalis muscle are removed with diathermy down to the level of the zygomatic arch.

Measurement of Cerebral Blood Flow, Cerebral Oxygen Utilization, and Cerebrovascular Resistance

A collimated scintillation crystal is placed over the temporal region of the exposed skull on the right side and angled in such a way that viewed only brain and overlying skull.

Approximately 260 µCi of ^{133}Xenon dissolved in 0.5 ml sterile heparinized saline (500 IU) is injected over 1 s into the catheter in the lingual branch of the carotid artery. The gamma-ray emission of the $_{133}$Xe are detected by the scintillation counter attached to a photomultiplier. The pulses are amplified and subjected to pulse height analysis (peak 81 KeV ± 8 KeV) to reduce Compton scatter before fed into a rate meter and scaler. The output from the rate meter is displayed on a chart recorder. Cerebral blood flow is calculated from the height/area equation (Høedt-Rasmussen et al. 1966). The formula used is

$$F = (H_2 - H_{10}) \times 100/\lambda A_{10}\,,$$

where F is CBF in ml blood per 100 g brain tissue per min; λ = brain tissue/blood partition coefficient for ^{133}Xe (the figure of 1.1 is used [Veall and Mallett 1966]); H_i = maximum initial height of the ^{133}Xe clearance curve in counts per min as taken from the chart recorder; H_{10} = height of the clearance curve 10 min after the peak height in counts per s; A_{10} = total integrated counts over the 10 min of clearance as taken from the scaler and corrected for background activity over that period.

Cerebral oxygen consumption is measured from the product of the DBF and the difference in oxygen content between the arterial blood and cerebral venous blood sampled from the sagittal sinus. Blood oxygen is measured by a charcoal-fuel cell system (Lex-O_2-Con).

An estimate of cerebrovascular resistance is obtained by subtracting the mean sagittal sinus pressure from the mean arterial pressure and dividing this pressure difference by the CBF.

EEG Recording

Electroencephalographic readings are recorded bilaterally throughout the experiment. A series of holes are drilled in the calvarium 10 mm apart in two rows. Each row is 14 mm lateral to the sagittal suture. The holes are threaded to receive nylon screws in which silver-silver chloride ball electrodes are fixed loosely. The electrodes are positioned epidurally and the free ends are soldered to a multi-channel socket which is mounted on the calvarium with plaster of Paris.

Experimental Procedure

Following completion of the surgery, the animals are left undisturbed for 1 h. At least three control estimations of CBF and other parameters are made until steady conditions of flow, arterial blood pressure, and blood gas tensions are obtained.

Protocols

Intravenous administration. After stable control values have been established, the infusion is started. The CBF is measured at 5 min after the start and again at 25 min. The infusion is stopped 10 min after this flow period, giving a total infusion time of 35 min. Further flow measurements are made at 10, 30, and 50 min after stopping the infusion.

Intracarotid administration. After establishing control values, the intracarotid infusion of the test drug is begun. The CBF is measured at 10, 30, 50 and 70 min. The infusion is stopped 80 min after commencing, and post infusion measurements are made after 20, 40, and 60 min.

EVALUATION

Data are presented as mean values ± SEM Evaluation of statistical significance is performed by means of Student's *t*-test with Bonferroni correction.

MODIFICATIONS OF THE METHOD

Kozniewska et al. (1992), Wang et al. (1992) measured cerebral blood flow in **rats** by intracarotid injection of ^{133}Xe.

Solomon et al. (1985) and Clozel and Watanabe (1993) induced cerebral vasoconstriction by injection of autologous blood in the cisterna magna of rats. Cerebral blood flow was measured with the radioactive microsphere technique.

Lin et al. (2003) described a model of subarachnoid hemorrhage-induced cerebral vasospasm in **mice**. Adult mice received injections of autologous blood into the cisterna magna. The diameters of large intracranial vessels were measured 1 h to 7 days after the subarachnoid hemorrhage. A diffuse blood clot was evident in both the anterior and posterior circulations. Vascular wall thickening, lumenal narrowing, and corrugation of the internal elastic lamina were observed. Both acute (6–12 h) and delayed (1–3 days) phases of vasoconstriction occurred after subarachnoid hemorrhage. Overall mortality was only 3%. The model is recommended for screening of therapeutic candidates.

Delayed cerebral vasospasm was induced in anesthetized **dogs** by removal of 4 ml cerebrospinal fluid and injection of the same volume of fresh autologous arterial nonheparinized blood into the cisterna magna by Varsos et al. (1983) as a model of subarachnoid hemorrhage. The procedure was repeated on the third day and angiograms were taken of the vertebral-basilar vessels. The reduction of diameter of the basilar artery was taken as endpoint.

Imaizumi et al. (1996) produced experimental subarachnoid hemorrhage by intracisternal injection of arterial blood in **rabbits**. The degree of vasospasm and the effect of calcitonin gene-related peptide were evaluated angiographically by measuring the basilar artery diameter.

Inoue et al. (1996) produced experimental subarachnoid hemorrhage in **cynomolgus monkeys** by placing a clot around the internal carotid artery. A series of angiographic analyses were performed, before subarachnoid hemorrhage and on days 7 and 14 after treatment with calcitonin gene-related peptide to examine changes in the diameter of the ipsilateral internal carotid artery, middle cerebral artery, and anterior cerebral artery.

Hughes et al. (1994) adapted the ^{133}xenon clearance technique for simultaneous measurement of cutaneous blood flow in **rabbits** at a large number of skin sites within the same animal.

REFERENCES AND FURTHER READING

Clozel M, Watanabe H (1993) BQ-123, a peptidic endothelin ET_A receptor antagonist, prevents the early cerebral vasospasm following subarachnoid hemorrhage after intracisternal but not after intravenous injection. Life Sci 52:825–834

Grome JJ, Rudolphi K, Harper AM (1985) Cerebrovascular effects of a xanthine derivative propentofylline (HWA 285). Drug Dev Res 5:111–121

Høedt-Rasmussen K, Sveinsdottir E, Lassen NA (1966) Regional cerebral blood flow in man determined by intra-arterial injection of radioactive inert gas. Circ Res 18:237

Hughes S, Brain S, Williams G, Williams T (1994) Assessment of blood flow changes at multiple sites in rabbit skin using a ^{133}xenon clearance technique. J Pharmacol Toxicol Meth 32:41–47

Imaizumi S, Shimizu H, Ahmad I, Kaminuma T, Tajima M, Yoshimoto T, Megyesi J, Findlay JM, Kikuchi H, Nozaki K (1996) Effect of calcitonin gene-related peptide on delayed vasospasm after experimental subarachnoid hemorrhage in rabbits. Surg Neurol 46:263–271

Inoue T, Shimizu H, Kaminuma T, Tajima M, Watabe K, Yoshimoto T, Dacey RG Jr, Solomon RA, Selman WR (1996) Prevention of cerebral vasospasm by calcitonin gene-related peptide slow-release tablet after subarachnoidal hemorrhage in monkeys. Neurosurgery 39:984–990

Kozniewska E, Oseka M, Stys (1992) Effects of endothelium-derived nitric oxide on cerebral circulation during normoxia and hypoxia in the rat. J Cerebr Blood Flow Metab 12:311–317

Lacombe P, Meric P, Seylaz J (1980) Validity of cerebral blood flow measurements obtained with quantitative tracer techniques. Brain Res Rev 2:105–169

Lin CL, Calisaneller T, Utica N, Dumont AS, Kassell NF, Lee KS (2003) A murine model of subarachnoid hemorrhage-induced cerebral vasospasm. J Neurosci Meth 123 :89–97

Nielsen SL, Lassen NA, Elmqvist D (1975) Muscle blood flow in man studied with the local radioisotope method. In: Kunze K, Desmedt JE (eds) Studies on Neuromuscular Diseases. Proc Intern Sympos, Giessen 1973, Karger, Basel, pp 79–81

Solomon RA, Antunes JL, Chen RYZ, Bland L, Chien S (1985) Decrease in cerebral blood flow in rats after experimental sub-archnoid hemorrhage: a new animal model. Stroke 16:58–64

Varsos VG, Liszczak TM, Han DH, Kistler JP, Vielma J, Black PM, Heros RC, Zervas NT (1983) Delayed cerebral vasospasm is not reversible by aminophylline, nifedipine, or papaverine in a "two-hemorrhage" canine model. J Neurosurg 58:11–17

Veall N, Mallett BL (1966) Regional cerebral blood flow determination by ^{133}Xe inhalation and external recording: The effect of arterial recirculation. Clin Sci 30:353

Wang Q, Paulson OB, Lassen NA (1992) Effect of nitric oxide blockade by N^G-nitro-L-arginine on cerebral blood flow response to changes in carbon dioxide tension. J Cerebr Blood Flow Metab 12:947–953

A.8.1.10 Effect on Cerebral Blood Flow in Cats (Fluvography)

PURPOSE AND RATIONALE

Cerebral blood flow in anaesthetized cats an be measured by fluvography. The heat transfer coefficient of

the brain can be measured with a probe representing an indirect blood flow value.

In this method, the measuring device consists of a thermo probe, which is attached to the tissue in order to record continually the heat transport (Hensel, 1956). The device depends on having an electrically heated part and an unheated reference point. The difference in temperature between the heated part of the device and the unheated reference point is a function of local blood flow. An increase in flow tends to lower the local temperature by carrying away the heat gain and vice versa.

PROCEDURE

Cats of either sex weighing 2.5–4.0 kg are anaesthetized by intraperitoneal administration of pentobarbital sodium (35 mg/kg) and intubated with a tracheal tube. The left femoral vein and the right femoral artery are cannulated for i.v. drug administration and determination of arterial blood pressure, respectively. The arterial cannula is connected to a Statham transducer P 23 Db.

For intraduodenal drug administration the duodenum is cannulated following laparotomy.

Before actually starting the experiment, the arterial blood gas concentrations are determined.

Animals are only used for further testing if they show normal blood gas concentrations. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

The head of the animal is fixed in a stereotactic device. The skull cap and the dura are opened, the probes are placed on the surface of the cortex in the region of the marginal frontal gyrus, and the exposed brain is covered with moist swabs. The Fluvograph (Hartmann+Braun, Frankfurt) is used with the appropriate thermo probes.

To test the correct position of the thermocouple and the response of the animal, inhalations of carbon dioxide/ air or injections of epinephrine (adrenaline) are used, leading to a distinctive increase in cerebral blood flow. Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is administered.

Standard compound:

- pentoxifylline
  1 and 3 mg/kg (i.v. administration)
  10 and 30 mg/kg (i.d. administration)

EVALUATION

The percent change in the heat transfer coefficient is used as an indirect measure for the change of cerebral blood flow. Statistics: Student's *t*-test is performed by unpaired comparison.

REFERENCES AND FURTHER READING

Betz E (1965) Local heat clearance from the brain as a measure of blood flow in acute and chronic experiments. Acta Neurol Scand Suppl 14:29–37

Golenhofen K, Hensel H, Hildebrandt G (1963) Durchblutungsmessungen mit Waerme-Elementen. Georg Thieme Verlag, Stuttgart

Lacombe P, Meric P, Seylaz J (1980) Validity of cerebral blood flow measurements obtained with quantitative tracer techniques. Brain Res Rev 2:105–169

A.8.1.11
Effect on Cerebral Blood Flow and in Ischemic Skeletal Muscle in Rats (Laser-Doppler-Effect)

PURPOSE AND RATIONALE

The principle of the Laser Doppler effect is based on the fact that a laser light beam directed on tissue is scattered in static structures as well as in moving cells. Light beams scattered in moving red cells undergo a frequency shift according to the Doppler effect, while beams scattered in static tissue alone remain unshifted in frequency. The number of Doppler shifts per time is recorded as a measure for erythrocyte flow in a given volume. This means, that the direction of flow cannot be determined, but relative changes in micro circulatory blood flow can be recorded. The procedure can be used to detect test compounds that improve the blood supply of the brain or the flow of red blood cells in the ischemic skeletal muscle.

PROCEDURE

Male Sprague-Dawley rats weighing 300–500 g are anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The trachea is exposed and intubated with a short tracheal tube to allow ventilation. The following vessels have to be cannulated: The femoral vein is cannulated for test drug administration. The femoral artery is cannulated for blood pressure recording and blood gas analysis.

Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further testing if they show normal blood gas concentrations (pa CO_2: 32–42 mm Hg; pa O_2: 70–110 mm Hg). The mean arterial blood pressure should not drop below 100 mm Hg. During the course of the experiment, blood flow, blood pressure and blood gas concentrations are regularly monitored.

For Cerebral Blood Flow

The head of the animal is fixed in a stereotactic device. After trepanation of the skull (opening 3 mm in diameter), the Laser Doppler probe is placed 1 mm above the surface of the brain. Values are measured with the Laser Doppler apparatus (Periflux F2, Perimed KB, Stockholm).

Following stabilization of the parameters mentioned above, the standard compound is administered and the change in blood flow is recorded. Five min after obtaining the original values, the test compound is infused. Following two administrations of the test compound, the standard compound is administered again. Duration of the effect is measured as half life in seconds.

For Peripheral Blood Flow

A small area of the femoral artery of the right hind limb is exposed and the Laser Doppler probe is placed 1 mm above the muscle surface. Before actually starting the experiment, the arterial blood gas concentrations are determined. Animals are only used for further proceeding if they show normal blood gas concentrations. During the course of the experiment, the blood pressure is recorded. The RBC flux is recorded continuously and after stabilization of the output signal, the femoral artery is occluded leading to underperfusion of the muscles of the right pelvic limb. At this stage, the test compound is administered by intravenous infusion for 10 min (0.05 ml/min).

Standard compound for cerebral blood flow is propentofylline (1 mg/kg, i.v.). The percent increase in blood flow produced by propentofylline ranges between 40% and 60%.

EVALUATION

The percent increase in blood flow after test drug administration is determined (compared to the value before drug administration).

Statistics: Student's *t*-test by unpaired comparison, test substance versus standard.

MODIFICATIONS OF THE METHOD

Iadecola (1992), Prado et al. (1992), Raszkiewicz et al. (1992) measured the influence of nitric oxide on cortical cerebral blood flow in anesthetized rats by Laser-Doppler flowmetry.

Benessiano et al. (1985) measured aortic blood flow with range-gated Doppler flowmeter in anesthetized rats.

Partridge (1991) measured nerve blood flow in the sciatic nerve of anesthetized rats with a Laser Doppler Flowmeter after application of local anesthetics and of epinephrine.

REFERENCES AND FURTHER READING

Benessiano J, Levy BI, Michel JB (1985) Instantaneous aortic blood flow measurement with range-gated Doppler flowmeter in anesthetized rat. J Pharmacol Meth 14:99–110

Iadecola C (1992) Does nitric oxide mediate the increases in cerebral blood flow elicited by hypercapnia? Proc Natl Acad Sci USA 89:3913–3916

Kunze K, Berk H (1975) Oxygen supply and muscle blood flow in normal and diseased muscle. In: Kunze K, Desmedt JE (eds) Studies on Neuromuscular Diseases. Proc Intern Sympos, Giessen 1973, Karger, Basel, pp 82–91

LeNoble JLML, Struyker-Boudier HAJ, Smits JFM (1987) Differential effects of general anesthetics on regional vasoconstrictor responses in the rat. Arch Int Pharmacodyn 289:82–92

Partridge BL (1991) The effects of local anesthetics and epinephrine on rat sciatic nerve blood flow. Anesthesiology 75:243–251

Prado R, Watson BD, Kuluz J, Dietrich WD (1992) Endothelium-derived nitric oxide synthase inhibition. Effects on cerebral blood flow, pial artery diameter, and vascular morphology in rats. Stroke 23:1118–1124

Raszkiewicz JL, Linville DG, Kerwin JF, Wagenaar F, Arneric SP (1992) Nitric oxide synthase is critical in mediating basal forebrain regulation of cortical cerebral circulation. J Neurosci Res 33:129–135

Rudquist I, Smith QR, Michel ME, Ask P, Öberg P, Rapoport SI (1985) Sciatic nerve blood flow measured by laser Doppler flowmetry and [^{14}C]iodoantipyrine. Am J Physiol 248:H311–H317

A.8.1.12 Traumatic Brain Injury

PURPOSE AND RATIONALE

A major goal in research into mechanisms of brain damage and dysfunction in patients with severe head injury and in discovery of potential therapies is the development of a suitable animal model. While a variety of experimental techniques have been developed (Denny-Brown and Russell 1941; Gurdjian et al. 1954; Ommaya and Gennarelli 1974; Sullivan et al. 1976; Nilsson et al. 1977; Hayes et al. 1984; Marmarou et al. 1994; Mésenge et al. 1996), the most widely employed technique is fluid percussion, which produces brain injury by rapid injection of fluid into the closed cranial cavity (Sullivan et al. 1976; McIntosh et al. 1989, 1990; Faden et al. 1989; Dixon et al. 1991; Sun and Faden 1995; Petty et al. 1996; Laurer et al. 2000; Maegele et al. 2005). Most authors used rats, however, other species, such ad cats (Sullivan et al. 1976; Hayes et al. 1983, 1984), rabbits (Lindgren and Rinder 1969) and mice (Hall et al. 1988; Mésenge et al. 1996) were employed.

PROCEDURE

Surgical Preparation

Male Sprague Dawley rats weighing from 400 to 500 g are anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). During surgical preparation and throughout the experiment, all wounds are infused with a topical anesthetic (lidocaine hydrochloride 2.0%). Catheters are inserted into the femoral vein for drug administration and into the femoral artery for blood pressure/blood gas monitoring. A 2.0 mm hollow female Leur-Loc fitting (to induce trauma) is rigidly fixed with dental cement to the animal's skull in a craniectomy centered over the left parietal cortex 5 mm from lambda, 5 mm from bregma, 4 mm from sagittal suture. The dura is left intact at this opening. Immediately following surgical preparation, a constant i.v. infusion of sodium barbital (15 mg/kg/h) is begun and maintained for the duration of the studies.

Drug Administration

Drugs or equal volumes of saline are administered through the femoral vein over 10 min by constant infusion beginning 15 min before trauma.

Fluid-Percussion Injury

The fluid-percussion device consists of a Plexiglas cylindrical reservoir, 60 cm long and 4.5 cm in diameter, bounded at one end by a Plexiglas, cork-covered piston mounted on O-rings. The opposite of the reservoir is fitted with a 2-cm-long metal housing on which a transducer is mounted and connected to a 5-mm tube (2 mm inner diameter) that terminates with a male Luer-Loc fitting. At the time of injury the tube is connected with the female Luer-Loc fitting that has been chronically implanted over the exposed dura of the rat. After the entire system is filled with 37°C isotonic saline, injury is induced by a metal pendulum which strikes the piston of the device from a predetermined height. The device produces a pulse of increased intracranial pressure of fairly constant duration (21–23 ms) by injecting various volumes of saline into the closed cranial cavity. Brief displacement and deformation of neural tissue results from the rapid epidural injection of saline. The magnitude of injury is regulated by varying the height of the pendulum, which results in corresponding variations of the intracranial pressure expressed in atmospheres. The pressure pulses are measured extracranially by a transducer and recorded on a storage oscilloscope.

EVALUATION

Behavioral Outcome

Posttraumatic deficits are evaluated at 24 h, 1 week and 2 weeks following trauma. Outcomes include forelimb flexion (right and left), lateral pulsion (right and left) and angle score (left, right and vertical position). Scores range from 0 (maximal deficits) to 5 (normal) for each task, By combining scores of all tests, a composite neuroscore is determined, ranging from 0 to 35 (Faden 1993).

Histopathology

At 2 weeks, following final neurological scoring, the rats are sacrificed by decapitation. The brain is removed, quickly frozen in isopentane and stored in a –80°C freezer until sectioning. Coronal brain sections are selected to span the longitudinal axis of the dorsal hippocampus between –3.2 and –3.8 Bregma. Sections (16 mm thick) are cut at –18°C in a microtome-cryostat and thaw-mounted onto chrome-gelatin rubbed microscope glass slides and kept at –80°C for histological study.

Sections are stained with Crystal violet. CA1 and CA3 pyramidal cells with a distinct nucleus and nucleolus are counted as viable neurons, in one reticle within CA3 and in three reticles (R1, R2 and R3) within the subfield of the hippocampus, in both right and left hemispheres. The number of viable neurons is counted twice at 400 × microscope field.

An immunochemical method is used to detect glial fibrillary acidic protein (GFAP)-positive astrocytes in the hippocampus (Faddis and Vijayan 1988). Counting of the number of cells is done under 400 × light microscopy in the dorsal CA1 subfield between medial and lateral regions.

Statistical Analysis

Neuroscores from forelimb flexion tests, lateral pulsion tests and angle board tests are statistically analyzed by non-parametric Mann-Whitney U-tests. Histological data are analyzed by one way ANOVA test, followed by Scheffe's test.

MODIFICATIONS OF THE METHOD

Shohami et al. (1995) tested the effect of a non-psychotropic cannabinoid which acts as a non-competitive NMDA antagonist on motor and memory functions after closed head injury in the **rat**.

Fox et al. (1998) developed a **mouse** model of traumatic brain injury using a device that produces controlled cortical impact, permitting independent manipulation of tissue deformation and impact velocity and

resulting in sustained sensory/motor and cognitive defects.

Tang et al. (1997) reported impairment in learning and memory in an experimental model of concussive brain injury in **mice**.

Bemana and Nago (1998) induced acute intercranial hypertension in **cats** by continuous inflation of an extradural balloon with physiological saline at a constant rate of 0.5 ml/h for 3 h. At this point, inflation was discontinued and the balloon remained expanded for an additional hour after which it was deflated.

A model of **traumatic injury to the spinal cord** was used by Springer et al. (1997). Female Long Evans rats weighing 200–250 g were anesthetized with pentobarbital and a dorsal laminectomy was performed to expose the spinal cord at thoracic level T 10. The vertebral column was stabilized by clamping the column at vertebra 8 and 11. The New York University (NYU) impactor device was used which produces accurate and reproducible damage to the rat spinal cord (Gruner 1992; Basso et al. 1996). This device is a weight drop apparatus that uses optical potentiometers to record the movement of a 10-g impact rod and the vertebral column following impact and is connected to a PC that monitors rod and vertebral movements during impact.

REFERENCES AND FURTHER READING

Basso DM, Beattie MS, Bresnahan JC (1996) Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Exp Neurol 139:244–256

Bemana I, Nagao S (1998) Effects of Niravoline (RU 51599), a selective kappa-opioid receptor agonist on intracranial pressure in gradually expanding extradural mass lesion. J Neurotrauma 15:117–124

Denny-Brown D, Russell WR (1941) Experimental cerebral percussion. Brain 64:93–164

Dixon CE, Clifton GL, Lighthall JW, Yagjmai AA, Haynes RL (1991) A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Meth 39:253–262

Faddis B, Vijayan VK (1988) Application of glial fibrillary acidic protein histochemistry in the quantification of astrocytes in the rat brain. Am J Anat 183:316–322

Faden AI (1993) Comparison of single and combination drug treatment strategies in experimental brain trauma. J Neurotrauma 10:91–100

Faden AI, Demediuk P, Panter SS, Vink R (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244:798–800

Fox GB, Fan L, Lavasseur RA, Faden AI (1998) Sustained sensory/motor and cognitive deficits with neuronal apoptosis following controlled impact brain injury in the mouse. J Neurotrauma 15:599–614

Gruner JA (1992) A monitored contusion model of spinal cord injury in the rat. J Neurotrauma 9:123–128

Gurdjian ES, Lissner HR, Webster JE (1954) Studies on experimental concussion. Relation of physiological effect to time duration of intracranial pressure increase at impact. Neurology 4:674–681

Hall ED, Yonkers PA, McCall JM (1988) Effects of the 21-amino-steroid U74006F on experimental head injury in mice. J Neurosurg 68:456–461

Hayes RL, Lewelt W, Yeatts ML (1983) Metabolic, behavioral and electrophysiological correlates of experimental brain injury in the cat. J Cerebr Blood Flow Metab 3:38–40

Hayes RL, Pechura CM, Katayama Y, Povlishock JT, Giebel ML, Becker DP (1984) Activation of pontine cholinergic sites implicated in unconsciousness following cerebral percussion in cats. Science 223:301–303

Laurer HL, Lenzlinger PM, McIntosh TK (2000) Models of traumatic brain injury. Eur J Trauma 26:95–100

Lindgren S, Rinder L (1969) Production and distribution of intracranial and intraspinal pressure changes at sudden extradural fluid volume input in rabbits. Acta Physiol Scand 76:340–351

Maegele M, Gruener-Lippert M, Ester-Bode T, Garbe J, Bouillon B, Neugebauer E, Klug N, Lefering R, Neiss WF, Angelov DN (2005) Multimodal early onset stimulation combined with enriched environment is associated with reduced CNS lesion volume and enhanced reversal of neuromotor dysfunction after traumatic brain injury in rats. Eur J Neurosci 21:2406–2418

Marmarou A, Foda MAAE, van den Brink W, Campell J, Kita H, Demetriadu K (1994) A new model of diffuse brain injury in rats. J Neurosurg 80:291–300

McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, Faden AL (1989) Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neurosci 28:233–244

McIntosh TK, Vink R, Soares H, Hayes R, Simon R (1990) Effect of noncompetitive blockade of N-methyl-D-aspartate receptors on the neurochemical sequelae of experimental brain injury. J Neurochem 55:1170–1179

Mésenge Ch, Verrecchia C, Allix M, Boulu RR, Plotkine M (1996) Reduction of the neurological deficit in mice with traumatic brain injury by nitric oxide synthase inhibitors. J Neurotrauma 13:209–214

Nilsson B, Ponten U, Voigt G (1977) Experimental head injury in the rat. Part 1. Mechanics,

Ommaya AK, Gennarelli TA (1974) Cerebral concussion and traumatic unconsciousness. Correlation of experimental and clinical observations on blunt head injuries. Brain 97:633–654

Petty MA, Poulet P, Haas A, Namer IJ, Wagner J (1996) Reduction of traumatic brain injury-induced cerebral oedema by a free radical scavenger. Eur J Pharmacol 307:149–155

Shohami E, Novikov M, Bass R (1995) Long-term effect of HU211, a novel non-competitive NMDA antagonist, on motor and memory functions after closed head injury in the rat. Brain Res 674:55–62

Springer JE, Azbill RD, Mark RJ, Begley JG, Waeg G, Mattson MP (1997) 4-Hydroxynonenal, a lipid peroxidation product, rapidly accumulates following traumatic spinal cord injury and inhibits glutamate uptake. J Neurochem 68:2469–2476

Sullivan HG, Martinez J, Becker DP, Miller JD, Griffith R, Wist AO (1976) Fluid-percussion model of mechanical brain injury in the cat. J Neurosurg 45:520–534

Sun FY, Faden AI (1995) Neuroprotective effects of 619C89, a use-dependent sodium channel blocker, in rat traumatic brain injury. Brain Res 673:133–140

Tang Y-P, Noda Y, Hasegawa T, Nabeshima T (1997) A concussive-like brain injury model in mice: impairment in learning and memory. J Neurotrauma 14:851–862

A.8.1.13 Cerebral Blood Flow Measured by NMRI

PURPOSE AND RATIONALE

Over the past several years, magnetic resonance imaging (MRI) has become an established tool in the drug discovery and development process. The main advantages of MRI are its high resolution, non-invasiveness and versatility, which allow comprehensive characterization of a disease state and the effects of drug intervention. Recent advances now allow the application of this technique to the characterization of models of lung inflammation in rats and to the profiling of anti-inflammatory drugs. Repeated measurements can be carried out on the same animal, and time-courses of events can be easily assessed. Furthermore, the prospect of using MRI to detect non-invasively a sustained mucus hypersecretory phenotype induced by endotoxin brings an important new perspective to models of chronic obstructive pulmonary disease in animals. Importantly, it might be possible to extend the use of this technique to the clinical study of inflammation in the lung and the consequences of drug treatment (Beckmann et al. 2003, 2004).

Functional magnetic resonance imaging has been developed as pharmacological magnetic resonance imaging for pharmacodynamic assays and to establish brain-penetrating parameters (Leslie and James 2000). Although the regional cerebral metabolic rate of glucose is strongly increased during cerebral activity, the cerebral metabolic rate of O_2 is not increased in direct proportion. The result is that the relative uptake of O_2 from blood actually decreases. The veins and capillaries draining from the activated region are "arterialized" and their deoxyhemoglobin concentration is reduced. Deoxygenated and oxygenated hemoglobin have different magnetization properties; thus the changes in the ratio of these two entities can be detected by blood-oxygen-level-dependent (BOLD) magnetic resonance imaging. At least for studies in animals, these methods may be preferred to positron emission tomography (Cherry and Phelps 1996).

Petty et al. (2003) studied the *in vivo* neuroprotective effects of a systemically active antagonist of the NMDA receptor glycine site by magnetic resonance imaging in ischemic stroke in rats.

PROCEDURE

Transient Model of Focal Ischemia in Rats

In the right common carotid artery was ligated. The transient model of middle cerebral artery occlusion was based on techniques described by Zea Longa et al. (1989) and Belayev et al. (1996). By means of an operating microscope, the right common carotid artery was exposed through a midline neck incision. The right superior thyroid, occipital, and pterygopalatine arteries were ligated and cut. A poly-L-lysine-coated 3–0 nylon monofilament with a heat-blunted tip was inserted through the proximal external carotid artery into the internal carotid artery and pushed forward a distance of 19 or 20 mm from the carotid bifurcation, depending on the weight of the rat, so as to occlude the origin of the middle cerebral artery. After suture placement, the neck incision was closed and the animal was allowed to regain consciousness. Two hours following occlusion of the artery, the rats were tested on a standard neurological battery to confirm the presence of a neurological deficit. Animals that did not exhibit a forelimb flexion were excluded from further study. At this time, the rats were re-anesthetised and the intraluminal suture was completely withdrawn to restore the blood supply.

Magnetic Resonance Imaging (MRI) Measurements

Measurements were performed 30 min, 3 h, and 24 h after the onset of ischemia on a 7-T Bruker BIOSPEC experimental scanner (DBX; Bruker Medical, Ettlingen, Germany) with a 30-cm bore magnet and actively shielded gradient coils (200 mT/m; rise time < 80 µs).

A 72-mm resonator was used for *rf* transmission; signals were detected with a 35-mm inductively coupled surface coil placed over the skull of the animal. The *rf* coils were decoupled from each other – the transmitter coil actively and the receiver coil passively.

Using gradient-echo imaging, sagittal pilot scans were performed to ensure accurate positioning of the animal in the magnet. For this purpose, the coronal plane 5.9 mm posterior to the rhinal fissure was placed in the isocenter of the magnet, thus focusing on the center of the ischemic territory resulting from the middle cerebral artery occlusion.

For the determination of the temporal evolution of the ischemic lesion, two NMR imaging modalities were used. A field of view of 3.2 cm, a slice thickness of 1.5 mm, and an interslice distance of 2 mm were used for both sequences. Multislice packages were recorded by placing the center of the multislice imaging packet 5.9 mm posterior to the rhinal fissure.

Diffusion-weighted imaging was performed using a Stejskal–Tanner spin-echo sequence [echo time (TE) = 37.2 ms, repetition time (TR) = 2325 ms, eight slices] in six rats per group. To enable quantification of the apparent diffusion coefficient (ADC) of brain water, three b values were used (b = 50, 825,

and 1600 s/mm^2). ADC maps were calculated pixelwise using the monoexponential model (Le Bihan et al. 1986).

Perfusion-weighted imaging was performed with an ultrafast version of the arterial spin tagging technique (Kerskens et al. 1996; Franke et al. 2000) in four rats per group. In independent experiments, three coronal slices were recorded, thus covering the central part of the ischemic lesion. Measurement parameters were: TE = 3.5 ms, TR = 7.4 ms, MATRIX = 128 × 64, AVERAGE = 8. Each experiment consisted of two image acquisitions separated by a recovery period. During the first acquisition, blood flowing through the neck was adiabatically inverted (preparation TIME = 3 s; *z*-gradient = 5 mT/m; B1 FIELD = 150 mG; off-resonance FREQUENCY = 6000 Hz; mean preparation DISTANCE = 2.8 cm upstream from the imaging plane); in the second acquisition, inflowing spins were left undisturbed. Both phases were separated by a recovery period of 10 s. In each perfusion experiment, the two images suffered the same signal loss due to magnetization transfer effects but differed in the magnetization of the inflowing blood. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion. In the second acquisition, inflowing spins were left undisturbed. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion.

EVALUATION

Data were transferred to a PC and image analysis was carried out using the image processing software Scion Image for Windows (Scion Corporation, Frederick, Md., USA). Lesion volumes were calculated using ADC maps, as the ischemic lesion area was estimated by summing up all pixels with a relative ADC < 80% compared to the healthy, contralateral hemisphere (Hoehn-Berlage et al. 1995). Perfusion signal intensities are referred to the homologous contralateral regions and given as ratios of ipsilateral to contralateral values.

MODIFICATIONS OF THE METHOD

Edema following middle cerebral artery occlusion in spontaneously hypertensive rats was measured by magnetic resonance imaging (Seega and Elger 1993; Elger et al. 1994a). Magnetic resonance imaging was also used to determine the size of intracerebral hemorrhage in rats induced by stereotactic microinfusion of collagenase into the caudate putamen (Elger et al. 1994b).

Reese et al. (2000) visualized regional brain activation by bicucculine by functional magnetic resonance imaging. Time-resolved assessment of bicucculine-induced changes in local cerebral blood volume was achieved using magnetite nanoparticles as an intravascular contrast agent.

Pevsner et al. (2001) described a photothrombotic method of acute small stroke induction in rats with histopathologic and *in vivo* magnetic resonance imaging (MRI) observations from 3 to 6 h after irradiation, which is homologous to a human autopsy specimen. Utilizing 30 min of irradiation with minimal beam intensity (0.1 W/cm^2) cold white light in conjunction with 20 mg of intravenous (i.v.) rose bengal as a rapid infusion, small infarcts were induced photochemically in the frontal lobes of rats.

Using *in vivo* and *ex vivo* magnetic resonance imaging, Ohlstein et al. (2000) evaluated the effects of tranilast, an antiallergic drug, on neointima formation following balloon angioplasty of the rat coronary artery.

Swain et al. (2003) employed T-two-star (T_2*)-weighted and flow-alternating inversion recovery (FAIR) functional magnetic resonance imaging to assess chronic changes in blood volume and flow as a result of exercise in rats. Prolonged exercise induced angiogenesis and increased cerebral blood flow in primary motor cortex.

Using MRI evaluation of brain damage, Banfi et al. (2004) demonstrated that pentoxifylline prevents spontaneous brain ischemia in stroke-prone rats.

Henderson et al. (2004) studied functional magnetic resonance imaging during hypotension in young and adult cats.

In a magnetic resonance imaging study Shirhan et al. (2004) found that spermine reduces infarction and neurological deficit a rat model of middle cerebral artery occlusion.

Paczynski et al. (2000) studied the effects of fluid management on edema volume and midline shift in a rat model of ischemic stroke. MRI were obtained 24 h after the onset of ischemia so that the ratio of hemispheric volumes ipsilateral and contralateral to the infarct and the extent of midline shift could be obtained.

Cash et al. (2001) evaluated the effectiveness of aminoguanidine as a neuroprotective agent in a rat model of transient middle cerebral artery occlusion using serial magnetic resonance imaging.

The MRI protocol consisted of three interleaved imaging regimens: proton density-weighted imaging (PDWI), T_2-weighted imaging (T_2WI) and diffusion-weighted imaging (DWI). Repetition time (TR) was 3 s for each of the three regimens and echo times (TE)

were 70 ms for the T_2WI and DWI and 28 ms for the PDWI. For DWI, diffusion-sensitizing gradients (*b*-value 590×10^{-3} s/mm^2) were applied along the inferior to superior axis of the brain. Two averages were acquired per phase encode step. The images were collected at an in-plane resolution of 0.31×0.31 mm from 18 contiguous, 1-mm-thick slices, with a total acquisition time of 43 min.

REFERENCES AND FURTHER READING

Banfi C, Sironi L, de Simoni G, Gelosa P, Barcella S, Perego C, Gianazza E, Guetrini U, Tremoli E, Mussoni L (2004) Pentoxifylline prevents spontaneous brain ischemia in stroke-prone rats. J Pharmacol Exp Ther 310:890–895

Beckmann N, Tigani B, Mazzoni L, Fozzard JR (2003) Techniques: magnetic resonance imaging of the lung provides potential for non-invasive preclinical evaluation of drugs. Trends Pharmacol Sci 24:550–554

Beckmann N, Laurent D, Tigani B, Panizzutti R, Rudin M (2004) Magnetic resonance imaging in drug discovery: lessons from disease areas. Drug Dis Today 9:35–42

Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD (1996) Middle cerebral artery occlusion in the rat by intraluminal suture: neurological and pathological evaluation of an improved model. Stroke 27:1616–1623

Cash D, Beech JS, Rayne RC, Bath PMW, Meldrum BS, Williams SCR (2001) Neuroprotective effect of aminoguanidine on transient focal ischaemia in the rat brain. Brain Res 905:91–103

Cherry SR, Phelps ME (1996) Imaging brain function with positron emission tomography. In: Toga AW, Mazziotta JC (eds) Brain mapping: the methods. Academic Press, New York, pp 191–221

Elger B, Seega J, Raschack M (1994a) Oedema reduction by levemopamil in focal cerebral ischaemia of spontaneously hypertensive rats studied by magnetic resonance imaging. Eur J Pharmacol 254:65–71

Elger B, Seega J, Brendel R (1994b) Magnetic resonance imaging study on the effect of levemopamil on the size of intracerebral hemorrhage in rats. Stroke 25:1836–1841

Franke C, van Dorsten FA, Olah L, Schwindt W, Hoehn M (2000), Arterial spin tagging perfusion imaging of rat brain: dependency on magnetic field strength. Magn Reson Imaging 18:1109–1113

Henderson LA, Macey PM, Richard CA, Runquist ML, Harper RM (2004) Functional magnetic resonance imaging during hypotension in the developing animal. J Appl Physiol 97:2248–2257

Hoehn-Berlage M, Norris DG, Kohno K, Mies G, D. Leibfritz D, Hossmann KA (1995) Evolution of regional changes in apparent diffusion coefficient during focal ischemia of rat brain: the relationship of quantitative diffusion NMR imaging to reduction of cerebral blood flow and metabolic disturbances. J Cereb Blood Flow Metab 15:1002–1011

Kerskens CM, Hoehn-Berlage M, Schmitz B, Busch E, Bock C, Gyngell ML, Hossmann KA (1996) Ultrafast perfusion-weighted MRI of functional brain activation in rats during forepaw stimulation: comparison with T_2*-weighted MRI. NMR Biomed. 8:20–23

Le Bihan D, Breton E, Lallemand D, Grenier P, E. Cabanis E, Laval-Jeantet M (1986) MR imaging of intravoxel incoherent motions: application to diffusion and perfusion in neurologic disorders. Radiology 161:401–407

Leslie RA, James MF (2000) Pharmacological magnetic resonance imaging: a new application for functional MRI. Trends Pharmacol Sci 21:314–318

Ohlstein EH, Romanic AM, Clark LV, Kapadia RD, Sarkar SK, Gagnon R, Chandra S (2000) Application of in vivo and ex vivo magnetic resonance imaging for the evaluation of tranilast on neointima formation following balloon angioplasty of the rat coronary artery. Cardiovasc Res 47:759–768

Paczynski RP, Venkatesan R, Diringer MN, He YY, Hsu CY, Lin W (2000) Effects of fluid management on edema volume and midline shift in a rat model of ischemic stroke. Stroke 31:1702–1708

Petty MA, Neumann-Haefelin C, Kalisch J, Sarhan S, Wettstein JG, Juretschke HP (2003) In vivo neuroprotective effects of ACEA 1021 confirmed by magnetic resonance imaging in ischemic stroke. Eur J Pharmacol 474:53–62

Pevsner PH, Eichenbaum JW, Miller DC, Pivawer G, Eichenbaum KD, Stern A, Zakian KL, Koutcher JA (2001) A photothrombotic model of small early ischemic infarcts in the rat brain with histologic and MRI correlation. J Pharm Toxicol Meth 45:227–233

Reese T, Bjelke B, Porszacz R, Baumann D, Bochelen D, Sauter A, Rudin M (2000) Regional brain activation by bicucculine visualized by functional magnetic resonance imaging. Time-resolved assessment of bicucculine-induced changes in local cerebral blood volume using an intravascular contrast agent. NMR Biomed 13:43–49

Seega J, Elger B (1993) Diffusion- and *T*2-weighted imaging: evaluation of oedema reduction in focal cerebral ischaemia by the calcium and serotonin antagonist levemopamil. Magnet Reson Imag 11:401–409

Shirhan MD, Moochala SM, Ng PY, Lu J, Ng KC, Teo L, Yap E, Ng I, Hwang P, Lim T, Sitoh YY, Rumpel H, Jose R, Ling E (2004) Spermine reduces infarction and neurological deficit following a rat model of middle cerebral artery occlusion: a magnetic resonance imaging study. Neuroscience 124:299–304

Swain RA, Harris AB, Wiener EC, Dutka MV, Morris HD, Theien BE, Konda S, Engberg K, Lauterbur PC, Greenough WT (2003) Prolonged exercise induces angiogenesis and increases cerebral blood flow in primary motor cortex of the rat. Neuroscience 117:1037–1046

Zea Longa E, Weinstein PR, Carlson S, Cummins RW (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84–91

A.8.2 Peripheral Blood Supply

A.8.2.1 Perfused Hindquarter Preparation with Sympathetic Nerve Stimulation in Rats

PURPOSE AND RATIONALE

Perfusion of the hindquarter in rats with a constant flow rate allows the evaluation of the effect of drugs on the peripheral vascular bed. Since constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

PROCEDURE

Male Wistar rats weighing between 250 and 300 g are pre-treated with heparin (1000 units/kg i.v.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.) The animals are intubated with a tracheal tube and positive pressure ventilation is maintained with a Harvard Rodent Respirator at 4–6 ml/stroke and 50 strokes/min. The right jugular vein is cannulated with polyethylene tubing for administration of drugs.

The lumbar sympathetic chain is isolated dorsal to the inferior mesenteric branches via an abdominal midline incision. The aorta is freed from the vena cava and two silk ligatures are placed around the aorta. The aorta is ligated and cannulated proximal as well as distally with polyethylene tubings. A short piece of rubber tubing is inserted at the distal end to allow intraarterial injections of drugs. Two "T" junctions allow the measurement of arterial pressure and perfusion pressure by Statham P 23 Db pressure transducers being recorded through a Hellige physiological recorder. From the proximal part of the aorta, blood is forced to its distal part by a peristaltic pump (Desaga) through a glass coil kept at 40°C. Flow rate is adjusted to produce a stable perfusion pressure as close to the systemic pressure as possible. After initial adjustment, flow rate is not altered for the remainder of the experiment.

Following perfusion pressure stabilization, the sympathetic chain is isolated and a small (1 mm wide, 2 mm long) curved bipolar electrode is placed around the nerve for electrical stimulation. Square-wave pulses from a Grass stimulator are used to activate the nerve with a constant current of 2.5 milliamps, supramaximal voltage and varying frequencies of 5 ms duration.

A dose-response curve is established for norepinephrine by giving doses of 0.01 µg, 0.03 µg, 0.1 µg, 0.3 µg, 1.0 µg, and 3.0 µg intra-arterially and measuring perfusion pressure changes. Similarly, a frequency-response curve to nerve stimulation is established by stimulation at 3 Hz, 6 Hz, and 10 Hz for 30 s. Two predrug readings are taken to insure consistent responses.

A minimum of four animals is used for each test compound.

EVALUATION

The first predrug dose-response curves are compared with the second predrug, 5 min and 60 min postdrug dose-response curves. From regression equations for norepinephrine and nerve stimulation, mean responses and potency values with 95% confidence limits are calculated.

MODIFICATIONS OF THE METHOD

Folkow et al. (1970) perfused the hindquarters of spontaneously hypertensive rats and control rats at a constant rate of flow with an oxygenated plasma substitute in order to study the increased flow resistance and vascular reactivity. The hindquarters were isolated from the upper part of the body by standardized mass ligatures at identical levels until the aorta and the inferior caval vein provided the only intact circulatory connections between the two parts of each animal.

Thimm et al. (1984) described reflex increases in heart-rate induced by perfusing the hind leg of the rat with solutions containing lactic acid.

Thimm and Baum (1987) obtained spike recordings from chemosensitive nerve fibres of group III and IV of the rat nervus peroneus. Applications were performed either by perfusion of the circulatory isolated hindleg or by superfusion of the isolated musculus extensor digitorum longus.

Kitzen et al. (1978) used the perfused hind limb of the **dog** with sympathetic nerve stimulation for cardiovascular analysis.

Reitan et al. (1991) developed a near anesthetic-free isolated hindlimb model in the **dog** and studied the effects of halothane and atropine sulfate on vascular resistance.

Wiegershausen and Deptalla (1969) used the isolated perfused hindlimb of the **cat** to study the influence of local anesthetics on the vasoconstrictor actions induced by bradykinin, epinephrine and histamine.

Santiago et al. (1994) analyzed the responses to bradykinin in the hindquarters vascular bed of the cat.

Champion et al. (1996, 1997) analyzed the responses of human synthetic adrenomedullin, an analog of adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat.

REFERENCES AND FURTHER READING

Bhattacharya BK, Dadkar NK, Dohadwalla AN (1977) Vascular reactivity of perfused vascular bed in spontaneously hypertensive and normotensive rats. Br J Pharmacol 59:243–246

Bomzon A, Naidu SG (1985) Perfusion of the isolated rat hind limb. An analysis of the technique. J Pharmacol Meth 14:285–296

Brody MJ, Shaffer RA, Dixon RL (1963) A method for the study of peripheral vascular responses in the rat. J Appl Physiol 18:645–647

Champion HC, Duperier CD, Fitzgerald WE, Lambert DG, Murphy WA, Coy DH, Kadowitz PJ (1996) [Mpr^{14}]-rADM(14–50), a novel analog of adrenomedullin, possesses potent vasodilator activity in the hindlimb vascular bed of the cat. Life Sci 59: PL1–7

Champion HC, Akers DL, Santiago JA, Lambert DG, McNamara DB, Kadowitz PJ (1997) Analysis of the responses to human synthetic adrenomedullin and calcitonin gene-

related peptides in the hindlimb vascular bed of the cat. Mol Cell Biochem 176:5–11
Dadkar NK, Dohadwalla AN, Bhattacharya BK (1977) The effect of tyramine on peripheral vasculature of the spontaneously hypertensive rat. J Pharm Pharmacol 29:48–49
Folkow B, Hallbäck M, Lundgren Y, Weiss L (1970) Background of increased flow resistance and vascular reactivity in spontaneously hypertensive rats. Acta Physiol Scand 80:93–106
Kitzen JM, Long JP, Cannon JG (1978) Pharmacology of 6,7-dihydroxy-2-dimethylaminotetralin (TL-99). I. Cardiovascular activity in the dog and cat. J Pharm Exp Ther 206:239–247
Reitan JA, Kien ND, Martucci RW, Thorup SJ, Dennis PJ (1991) Development of a near anesthetic-free isolated canine hindlimb model. The effects of halothane and atropine sulfate on vascular resistance. J Pharmacol Meth 26:223–232
Ross BD (1972) Hind-limb perfusion. In: Perfusion techniques in biochemistry. A laboratory manual in the use of isolated perfused organs in biochemical experimentation. Clarendon Press, Oxford, pp 308–320
Ruderman NB, Houghton CRS, Hems R (1971) Evaluation of the isolated perfused rat hind-quarter for the study of muscle metabolism. Biochem J 124:639–651
Santiago JA, Garrison EA, Kadowith PJ (1994) Analysis of responses to bradykinin: effects of Hoe-140 in the hindquarters vascular bed of the cat. Am J Physiol 267 (Heart Circ Physiol 36): H828–H836
Thimm F, Baum K (1987) Response of chemosensitive nerve fibres of group III and IV to metabolic changes in rat muscles. Pflügers Arch 410:143–152
Thimm F, Carvalho M, Babka M, Meier zu Verl E (1984) Reflex increases in heart-rate induced by perfusing the hind leg of the rat with solutions containing lactic acid. Pflügers Arch 400:286–293
Werber AH, Fink GD (1985) Continuous measurement of hindquarter resistance changes to nerve stimulation and intra-arterial drug administration in rats. J Pharmacol Meth 13:67–82
Wiegershausen B, Deptalla H (1969) Der Einfluß einiger Lokalanästhetika auf die erregende Wirkung von Bradykinin am glatten Muskel. 1. Mitteilung: Die Verstärkung der vasokonstriktorischen Wirkung von Bradykinin an der isolierten Hinterpfote der Katze. Arch Int Pharmacodyn 177:278–286

A.8.2.2 Effect on Peripheral Blood Flow in Rats

PURPOSE AND RATIONALE

Various methods exist to measure peripheral blood flow in rats, such as the microsphere technique, electromagnetic flowmetry and Doppler ultrasonic flowmetry.

Radioactive microspheres are used to calculate the distribution of blood from the heart to various organs and tissues before and after the administration of test compounds. In this method, microspheres are injected into the left cardiac ventricle. It is postulated that the first contraction of the cardiac muscle will expel these spheres into the circulation. Consequently the microspheres can be trapped in different organs according to the organ's perfusion rate. It is not a primary screening method but it is a useful test for distinguishing compounds with blood flow altering activities.

The microspheres used are 14 µ in diameter. They are marked with isotopes. In this test, four different radioactive elements are used (Cr^{51}, Sr^{85}, Sc^{46}, Ce^{141}), allowing the determination of blood flow before dosing and after the administration of 3 different compounds or 3 different doses (of the same compound).

PROCEDURE

Male Wistar rats weighing 500–550 g are anaesthetized with pentobarbital. The trachea is exposed and intubated with a short endotracheal tube to allow ventilation. Prior to testing the jugular vein is cannulated for administration of test drugs. The carotid artery is cannulated and later on the catheter is passed retrograde into the ventricle. Ventricular pressure is recorded to assure the correct emplacement of the catheter tip in the ventricle. This catheter is connected to another catheter allowing the injection of microspheres into the left ventricle later on. The right arteria brachialis is cannulated and connected to a Hellige blood pressure recorder. During the course of the test, blood pressure will be measured continuously. The left femoral artery is cannulated and connected to an infusion pump. During the experiment blood will be withdrawn from this artery.

After these preparations the rat is allowed 15–30 min to recover. Before actually starting the experiment, the arterial blood gas concentration of each animal is measured. Animals are only used for the experiment if they show normal blood gas concentrations. During the following procedure blood pressure, ventricular pressure and the heart rate are continuously recorded. To determine baseline blood flow animals receive 0.2 ml vehicle/min over a 3 min period. In the 4th minute rats receive the first injection of microspheres (Cr^{51}). Simultaneously 0.5 ml/min blood is withdrawn from the femoral artery catheter for one minute, the pump thus being used as a reference organ. The animals are allowed 20 min to recover before the administration of drugs. The test compound is infused into the jugular vein at a rate of 0.2 ml/min for 3 min followed by injection of the second microsphere (Sr^{85}). The same procedure is repeated using the other two microspheres (Sc^{46} and Ce^{141}) following administration of the second and third test compound.

At the end of the experiment blood gas concentrations are measured. The animals are killed and their organs are removed. Usually blood flow is determined in the following organs:

- brain (right and left hemisphere; right hemisphere showing slight ischemia due to cannulation of carotis)
- cerebellum
- lungs
- heart
- kidney (right and left)
- skeletal muscle (right hind extremity)
- duodenum
- stomach
- spleen
- diaphragm
- adrenal gland (right and left)

To determine effects of test compounds on the blood flow in the underperfused skeletal muscle the same experiment can be performed with the right femoral artery being clamped. In this way effects of the test drug on the ischemic and normal skeletal muscle (left thoracic limb) can be compared in the same animal.

EVALUATION

The rate of blood flow/tissue at a certain time is determined by measuring radioactivity in the different tissues and comparing the results to that of the blood sample.

MODIFICATIONS OF THE METHOD

Blood flow in various peripheral organs, e. g., renal blood, flow can be measured with electromagnetic flowmeters (e. g., Transflow 601, Skalar Medical, Holland) or with Doppler ultrasonic flowmetry (Shaffer and Medvedev 1991).

Lappe et al. (1986) studied regional vascular resistance in conscious spontaneously hypertensive rats which were chronically instrumented with pulse Doppler flow probes to allow measurement of renal, mesenteric and hind quarters blood flow.

Hartman et al. (1994) validated a transit-time ultrasonic volume flow meter by simultaneous measurements with an electromagnetic flow metering method.

Lepore et al. (1999) used electron paramagnetic resonance to investigate the time course of nitric oxide generation and its susceptibility of nitric oxide synthase in ischemia-reperfusion injury to rat skeletal muscle *in vivo*. Total hind limb ischemia was applied for 2 h using a rubber band tourniquet method. At the end of ischemia the tourniquet was removed and the limb allowed to reperfuse for various time intervals.

Beattie et al. (1995) measured carotid arterial vascular resistance in anesthetized **rabbits**. Carotid blood flow was measured by a Doppler flow probe placed around the right common carotid artery. Dose-response curves of reduction of carotid arterial vascular resistance were constructed after injection of various doses of substance P-methyl ester via the right lingual artery. Intravenous injection of various doses of a selective tachykinin NK_1 receptor antagonist inhibited this effect dose-dependently.

REFERENCES AND FURTHER READING

Beattie DT, Beresford IJM, Connor HE, Marshall FH, Hawcock AB, Hagen RM, Bowers J, Birch PJ, Ward P (1995) The pharmacology of GR203040, a novel, potent and selective tachykinin NK_1 receptor antagonist. Br J Pharmacol 116:3149–3157

Hartman JC, Olszanski DA, Hullinger TG, Brunden MN (1994) *In vivo* validation of a transit-time ultrasonic volume flow meter. J Pharmacol Toxicol Meth 31:153–160

Heiss WD, Traupe H (1981) Comparison between hydrogen clearance and microsphere technique for rCBF measurement. Stroke 12:161–167

Hof RP, Wyler F, Stalder G (1980) Validation studies for the use of the microsphere method in cats and young minipigs. Basic Res Cardiol 75:747–756

Lappe RW, Todt JA, Wendt RL (1986) Effect of fenoldopam on regional vascular resistance in conscious spontaneously hypertensive rats. J Pharm Exp Ther 236:187–191

Lepore DA, Kozlov AV, Stewart AG, Hurley JV, Morrison WA, Tomasi A (1999) Nitric oxide synthase-independent generation of nitric oxide in rat skeletal muscle ischemic-perfusion injury. Nitric Oxide: Biol Chem 3:75–84

Marcus ML, Heistad DD, Ehrhardt JC, Abboud FM (1976) Total and regional cerebral blood flow measurements with 7–10, 15, 25 and 50 µm microspheres. J Appl Physiol 40:501–507

Shaffer RA, Medvedev OS (1991) New applications of a 20-Mhz Doppler ultrasonic flowmeter. In: 7th Freiburg Focus on Biomeasurement. Cardiovascular and Respiratory *in vivo* Studies. Biomesstechnik-Verlag March GmbH, 79232 March, Germany, pp 142–147

Vetterlein F, Halfter R, Schmidt G (1979) Regional blood flow determination in rats by the microsphere method during i.v. infusion of vasodilating agents. Arzneim-Forsch/Drug Res 29:747–751

A.8.2.3 Effect on Peripheral Blood Flow in Anesthetized Dogs

PURPOSE AND RATIONALE

Effects on blood pressure have to be analyzed whether they are mediated by central, cardial, or peripheral action. By injecting small doses of the test compound directly into a vascular bed, thus avoiding changes of central hemodynamics, peripheral vasodilating activity of a compound can be tested. This test is used to evaluate direct vasodilating or constricting activities of drugs *in vivo*-measurements of blood flow.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used. The dogs are premedi-

cated intravenously with heparin (bolus of 500 IU/kg and successive injections of 50 IU/kg every 30 min) and anesthetized by intravenous injection of thiobarbital sodium (0.5 mg/kg i.v.), chloralose (20 mg/kg i.v.) and urethane (250 mg/kg i.v.). Respiration is maintained with room air through a tracheal tube using a positive pressure respirator. Blood gas analysis is performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

To measure peripheral blood flow and to administer the test substance, a bypass is inserted into a femoral artery incorporating an electromagnetic flow probe and a port for injections. The other femoral artery is also equipped with a bypass used for the administration of a reference compound or a second test drug.

For recording of peripheral blood pressure and heart rate, one of the bypasses is connected to a pressure transducer (Statham P 23 BD).

All parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 20 min, the vehicle is administered (control), and 10 min later the test compound. Immediately after each administration, the port is flushed with physiological saline. Successive doses are administered after recovery to baseline values.

Readings are taken at times 0, 0.5, 1, 2, 5 and 10 min, and, if necessary, at additional 10 min-intervals following drug administration.

Standard compound:

- carbocromene 1 mg/kg

Characteristics:

- blood pressure
  – systolic, BPs
  – diastolic, BPd
- heart rate, HR
- peripheral blood flow, PF

EVALUATION

Changes in blood pressure, heart rate and peripheral blood flow at different times after drug administration are compared to vehicle control values obtained in the 10 min pre-drug period.

With $n > 2$, results are presented as mean $\pm$ SEM. Statistical significance is assessed by means of the paired *t*-test. Scores are compared to the efficacy of standard compounds for intensity and for duration of the effect.

MODIFICATIONS OF THE METHOD

Regional blood flow can be determined by the use of microspheres (Rudolph and Heyman 1967). The method is based on the principle that biologically inert microspheres will be trapped due their diameter in the microvasculature (Hales and Cliff 1977). The use of radioactive microspheres has some disadvantages (Buckberg et al. 1971). The use of fluorescent labeled microspheres for measurement of regional organ perfusion has been recommended (Glenny et al. 1993; Prinzen and Glenny 1994; van Oosterhout et al. 1995). Raab et al. (1999), Thein et al. (2000) described the automation of the use of fluorescent microspheres using a special sample processing unit. A Zymate-Robotic System (Zymark, Idstein, Germany) was modified to handle a special filtration device.

Ebara et al. (1994) measured renal blood flow in dogs after intrarenal arterial infusion of adrenomedullin.

REFERENCES AND FURTHER READING

Buckberg GD, Luck JC, Payne DB, Hoffmann JIE, Archie JP, Fixler DE (1971) Some sources of error in measuring blood flow with radioactive microspheres. J Appl Physiol 31:589–604

Ebara T, Miura K, Okumura M, Matsuura T, Kim S, Yukimura T, Iwao H (1994) Effect of adrenomedullin on renal hemodynamics and function in dogs. Eur J Pharmacol 263:69–73

Glenny RW, Bernard S, Brinkley M (1993) Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. J Appl Physiol 74:2585–2597

Hales JRS, Cliff WJ (1977) Direct observations of the behavior of microspheres in the microvasculature. Bibliotheca Anatom 15:87–91

Prinzen FW, Glenny RW (1994) Development of non-radioactive microsphere technique for blood flow measurement. Cardiovasc Res 28:1467–1475

Raab S, Thein N, Harris AG, Messmer K (1999) A new sample-processing unit for the fluorescent microsphere method. Am J Physiol 276 (Heart Circ Physiol 45): H1801–H1806

Rudolph A, Heyman MA (1967) The circulation of the fetus in utero; methods for studying distribution of cardiac output and organ blood flow. Circ Res 21:163–184

Thein E, Raab S, Harris AG, Messmer K (2000) Automation of the use of fluorescent microspheres for the determination of blood flow. Comp Meth Progr Biomed 61:11–21

Turner RA (1971) β-adrenergic blocking agents. In: Turner RA, Hebborn P (eds) Screening methods in pharmacology. Vol II, pp 21–40. Academic Press, New York and London

Van Oosterhout MF, Willigers HM, Reneman RS (1995) Fluorescent microspheres to measure organ perfusion: validation of o simplified sample processing technique. Am J Physiol 269 (Heart Circ Physiol 38):H725–H733

A.8.2.4
Effect on Peripheral Blood Supply Measured by Local Oxygen Pressure

PURPOSE AND RATIONALE

Local oxygen pressure is directly related to oxygen supply to peripheral organs, e. g., muscle (Luebbers 1969; Kessler 1969). The local oxygen pressure (PO_2) is recorded directly on the muscle surface. In the following procedure the effect of test compounds on the local oxygen pressure (PO_2) of the normal and the ischemic skeletal muscle is determined.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The dog is anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal). Prior to testing, the following vessels have to be cannulated: The V. femoralis of the left pelvic limb is cannulated for administration of test compounds. The A. femoralis of the left pelvic limb is cannulated for blood pressure recording. The V. femoralis of the right pelvic limb is cannulated. During the course of this test blood will be withdrawn from this vein to monitor lactate concentrations.

Small areas of muscles of the right pelvic limb and the right thoracic limb are exposed. Muscle relaxation is induced by intravenous injection of 0.1 mg/kg alcuronium chloride (Alloferin) and maintained by i.p. administration of 0.05 mg/kg Alloferin at 30 min intervals. The trachea is exposed and intubated to assist the dog's respiration.

A PO_2 electrode is placed on the exposed muscle area of the right hind limb. After stabilization of PO_2 curves, the femoral artery of the right hind limb is occluded by putting a clip around the vessel. Muscle PO_2 drops rapidly. Following stabilization, test compounds are given by intravenous infusion for 10 min or by intraduodenal administration at this stage. The PO_2 of the non ischemic muscle is recorded simultaneously via a second electrode on the right thoracic extremity. The clip is removed after maximally one hour. This procedure can be repeated up to four times in one animal. Blood gas concentrations and pH are determined at the beginning and end of each experiment.

Standard compound:

- pentoxifylline

EVALUATION

The following parameters are determined:

- Maximal increase in PO_2 (mm Hg) after administration of test drug
- duration of effect by determining the half life

REFERENCES AND FURTHER READING

Dawson JM, Okyayuz-Baklouti I, Hudlická O (1990) Skeletal muscle microcirculation: the effects of limited blood flow and treatment with torbafylline. Int J Microcirc Exp 9:385–400

Ehrly AM, Schroeder W (1976) Sauerstoffdruckwerte im ischaemischen Muskelgewebe von Patienten mit chronischen arteriellen Verschlußerkrankungen. Verh Dtsch Ges Kreislaufforsch 42:380–384

Kessler M, Grunewald W (1969) Possibilities of measuring oxygen pressure fields in tissue by multiwire platinum electrodes. Progr Resp Res 3:147–152

Kessler M, Hoeper J, Krumme BA (1976) Monitoring of tissue perfusion and cellular function. Anesthesiology 45:184–197

Kunze K, Berk H (1975) Oxygen supply and muscle blood flow in normal and diseased muscle In: Kunze K, Desmedt JE (eds) Studies on Neuromuscular Diseases. Karger, Basel p 82–91

Luebbers DW, Baumgärtl H, Fabel H, Huch A, Kessler M, Kunze K, Riemann H, Seiler D, Schuchardt S (1969) Principle of construction and application of various platinum electrodes. Progr Resp Res 3:136–146

A.8.2.5
Effect on Mesenteric Blood Flow in Rats

PURPOSE AND RATIONALE

Blood flow in the mesenteric vascular bed *in situ* (Bhattacharya et al. 1977; Eikenburg 1984; Randall et al. 1989; Jackson and Inagami 1990) can be studied in rats in a way similar to that of the perfused hindquarter.

PROCEDURE

Wistar rats of either sex weighing 250–300 g are anesthetized with a combination of urethane (500 mg/kg i.v.) and sodium pentobarbitone (30 mg/kg). The abdomen is opened by a midline incision and a segment of the superior mesenteric artery is exposed by careful dissection of the surrounding tissue. Care is taken to avoid damage to the accompanying nerve terminals. One cannula is inserted into the carotid artery and the other into the superior mesenteric artery. Blood from the carotid artery is forced by a peristaltic pump (Desaga) into the superior mesenteric artery, using a glass coil kept at 40°C. Blood required to fill the tubing initially is obtained from donor rats. Heparin is administered intravenously to the animals prior to cannulation of the mesenteric artery. The blood pressure and the perfusion pressure are measured by Statham P 23 Db pressure transducers and recorded through a 2-channel Hellige recorder. The pump speed is initially

adjusted so that the perfusion pressure equals the systemic blood pressure. Intraarterial injections into the mesenteric vascular bed are made by puncturing the tubing going towards the periphery. Intravenous injections are made through a cannula inserted into the external jugular vein.

EVALUATION

Changes in the vascular resistance are measured by comparing perfusion pressure before and after drug administration. If constant blood flow is maintained, changes in the vascular resistance of the perfused bed are directly proportional to changes in the perfusion pressure.

MODIFICATIONS OF THE METHOD

Reactivity in the mesenteric vascular bed can be tested in an isolated preparation (McGregor 1965; Kawasaki and Takasaki 1984; Laher and Triggle 1984; McAdams 1984; Foy and Nuhu 1985; Longhurst and Head 1985; Soma et al. 1985; Hsueh et al. 1986; Longhurst et al. 1986; Manzini and Perretti 1988; Nassar et al. 1988; Randall and Hiley 1988). The abdomen of anesthetized rats is opened and the superior mesenteric artery is separated from surrounding tissue in the region of the aorta. A cannula is inserted into the superior mesenteric artery at its origin from the abdominal aorta. The cannula is filled with heparinized Krebs solution. The ileocoeliac branch of the artery is tied off and the intestine separated from the mesentery by cutting close to the intestinal border of the mesentery. The cannulated artery and its vascular bed are dissected out and mounted in an organ bath. The preparation is perfused with oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. Perfusion pressure is recorded via a side arm of the arterial cannula using a Statham pressure transducer. The flow rate is adjusted to give a baseline perfusion pressure of 20–30 mm Hg. The test substances are infused into another side arm of the arterial cannula for 15 s using an infusion pump. After three stimuli with norepinephrine (1 µg) or potassium chloride (1 mg), the test drugs are infused followed by further stimulation. The inhibition of increase of perfusion pressure after test drugs is expressed as percentage of control.

Nuki et al. (1994) compared the vasodilating activity of chicken calcitonin gene-related peptide with human α-CGRP and rat CGRP in the precontracted mesenteric vascular bed of rats.

The **rabbit** isolated arterially perfused intestinal segment preparation was used by Brown et al. (1983) as a model for vascular dopamine receptors.

Komidori et al. (1992) recommended the isolated rat mesenteric vascular-intestinal loop preparation as an excellent model fort demonstrating resistance changes in isolated vascular beds while simultaneously measuring endogenous catecholamine overflow.

Pelissier et al. (1992) showed that perfusion with hypotonic solutions removed the endothelial layer in the isolated perfused mesenteric vascular bed of the rat, allowing the study of endothelial-dependent vascular responses.

Santiago et al. (1993) used the mesenteric vascular bed of the **cat** to study the inhibitory effects of the bradykinin receptor antagonist Hoe 140 on vascular responses to bradykinin.

The responses of adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat were compared by Santiago et al. (1995).

Chu and Beilin (1994) studied the mesenteric vascular reactivity which is reduced in pregnant rats after application of bradykinin and the bradykinin receptor antagonist Hoe 140.

Mulavi and Halpern (1977), Qiu et al. (1995) studied the mechanical and contractile properties of *in situ* localized mesenteric arteries in normotensive and spontaneously hypertensive rats.

REFERENCES AND FURTHER READING

Bhattacharya BK, Dadkar NK, Dohadwalla AN (1977) Vascular reactivity of perfused mesenteric vascular bed in spontaneously hypertensive and normotensive rats. Br J Pharmacol 59:243–246

Brown RA, O'Connor SE, Smith GW, Verity A (1983) The rabbit isolated arterially perfused intestinal segment preparation: A model for vascular dopamine receptors. J Pharmacol Meth 9:137–145

Chu ZM, Beilin LJ (1994) Effects of HOE 140 on systemic depressor responses to bradykinin and mesenteric vascular reactivity *in vivo* in pregnant Wistar–Kyoto rats. Clin Exp Pharmacol Physiol 21:137–140

Eikenburg DC (1984) Functional characterization of the pre- and postjunctional α-adrenoceptors in the *in situ* perfused rat mesenteric vascular bed. Eur J Pharmacol 105:161–165

Foy JM, Nuhu SZ (1985) Effect of three 'loop' diuretics and prostaglandins E_2 & I_2 on the isolated perfused rat mesenteric vasculature. Arch Int Pharmacodyn 273:237–250

Hsueh W, Gonzalez-Crussi F, Arroyave JL (1986) Release of leukotriene C_4 by isolated, perfused rat small intestine in response to platelet-activating factor. J Clin Invest 78:108–114

Jackson EK, Inagami T (1990) Blockade of the pre- and postjunctional effects of angiotensin *in vivo* with a non-peptide angiotensin receptor antagonist. Life Sci 46:945–953

Kawasaki H, Takasaki K (1984) Vasoconstrictor response induced by 5-hydroxytryptamine released from vascular adrenergic nerves by periarterial nerve stimulation. J Pharm Exp Ther 229:816–822

Komidori H, Yamamoto R, Nickols GA, Takasaki K (1992) Characterization of the isolated rat mesenteric vascular-intestinal loop preparation. J Pharmacol Meth 27:59–65
Laher I, Triggle CR (1984) Pharmacological studies of smooth muscle from Dahl salt-sensitive and salt-resistant rats. Can J Physiol Pharmacol 62:101–104
Longhurst PA, Head JH (1985) Responses of the isolated perfused mesenteric vasculature from diabetic rats: the significance of appropriate control tissues. J Pharm Exp Ther 235:45–49
Longhurst PA, Stitzel RE, Head RJ (1986) Perfusion of the intact and partially isolated mesenteric vascular bed: Application to vessels from hypertensive and normotensive rats. Blood Vessels 23:288–296
Manzini S, Perretti F (1988) Vascular effects of capsaicin in isolated perfused rat mesenteric bed. Eur J Pharmacol 148:153–159
McAdams RP (1984) The effect of temperature on the α-adrenoreceptor antagonist potency of indoramin and labetalol in the rat perfused mesenteric vascular bed. J Pharm Pharmacol 36:628–629
McGregor DD (1965) The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric blood vessels of the rat. J Physiol (London) 177:21–30
Mulavi NJ, Halpern W (1977) Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 41:19–26
Nassar BA, Huang YS, McDonald ATJ, Jenkins KD, Horrobin DF (1988) The influence of phenelzine and tranylcypromine on the release of prostaglandins from the rat mesenteric vascular bed. Can J Physiol Pharmacol 66:1206–1209
Nuki C, Kawasaki H, Takasaki K, Wada A (1994) Structure-activity study of chicken calcitonin gene-related peptide (CGRP) on vasorelaxation in rat mesenteric resistance vessels
Pelissier T, Miranda HF, Bustamante D, Paeile C, Pinardi G (1992) Removal of the endothelial layer in perfused mesenteric vascular bed of the rat. J Pharmacol Meth 27:41–44
Qiu HY, Valtier B, Struyker-Boudier HAJ, Levy BI (1995) Mechanical and contractile properties of *in situ* localized mesenteric arteries in normotensive and spontaneously hypertensive rats. J Pharmacol Toxicol Meth 33:159–170
Randall MD, Hiley CR (1988) Effect of phenobarbitone pretreatment upon endothelium-dependent relaxation to acetylcholine in rat superior mesenteric arterial bed. Br J Pharmacol 94:977–983
Randall MD, Douglas SA, Hiley CR (1989) Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat. Br J Pharmacol 98:685–699
Santiago JA, Osei SY, Kadowith PJ (1993) Inhibitory effects of Hoe 140 on vascular responses to bradykinin in the mesenteric vascular bed of the cat. Eur J Pharmacol 236:315–318
Santiago JA, Garrison E, Purnell WL, Smith RE, Champion HC, Coy DH, Murphy WA, Kadowitz PJ (1995) Comparison of responses to adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat. Eur J Pharmacol 272:115–118
Soma M, Manku DK, Horrobin DF (1985) Prostaglandins and thromboxane outflow from the perfused mesenteric vascular bed in spontaneously hypertensive rats. Prostaglandins 29:323–333

A.8.2.6
Effect on Pulmonary Blood Flow

PURPOSE AND RATIONALE

During controlled pulmonary blood flow, lobar arterial pressure can be measured in anesthetized cats (Lippton et al. 1984; Hyman et al. 1989; McMahon and Kadowitz 1993; deWitt et al. 1994).

PROCEDURE

Adult cats of either sex weighing 2.5 to 4.5 kg are sedated with ketamine hydrochloride (10–15 mg/kg i.m.) and anesthetized with pentobarbital sodium (30 mg/kg i.v.). The animals are fixed in supine position and supplemental doses of anesthetic are administered to maintain a uniform level of anesthesia. The trachea is intubated and the animals breath room air enriched with 95% O_2/5% CO_2. Systemic arterial pressure is measured from a catheter inserted into the aorta from a femoral artery, and intravenous injections are made from a catheter positioned in the inferior vena cava from a femoral vein.

For perfusion of the left lower lung lobe, a special designed 28-cm 6F triple-lumen balloon perfusion catheter (Arrow International, Reading, PA) is passed under fluoroscopic guidance from the left external jugular vein into the artery to the left lower lobe. The animal is heparinized by 1000 IU/kg i.v., and the lobar artery is isolated by distension of the balloon cuff on the perfusion catheter. The lobe is then perfused by way of the catheter lumen beyond the balloon cuff, with blood withdrawn from a femoral artery with a perfusion pump. Lobar arterial pressure is measured from a second port 5 mm from the cuff on the perfusion catheter. The perfusion rate is adjusted so that lobar arterial perfusion pressure approximates mean pressure in the main pulmonary artery. Left atrial pressure is measured with a 6F double-lumen catheter passed transseptally into the vein draining the left lower lobe. The catheter tip is positioned so that the left atrial pressure port on the distal lumen is 1–2 cm into the lobar vein and the second catheter port is near the venoatrial junction.

Lobar arterial pressure can be elevated to an high steady state level by the administration of N^{ω}-nitro-L-arginine$^{\omega}$, followed by an intralobar infusion of the stable prostaglandin/endoperoxide analogue U-46619.

EVALUATION

Dose-response curves after administration of graded doses of drugs, e. g., decrease of lobar arterial pressure

after various doses of bradykinin, are established. The effects of antagonists, e. g. HOE 140, can be studied.

MODIFICATIONS OF THE METHOD

Liu et al. (1992) used a blood-perfused rat lung preparation to study pulmonary vasoconstriction or endothelium-dependent relaxation.

Byron et al. (1986) studied the deposition and airway-to-perfusate transfer of disodium fluorescein from 3–4 μm solid aerosols in an isolated perfused lung preparation of rats.

Mor et al. (1990) determined angiotensin-converting enzyme activity in the isolated perfused **guinea pig** lung.

Franks et al. (1990) used in Beagle **dogs** a single breath technique employing freon-22 as the soluble marker gas simultaneously with measurement of aortic blood flow by an electromagnetic flowmeter.

Tanaka et al. (1992) measured lung water content in dogs with acute pulmonary hypertension induced by injection of glass beads.

Drake et al. (1978) studied filtration characteristics of the exchange vessels in isolated dog lung by calculating the volume conductance with use of different components of the weight-gain curve following changes in capillary pressure.

Heaton et al. (1995) studied the effects of human adrenomedullin on the pulmonary vascular bed of isolated, blood perfused rat lung.

DeWitt et al. (1994), Lippton et al. (1994) investigated the effects of adrenomedullin in the pulmonary and systemic vascular bed of the **cat**.

Nossaman et al. (1995) compared the effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat.

REFERENCES AND FURTHER READING

Baum MD, Kot PA (1972) Responses of pulmonary vascular segments to angiotensin and norepinephrine. J Thorac Cardiovasc Surg 63:322–328

Byron PR, Roberts NSR, Clark AR (1986) An isolated perfused rat lung preparation for the study aerosolized drug deposition and absorption. J Pharmaceut Sci 75:168–171

DeWitt BJ, Cheng DY, McMahon TJ, Nossaman BD, Kadowitz PJ (1994a) Analysis of responses to bradykinin in the pulmonary vascular bed of the cat. Am J Physiol, Heart Circ Physiol 266:H2256–H2267

DeWitt BJ, Cheng DY, Caminiti GN, Nossaman BD, Coy DH, Murphy WA, Kadowitz PJ (1994b) Comparison of responses to adrenomedullin and calcitonin gene-related peptide in the pulmonary vascular bed of the cat. Eur J Pharmacol 257:303–306

Drake R, Gaar KA, Taylor AE (1978) Estimation of the filtration coefficient of pulmonary exchange vessels. Am J Physiol 234:H266–H274

Franks PJ, Hooper RH, Humphries RG, Jones PRM, O'Connor SE (1990) Effective pulmonary flow, aortic flow and cardiac output: *in vitro* and *in vivo* comparison in the dog. Exper Physiol 75:95–106

Heaton J, Lin B, Chang J-K, Steinberg S, Hyman A, Lippton H (1995) Pulmonary vasodilation to adrenomedullin: A novel peptide in humans. Am J Physiol 268, Heart Circ Physiol 37:H2211–H2215

Hyman AL, Kadowitz PJ, Lippton HL (1989) Methylene blue selectively inhibits pulmonary vasodilator response in cats. J Appl Physiol 66:1513–1517

Lippton H, Chang J-K, Hao Q, Summer W, Hyman AL (1994) Adrenomedullin dilates the pulmonary vascular bed *in vivo*. J Appl Physiol 76:2154–2156

Lippton HL, Nandiwada PA, Hyman AL, Kadowitz PJ (1984) Influence of cyclo-oxygenase blockade on responses to isoproterenol, bradykinin and nitroglycerin in the feline pulmonary vascular bed. Prostaglandins 28:253–270

Liu SF, Dewar A, Crawley DE, Barnes PJ, Evans TW (1992) Effect of tumor necrosis factor on hypoxic pulmonary vasoconstriction. J Appl Physiol 72:1044–1049

McMahon TJ, Kadowitz PJ (1993) Analysis of responses to substance P in the pulmonary vascular bed of the cat. Am J Physiol 264 (Heart Circ Physiol 33): H394–H402

Mor L, Bomzon A, Frenkel R, Youdim MBH (1990) Angiotensin-converting enzyme activity in the isolated perfused guinea pig lung. J Pharmacol Meth 23:141–153

Nossaman BD, Feng CJ, Cheng DY, Dewitt BJ, Coy DH, Murphy WA, Kadowitz PJ (1995) Comparative effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and the rat. Life Sci 56:63–66

Tanaka H, Tajimi K, Matsumoto A, Kobayashi K (1992) Effects of milrinone on lung water content in dogs with acute pulmonary hypertension. J Pharmacol Toxicol Meth 28:201–208

A.8.2.7 Effect on Contractile Force of Ischemic Muscle

PURPOSE AND RATIONALE

Skeletal muscle is stimulated until it reaches its maximal force of contraction. This means that the muscle is forced to function at a level near exhaustion and has to use maximally the substrate supplied by the circulation. Then, the femoral artery is occluded, leading to underperfusion and a subsequent lack of substrate. As a consequence, the muscle's force of contraction decreases rapidly. Measuring the change in contractile force caused by drug administration reveals a drug's ability to restore ischemic muscle functions. In the following procedure, the drugs are tested for their effect on the force of contraction of the ischemic skeletal muscle.

PROCEDURE

Male Wistar rats weighing 400–450 g are anaesthetized by intraperitoneal administration of pentobarbital (Nembutal) (35 mg/kg). A tracheal tube is placed

to assist the rat's ventilation. The left carotid artery is cannulated for blood pressure recording and the left jugular vein is cannulated for the i.v. administration of test drugs. An incision is made to the skin of the right pelvic limb distal to the groin and the skeletal muscle is exposed down to the ankle. The skin is carefully trimmed away from the muscle to assure that contractions cannot be impaired by retraction of the skin. The major nerve supply is severed and a small length of the descending branch of the femoral artery is prepared free. The freely hanging muscle is attached to the force transducer (range 0–500 g, Z 6, Rhema, Germany) and a resting tension of 50 g is placed on the muscle. To prevent dehydration, the skin is left attached to the muscle and the muscle is kept moist by the continuous drip of a 0.9% NaCl-solution.

After these preparations, the rat is allowed to recover at least 30 min. Two needle electrodes are inserted into the muscle. Square impulses of 40 ms are generated with Stimulator 1 (Hugo Sachs Elektronik, Freiburg, Germany). The muscle is stimulated with a frequency of about 80 contractions per minute. The amplitude is increased gradually up to the muscle's maximal contractile force (usually between 2.0 and 3 mA). Following stabilization, the femoral artery is occluded with a clip for 5 min and subsequently reopened. After at least 15 min, test drugs are administered by intravenous infusion (0.075 ml/min) for 10 min. Five minutes after starting drug infusion, the artery is clamped again (for 5 min) while drug infusion is still going on. The force of contraction is continuously recorded. After declamping the artery, the rat is allowed to recover for at least 30 min before the whole procedure is repeated with another test drug. In this way, 3 different compounds can be tested in the same animal.

Standard compound:

- pentoxifylline

EVALUATION

The following parameters are measured:

- the percent inhibition of contractile force before drug administration (artery being clamped)
- the percent inhibition of contractile force after drug administration (artery being clamped)

The percent increase in contractile force after drug administration is calculated.

CRITICAL ASSESSMENT OF THE METHOD

An attempt is made to measure not only the effects of the drug on vasculature tonus but also on muscle metabolism.

MODIFICATIONS OF THE METHOD

Weselcouch and Demusz (1990) studied drug effects in the ischemic hindlimbs of **ferrets**. The hindlimb was stimulated to contract isometrically via supramaximal electrical stimulation of the sciatic nerve. Ischemia was induced by partial occlusion of the abdominal aorta. Pentoxifylline attenuated the loss of function in a dose-related manner.

Okyayuz-Baklouti et al. (1992) studied the functional, histomorphological and biochemical changes in atrophying skeletal muscle using a novel immobilization model in the rat.

Le Tallec et al. (1996) reported the effects of dimethylformamide on *in vivo* fatigue and metabolism in rat skeletal muscle measured by 31P nuclear magnetic resonance spectroscopy.

REFERENCES AND FURTHER READING

Angersbach D, Ochlich P (1984) The effect of 7-(2′-oxopropyl)-1,3-di-n-butyl-xanthine (BRL 30892 on ischaemic skeletal muscle pO_2, pH and contractility in cats and rats. Arzneim. Forsch/Drug Res 34:1274–1278

Le Tallec N, Lacroix P, de Certaines JD, Chagneau F, Lavasseur R, Le Rumeur E (1996) Effects of dimethylformamide on *in vivo* fatigue and metabolism in rat skeletal muscle measured by 31P-NMR. J Pharmacol Toxicol Meth 35:139–143

Okyayuz-Baklouti I (1989) The effects of torbafylline on blood flow, pO_2, and function of rat ischemic skeletal muscle. Eur J Pharmacol 166:75–86

Okyayuz-Baklouti I, Konrad-Clement S, Reifert P, Schmitt T, Schuck D (1992) Novel immobilization model in the rat: functional, histomorphological and biochemical changes in atrophying skeletal muscle. J Musc Res Mot 14:259–260

Ward A, Clissold SP (1987) Pentoxifylline, a review of its pharmacodynamic and pharmacokinetic properties. Drugs 34:50–97

Weselcouch EO, Demusz (1990) Drug effects on function in the ferret ischemic hindlimb. J Pharmacol Meth 23:255–264

A.8.2.8 Effect on Perfusion of Rabbit Ear (Pissemski Method)

PURPOSE AND RATIONALE

The procedure was described as early as 1914 by Pissemski, based on experiments of Krawkow (1913) in fish gills. It can be used to elucidate vasoactive properties (both vasoconstrictive and vasodilating) of

compounds. The isolated rabbit's ear is used to determine the effect of test compounds on its perfusion rate. Administration of norfenephrine induces vasoconstriction leading to a decrease in perfusion rate. A compound with vasodilatory properties will inhibit the norfenephrine induced fall in perfusion rate whereas a vasoconstrictor will potentiate this effect.

PROCEDURE

A rabbit of either sex weighing 1.5–3 kg is sacrificed by CO_2 narcosis and its ears are severed immediately. The ear is placed on a glass disc, the posterior auricular artery is exposed and cannulated. The cannula is connected to a tubing with a T-branch allowing the infusion of different solutions. Ringer's solution, kept at room temperature, is infused under 40 cm water column pressure via the cannula. The perfusion flow volume is recorded using a time ordinate recorder and a CONDON tipper or a photoelectric drop counter.

Prior to drug administration, the pH of the Ringer solution (containing test compound) must be determined. If the pH is greater than 8.5 or smaller than 6.5, it should be adjusted by adding a diluted NaOH- or HCl-solution.

Testing for Vasodilatory (Norfenephrine Antagonistic) Activity

Norfenephrine is infused at a concentration of 0.5 µg/ml until the maximal contraction is reached. The test compound is prepared in Ringer's solution at a concentration of 100 µg/ml. A volume of 30 ml is infused via the cannula over a 15 min period under constant pressure. The change of perfusion rate is determined. If there is a positive response (increase in perfusion rate), the test may be repeated using lower concentrations. If there is a negative response (further decrease in perfusion rate), the compound can be tested for vasoconstrictive activity.

Testing for Vasoconstrictive Activity

This test is repeated as described above without administration of norfenephrine.

Standard compounds:

- as vasodilator
  - dihydralazine
  - theophylline
  - pentoxyfylline 100 µg/ml
- as vasoconstrictor
  - norfenephrine (Novadral)

EVALUATION

Testing for Vasodilatory Activity

The perfusion rate of the ear vessel is determined during the course of the test:

R = perfusion rate of vehicle perfused vessel

RN = perfusion rate of norfenephrine constricted vessel

RNP = perfusion rate of norfenephrine constricted vessel following compound administration

The percent inhibition of norfenephrine induced decrease in perfusion pressure is calculated using the following formula:

$$\% \text{ inhibition} = \frac{(RN - RNP)}{R - RN} \times 100$$

Testing for Vasoconstrictive Activity

The normal perfusion rate (ear vessel perfused with Ringer's solution) is taken as 100%. The percent inhibition of perfusion rate following compound administration is determined.

MODIFICATIONS OF THE METHOD

Schlossmann (1927) used the isolated rabbit ear preparation according to Pissemski for determination of the adrenaline content in blood.

De la Lande and Rand (1965), de la Lande et al. (1967) described a method of perfusing the isolated central artery of the rabbit ear. Small segments of the artery, taken from the base of the ear, were perfused at a constant rate with Krebs solution at 37°C. To enable drugs to be applied either to the intima or the adventitia, the artery was double cannulated so that the intraluminal and extraluminal perfusion media did not mix. Constrictor responses were measured by the maximum rise in perfusion pressure.

Steinsland et al. (1973) studied the inhibition of adrenergic transmission by parasympathicomimetics in the isolated central ear artery of the rabbit. Perfusion was performed at a constant flow rate with Krebs' solution and perfusion pressure was recorded with a transducer.

Allen et al. (1973) incubated isolated segments of rabbit ear artery with (^{3}H)-(–)-noradrenaline and measured the amount of tritium released into the luminal perfusate and into the extraluminal superfusate.

Budai et al. (1990) used isolated proximal 3–4-cm segments of the rabbit ear artery or rat tail artery in a low volume perfusion-superfusion system for measurement of transmitter release from blood vessels *in vitro*.

Miyahara et al. (1993) used arterial rings rabbit ear arteries *in vitro* which were contracted by perivascular nerve stimulation, or 5×10^{-7} noradrenaline or high potassium (29.6 mM) solution. High doses of dexamethasone or clobetasol-17-propionate decreased the amplitude of contractions. Furthermore, the authors performed *in vivo* experiments in albino rabbits, whereby the fur was removed from the distal parts of the ear by applying a depilatory cream at least 24 h before the experiments. The apical regions of the ear were then stripped with adhesive tape 7 times to remove the keratinous epidermal layer. The rabbit was anesthetized and the experimental parts of the ear were placed under a high resolution magnifying camera and immobilized using bilayer adhesive tapes. The vascular reactions induced by topical application of corticosteroids were recorded chronically using videotapes.

Aoki and Chiba (1993) described a method for separate intraluminal and extraluminal perfusion of the **basilar artery** in dogs. A polyethylene roof was designed to cover the canine basilary artery so that an extraluminal superfusion stream could pass over the artery that simultaneously received an intraluminal perfusion.

REFERENCES AND FURTHER READING

Allen GS, Rand MJ, Story DF (1973) Techniques for studying adrenergic transmitter release in an isolated perfused artery. Cardiovasc Res 7:423–428

Aoki T, Chiba S (1993) A new cannula-inserting method for measuring vascular responsiveness. Separate intraluminal and extraluminal perfusion of canine basilar artery. J Pharmacol Toxicol Meth 29:21–27

Budai D, Buchholz JN, Duckles SP (1990) Low volume perfusion-superfusion system for measurement of transmitter release from blood vessels *in vitro*. J Pharmacol Meth 23:41–49

De la Lande IS, Rand MJ (1965) A simple isolated nerve-blood vessel preparation. Aust J Exp Biol Med 43:939–656

De la Lande IS, Frewin D, Waterson JG (1967) The influence of sympathetic innervation on vascular sensitivity to noradrenaline. Br J Pharmac Chemother 31:82–93

Green AF, Boura ALA (1964) Depressants of peripheral sympathetic nerve function. In: Laurence DR, Bacharach AL (eds) Evaluation of Drug Activities: Pharmacometrics. Academic Press, London and New York, 369–430

Griesbacher T, Lembeck F (1987) Actions of bradykinin antagonists on bradykinin-induced plasma extravasation, venoconstriction, prostaglandin E_2 release, nociceptor stimulation and contraction of the iris sphincter muscle of the rabbit. Br J Pharmacol 92:333–340

Krawkow NP (1913) Über die Wirkung von Giften auf die Gefässe isolierter Fischkiemen. Pflüger's Arch 151:583–603

Lembeck F, Griesbacher T, Eckhardt M, Henke S, Breipohl G, Knolle J (1991) New, long-acting, potent bradykinin antagonists. Br J Pharmacol 102:297–304

Miyahara H, Imayama S, Hori Y, Suzuki H (1993) Cellular mechanisms of the steroid-induced vascular responses in the rabbit ear artery. Gen Pharmacol 24:1155–1162

Pissemski SA (1914) Über den Einfluss der Temperatur auf die peripherischen Gefässe. Pflüger's Arch 156:426–442

Schlossmann H (1927) Untersuchungen über den Adrenalingehalt des Blutes. Naunyn Schmiedeberg's Arch Exp Path Pharmakol 121:160–203

Schneider G (1935) Einfluß von Novalgin und kolloidalem Eisen auf die Ödembildung am isolierten Kaninchenohr. Naunyn-Schmiedeberg's Arch exp Path Pharmakol 179:56–60

Steinsland OS, Furchgott RF, Kirpekar SM (1973) Inhibition of adrenergic transmission by parasympathicomimetics in the rabbit ear artery. J Pharmacol Exp Ther 184:346–356

Turner RA (1965) The perfused rabbit ear. In: Screening methods in pharmacology. Chapter 12: Sympatholytic agents. pp 150–151. Academic Press New York, London

A.8.2.9 Effect on Venous Tonus In Situ in Dogs

PURPOSE AND RATIONALE

Veins can be classified into two groups: those that respond and those that do not respond to epinephrine, acetylcholine and sympathetic nerve stimulation. As studies in dogs have shown (Rice et al. 1966) the reactive veins have a considerable amount of smooth muscle whereas the nonreactive ones lack any appreciable amount of smooth muscle fibres in the tunica media. A special preparation allows the registration of localized venous vasoconstriction.

PROCEDURE

Dogs weighing 20–30 kg are anesthetized with 35 mg/kg pentobarbital sodium i.v. The trachea is cannulated and the dog ventilated with a respiration pump. The femoral vein is cannulated for systemic injections. After administration of 5 mg/kg heparin sodium i.v., the saphenous vein and the femoral artery are cannulated. The venous cannula is placed approximately 1 cm distal to its junction with another vein. After the non-perfused branch of the junction is ligated, constant blood flow is maintained from the femoral artery by using a Sigmamotor pump. The flow is adjusted so that a normal physiologic pressure in the vein is maintained. Perfusion pressure is measured between the pump and the vein so that any changes in pressure reflect changes in venous resistance. The peak changes in perfusion pressure are used to measure pressure changes from recorded data. The blood flow is maintained on a constant level. Therefore, changes in pressure must reflect changes in resistance. Pressure is recorded with a polygraph using an Statham pressure transducer (P23AA). In addition to recording perfusion pressure, venous pressure is measured at two additional points along the vein. In order to

record venous pressure centrally from the site of perfusion, the shaft of a 27-gauge needle is placed into the end of a 10 cm piece of a thin Silastic tubing. At the other end, a 27 gauge needle is inserted and attached to a Statham pressure transducer (P23B). Pressure is recorded on a polygraph. One needle is inserted into the vein just proximal of the junction of the two veins. The second needle is placed into the vein so that the distance between the tip of the perfusion cannula and the first needle is the same as the distance between the two needles. In this way pressure decreases across the junction and an adjacent segment can be measured simultaneously. Injections of test compounds are made into the cannula between the pump and the saphenous vein. Changes in pressure measured by the three transducers are recorded. As standard, doses of 0.1–1.0 µg norepinephrine are injected.

EVALUATION

Responses to test drugs are measured in mm Hg and calculated as percentage of response to norepinephrine.

REFERENCES AND FURTHER READING

Rice AJ, Leeson CR, Long JP (1966) Localisation of venoconstrictor responses. J Pharmacol Exp Ther 154:539–545

A.8.3 Arterial Aneurysms

A.8.3.1 General Considerations

Abdominal aortic aneurysms represent a life-threatening condition characterized by chronic inflammation, destructive remodeling of the extracellular matrix and segmental dilatation of the aortic wall (Dobrin 1989; Ernst 1993). Several authors described animal models of aneurysms (Carrell et al. 1999; Dobrin 1999; Daugherty and Cassis 2004). Most studies were performed in mice. Abdominal aortic aneurysms are evoked by genetically defined defects in extracellular matrix maturation, increased degradation of elastin and collagen, aberrant cholesterol homeostasis, or enhanced production of angiotensin peptides.

The **Blotchy mouse** has an X-linked trait that leads to aortic aneurysms and subsequent fatal rupture in nearly all affected male mice (Brophy et al. 1988; Reilly et al. 1990).

Maki et al. (2002) showed that inactivation of the **lysyl oxidase gene Lox** leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice.

Aneurysm development has been noted in a number of mice with deficiencies in the components of the matrix metalloproteinase system (Silence et al. 2001, 2002).

The use of mice in atherosclerosis research was escalated by the development of mice that are deficient in either apoE or LDL receptors susceptible to aneurysm development (Ishibashi et al. 1994; Prescott et al. 1999).

Accelerated atherosclerosis, aortic aneurysms formation, and ischemic heart disease were found in apolipoprotein E and endothelial nitric oxide synthase double-knockout mice (Kuhlencordt et al. 2001). ACE inhibition reduces some symptoms of vascular pathology in apoE and eNOS compound-deficient mice (Knowles et al. 2000). Mice with deficiency of the LDL receptor-related protein (LRP) showed pathological changes in the aortic arch and abdominal aorta with substantial lengthening, dilatation, thickening and large aneurysms (Herz and Strickland 2001; Boucher et al. 2003).

Salt-sensitive aortic aneurysms and rupture in hypertensive transgenic mice that overproduce angiotensin II were described by Nishijo et al. (1998).

The infusion of elastase into the infrarenal segment has been used as model for abdominal aorta aneurysms in rats (Anidjar et al. 1990) and mice (Pyo et al. 2000).

Lee et al. (2001) described abdominal aortic aneu rysms in mice lacking expression of inducible nitric oxide synthase.

Periarterial application of calcium chloride was used to induce aneurysms of the **rabbit** common carotid artery (Gertz et al. 1988) and in the rabbit aorta (Freestone et al. 1997).

This technique has been used in **mice** by Chiou et al. (2001) and Longo et al. (2002).

MODIFICATIONS OF THE METHOD

Nomoto et al. (2003) described the effects of two inhibitors of renin-angiotensin system on attenuation of postoperative remodeling after **left ventricular aneurysm** repair in rats.

REFERENCES AND FURTHER READING

Anidjar S, Salzmann JL, Gentric D, Lagneau P, Camilleri JP, Michel JB (1990) Elastase-induced experimental aneurysms in rats. Circulation 82:973–981

Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J (2003) LRP: role in vascular wall integrity and protection from atherosclerosis. Science 3000:329–332

Brophy CM, Tilson JE, Braverman IM, Tilson MD (1988) Age of onset, pattern of distribution, and histology of aneurysm development in a genetically predisposed mouse. J Vasc Surg 8:45–48

Carrell TW, Smith A, Bumand KG (1999) Experimental techniques and models in the study of the development and treatment of abdominal aortic aneurysm. Br J Surg 86:305–312
Chiou AC, Chiu B, Pearce WH (2001) Murine aortic aneurysm produced by periarterial application of calcium chloride. J Surg Res 99:371–376
Daugherty A, Cassis LA (2004) Mouse models of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 24:429–434
Dobrin PB (1989) Pathophysiology and pathogenesis of aortic aneurysms. Current concepts. Surg Clin North Am 69:687–703
Dobrin PB (1999) Animal models of aneurysms. Ann Vasc Surg 13:641–648
Ernst CB (1993) Abdominal aortic aneurysm. N Engl J Med 328:1167–1172
Freestone T, Turner RJ, Higman DJ, Lever MJ, Powell JT (1997) Influence of hypercholesterolemia and adventitial inflammation on the development of aortic aneurysms in rabbits. Arterioscler Thromb Vasc Biol 17:10–17
Gertz SD, Kurgan A, Eisenberg D (1988) Aneurysms of the rabbit common carotid artery induced by periarterial application of calcium chloride in vivo. J Clin Invest 81:649–656
Herz J, Strickland DK (2001) LRP: a multifunctional scavenger and signaling receptor. J Clin Invest 108:779–784
Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK (1994) Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin Invest 93:1885–1893
Knowles JW, Reddick RL, Jenette JC, Smithies O, Maeda N (2000) Enhanced atherosclerosis and kidney dysfunction in *eNOS(-)/(-)Apo(-)/(-)* mice are ameliorated by enalapril treatment. J Clin Invest 105:451–458
Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL (2001) Accelerated atherosclerosis, aortic aneurysms formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. Circulation 104:44–454
Lee JK, Borhani M, Ennis TL, Upchurch GR Jr, Thompson RW (2001) Experimental abdominal aortic aneurysms in mice lacking expression of inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol 21:1391–1401
Longo GW, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT (2002) Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. J Clin Invest 110:625–632
Maki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, Soininen R (2002) Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Circulation 106:2503–2509
Nishijo N, Sugiyama F, Kimoto K, Taniguchi K, Murakami K, Suzuki S, Fukamizu A, Yagami K (1998) Salt-sensitive aortic aneurysms and rupture in hypertensive transgenic mice that overproduce angiotensin II. Lab Invest 78:1059–1066
Nomoto T, Nishina T, Tsuneyoshi H, Miwa S, Nishimura K, Komeda M (2003) Effects of two inhibitors of renin-angiotensin system on attenuation of postoperative remodeling after left ventricular aneurysm repair in rats. J Card Surg 18 [Suppl 2]:S61–S68
Prescott MF, Sawyer WK, Von Linden Reed J, Jeune M, Chou M, Caplan SL, Jeng AY (1999) Effect of matrix metalloproteinase inhibition on progression of atherosclerosis and aneurysm in LDL receptor-deficient mice overexpressing MMP-3, MMP-12, and MMP-13 and on restenosis in rats after balloon injury. Ann N Y Acad Sci 878:179–190
Pyo R, Lee HK, Shipley M, Curci JA, Mao D, Ziporin SJ, Ennis TL, Shapiro SD, Senior RM, Thompson RW (2000) Targeted disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental aortic aneurysms. J Clin Invest 105:1641–1649
Reilly JM, Savage EB, Brophy CM, Tilson MD (1990) Hydrocortisone rapidly induces aortic rupture in a genetically susceptible mouse. Arch Surg 125:707–709
Silence J, Lupu F, Collen D, Lijnen HR (2001) Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. Arterioscler Thromb Vasc Biol 21:1440–1445
Silence J, Collen D, Lijnen HR (2002) Reduced atherosclerotic plaque but enhanced aneurysm formation in mice with inactivation of the tissue inhibitor of metalloproteinase-1 (*TIMP-1*) gene. Circ Res 90:897–903

A.8.3.2 Angiotensin II-Induced Aortic Aneurysm in Mice

PURPOSE AND RATIONALE

Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice (Daugherty et al. 2000, 2001; Knowles et al. 2000; Wang et al. 2001; Manning et al. 2002, 2003; Deng et al. 2003; Martin-McNulty et al. 2003; Saraff et al. 2003).

Wang et al. (2005) reported that a Rho-kinase inhibitor attenuated angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis.

PROCEDURE

Animal Preparation

Osmotic minipumps (model 2004, Alzet) containing either PBS or Ang II (1.44 mg/kg per day) in PBS were implanted subcutaneously in 6-month-old apoE-KO male mice (Jackson Laboratories). Two days before saline and Ang II infusion, mice were provided with water (Ang IIgroup) or water containing test compound at a concentration of 0.5 mg/ml (low-dose group) or 1.0 mg/ml (high-dose group). Age-matched apoE-KO mice without any treatment were used as naïve controls. At the end of the 30-day treatment period, mice were euthanized, and the hearts were perfused with DEPC in saline. The arterial tree was rapidly dissected from fat and connective tissue and snap-frozen in liquid nitrogen.

MORPHOLOGICAL EXAMINATION

Quantification of Aneurysm Formation

After the aorta was dissected free from the surrounding connective tissue, images were recorded with a digital camera and later used to measure the outer diameter of the suprarenal aorta at the midpoint between the diaphragm and right renal artery. A commonly used

clinic standard to diagnose abdominal aortic aneurysm is an increase in aortic diameter of ≈50% (Johnston et al. 1991). The average diameter of the normal suprarenal aorta in naïve control mice is 0.8 mm. A threshold of 1.22 mm was set as evidence of an incidence of aneurysm formation. Aneurysm severity was assessed with a scoring system described by Daugherty et al. (2001): type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from naïve apoE-KO mice without Ang II treatment); type I, a dilated lumen with no thrombus; type II, remodeled tissue often containing thrombus; type III, a pronounced bulbous form of type II containing thrombus; and type IV, multiple aneurysms containing thrombus. To analyze this measurement semiquantitatively, the numerical score assigned to the type of aneurysm for each animal in a group was averaged to generate a pathology score for statistical comparison.

Quantification of Atherosclerotic Lesion Area

The left and right carotid arteries and the aortic arch were dissected, excised, opened longitudinally, and pinned down on wax-coated Petri dishes. Atherosclerotic lesions were visible without staining. Images of the open luminal surface of the vessels were captured with a digital camera (Sony) mounted on a dissecting microscope. The atherosclerotic lesion area was quantified by use of the C-Simple system (Compix) and expressed as a percentage of the total luminal surface area (Wang et al. 2000, 2001; Martin-McNulty et al. 2003).

Histology and Immunohistochemical Staining

Two representative suprarenal aortas from each group were fixed in formalin, embedded in paraffin, and cut into 5-μm-thick sections. The adjacent sections were stained with hematoxylin and eosin or by the immunohistochemical method. To identify macrophages in the aortic wall, a purified rat anti-mouse Mac-3 monoclonal antibody (BD Pharmingen) was used for immunochemistry staining (PhenoPath Laboratories). To localize cells undergoing nuclear DNA fragmentation, *in situ* terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) was performed using an *in situ* apoptosis detection kit (Roche Biochemicals) (Song et al. 2000; Feng et al. 2002). Paraffin sections were deparaffinized and rehydrated. Sections were then washed with PBS and incubated with proteinase K (20 μg/ml) for 20 min. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in methanol at room temperature. TdT, which catalyzes a template-independent addition of deoxyribonucleotide to 3-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragment *in situ*. The TUNEL signal was then detected with an anti-digoxigenin antibody conjugated with peroxidase and developed with diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and cleared before coverslips were placed. Both positive and negative control slides were processed at the same time in each experiment. The presence of apoptotic cells was scored as nuclear staining, with a distinctive morphological appearance associated with cell shrinkage and chromatin condensation.

EVALUATION

Results are presented as mean ± SE for the number of animals used. Statistical comparison for the incidence of abdominal aortic aneurysms was performed by χ^2 analysis. Multiple comparison of mean values was performed by ANOVA, followed by a subsequent Student-Newman-Keuls test for repeated measures. Differences were considered statistically significant at a value of $P < 0.05$.

REFERENCES AND FURTHER READING

Daugherty A, Manning MW, Cassis LA (2000) Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 195:1605–1612

Daugherty A, Manning MW, Cassis LA (2001) Antagonism of AT2 receptors augments angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. Br J Pharmacol 134:865–870

Deng GG, Martin-McNulty B, Sukovich DA, Freay A, Halks-Miller M, Thinnes T, Loskutoff DJ, Carmeliet P, Dole WP, Wang YX (2003) Urokinase-type plasminogen activator plays a critical role in angiotensin II-induced abdominal aortic aneurysm. Circ Res 92:510–517

Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP (2002) Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation 106:873–879

Johnston KW, Rutherford RB, Tilson MD, Shah DM, Hollier L, Stanley JC (1991) Suggested standards for reporting on arterial aneurysms: Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular Surgery. J Vasc Surg 13:452–458

Knowles JW, Reddick RL, Jenette JC, Smithies O, Maeda N (2000) Enhanced atherosclerosis and kidney dysfunction in *eNOS(-)/(-)Apo(-)/(-)* mice are ameliorated by enalapril treatment. J Clin Invest 105:451–458

Manning MW, Cassis LA, Huang J, Szilvassy SJ, Daugherty A (2002) Abdominal aortic aneurysms: fresh insights from a novel animal model of the disease. Vasc Med 7:45–54

Manning MW, Cassis LA, Daugherty A (2003) Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis

and abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 23:483–488
Martin-McNulty B, Tham DM, da Cunha V, Ho JJ, Wilson DW, Rutledge JC, Deng GG, Vergona R, Sullivan ME, Wang YX (2003) 17 β-estradiol attenuates development of angiotensin II-induced aortic abdominal aneurysm in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 23:1627–1632
Saraff K, Babamusta F, Cassis LAS, Daugherty A (2003) Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 23:1621–1626
Song W, Lu X, Feng Q (2000) Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. Cardiovasc Res 45:595–602
Wang YX, Halks-Miller M, Vergona R, Sullivan ME, Fitch R, Mallari C, Martin-McNulty B, da Cunha V, Freay A, Rubanyi GM, Kauser K (2000) Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E–deficient mice. Am J Physiol 278:H428–H434
Wang YX, Martin-McNulty B, Feay AD, Sujowich DA, Halks-Miller M, Li AA, Vergona R, Sullivan ME, Morser J, Dole WP, Deng GG (2001) Angiotensin II increases urokinase-type plasminogen activator expression and induces aneurysms in the abdominal aorta of apolipoprotein E-deficient mice. Am J Pathol 159:1455–1464
Wang YX, Martin-McNulty B, da Cinha V, Vincelette J, Lu X, Feng Q, Hlaks-Miller M, Mahmoudo M, Schroeder M, Subramanyam B, Tseng JL, Deng GD, Schirm S, Johns A, Kauser K, Dole WP, Light DR (2005) Fasudil, a Rho-kinase inhibitor, attenuates angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis. Circulation 111:2219–2226

A.8.4 Angiogenesis and Anti-Angiogenesis

A.8.4.1 General Considerations

Regulation of new blood vessel formation, angiogenesis, is precisely programmed throughout the lifetime of vertebrates. Besides the role of angiogenesis in normal function, it is an essential component of disease processes, including tumor growth, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Folkman and Shing 1992). Multiple factors that stimulate angiogenesis either directly or indirectly have been described, including the fibroblast growth factor family (Esch et al. 1985), vascular endothelial growth factor (Leung et al. 1990; Thomas 1996; Ferrara and Davis-Smyth 1997), epidermal growth factor (Gospadarowicz et al. 1979), transforming growth factor-α and -β (Schreiber et a 1986; Yang and Moses 1990), tumor necrosis factor-α (Leibovich et al. 1987), angiogenin (Fett et al. 1985), CYR61, a product of a growth factor-inducible immediate early gene (Babic et al. 1998) ect. The pharmacological inhibition of angiogenesis is of considerable interest in the development of new therapeutic modalities for the treatment of diseases such as diabetic retinopathy, atherosclerosis, hemangiomas, rheumatoid arthritis and cancer, in which pathological angiogenesis occurs (Ezekowitz et al. 1992; Folkman and Shing 1992; Fan and Brem 1992; O'Brien et al. 1994). Several natural inhibitors of angiogenesis were described, such as thrombospondin (Good et al. 1990), somatostatin (Barrie et al. (1993), angiostatin, isolated from a subclone of Lewis lung carcinoma, (O'Reilly et al. 1994, 1996), endostatin, a 20 kDa angiogenesis inhibitor from a murine hemangioendothelioma which is a C-terminal fragment of collagen XVIII (O'Reilly et al. 1997; Dhanabal et al. 1999), vasostatin (Pike et al. 1998).

Angiogenesis was studied *ex vivo* by culturing rat or mouse aortic rings in collagen gel (Zhu et al. 2003). Unlike rat aorta explants, unstimulated mouse aortic rings were unable to spontaneously produce an angiogenic response under serum-free conditions. They, however, responded to basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), generating networks of branching neovessels.

Couffinhal et al. (1998) published a mouse model of angiogenesis. The femoral artery of one hind-limb was ligated and excised. Laser Doppler perfusion imaging was employed to document the consequent reduction in hind-limb blood-flow, which typically persisted for up to 7 days. Neovascularization was shown to develop in association with augmented expression of VEGF mRNA and protein from skeletal myocytes as well as endothelial cells from the ischemic hind-limb.

REFERENCES AND FURTHER READING

Babic AM, Kireeva ML, Kolesnikova TV, Lau LF (1998) CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 95:6355–6360
Barrie R, Woltering EA, Hajarizadeh H, Mueller C, Ure T, Fletcher WS (1993) Inhibition of angiogenesis by somatostatin and somatostatin-like compounds is structurally dependent. J Surgical Res 55:446–450
Couffinhal T, Siver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM (1998) Mouse model of angiogenesis.. Am J Pathol 152:1667–1679
Dhanabal M, Volk R, Ramchandran R, Simons M, Sukhatme VP (1999) Cloning, expression, and *in vitro* activity of human endostatin. Biochem Biophys Res Commun 258:345–352
Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Bohlen P, Gillemin R (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. Proc Natl Acad Sci USA 82:6507–6511

Ezekowitz RA, Mulliken JB, Folkman J (1992) Interferon alfa-2a therapy for life-threatening hemangiomas in infancy. New Engl J Med 326:1456–1463
Fan TPD, Brem S (1992) in: Waring MJ, Ponder B (eds) The Search for New Anticancer Drugs: Cancer Biology Series, Vol 3, pp 185–229, Kluwer Publ., Lancaster
Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18:4–25
Fett JW, Strydom DJ, Lobb RR, Aldeman EM, Bethune JL, Riordan JF, Vallee BL (1985) Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry 24:5480–5486
Folkman J, Klagsbrun M (1987) Angiogenic factors. Science 235:442–447
Folkman J, Shing Y (1992) Angiogenesis. J Biol Chem 267:10931–10934
Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628
Gospodarowicz D, Bialecki H, Thakral GK (1979) The angiogenic activity of the fibroblast and epidermal growth factor. Exp Eye Res 28:501–514
Klagsbrun M, D'Amore PA (1991) Regulators of angiogenesis. Ann Rev Physiol 53:217–239
Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N (1987) Macrophage-induced angiogenesis is mediated by tumor necrosis factor alpha. Nature 329:630–632
Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1990) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309
O'Brien ER, Garvin MR, Dev R, Stewart DK, Hinohara T, Simpson JB, Shwartz SM, (1994) Angiogenesis in human coronary atherosclerotic plaques. Am J Pathol 145:883–894
O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J (1994) Angiostatin, a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315–328
O'Reilly MS, Holmgren L, Chen C, Folkman J (1996) Angiostatin induces and sustains dormancy of human primary tumors in mice. Nature Med 2:689–692
O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–285
Pike SE, Yao L, Jones KD, Cherney B, Appella E, Sakaguchi K, Nakhasi H, Teruya-Feldstein J, Wirth P, Gupta G, Tosato G (1998) Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. J Exp Med 188:2349–2356
Schreiber AB, Winkler ME, Derynk R (1986) Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. Science 232:1251–1253
Thomas KA (1996) Vascular endothelial growth factor, a potent and selective angiogenic agent. J Biol Chem 271:603–606
Yang EY, Moses HL (1990) Transforming growth factor 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. J Cell Biol 111:731–741
Zhu WH, Iurlaro M, MacIntyre A, Fogel E, Nicosia RF (2003) The mouse aorta model: Influence of genetic background and aging on bFG- and VEGF-induced angiogenic sprouting. Angiogenesis 6:193–199

A.8.4.2 Endothelial Cell Proliferation

PURPOSE AND RATIONALE

Human umbilical vein endothelial cells (HUVEC) were used by various authors to study endothelial cell proliferation (Bussolino et al. 1992; Benelli et al. 1995; Danesi et al. 1997; Hu 1998; Iurlaro et al. 1998; Vacca et al. 1999; Xin et al. 1999).

PROCEDURE

The HUV-EC-C human endothelial cells (American Type Culture Collection, Rockville, MD) are cultured at 37°C and 5% CO_2 in 90% Ham's F12K, 10% fetal bovine serum, 30 µg/ml endothelial cell growth factor, 100 µg/ml heparin, and 4 mM L-glutamine. The effect of test compound on HUV-EC-C cell proliferation is evaluated on 3×10^3 cells/well in 24-well plates. After 24 h, the test compound in various concentrations of the vehicle are added, and plates are incubated for 72 h. Cells are then harvested with trypsin/EDTA and counted by an hemocytometer.

EVALUATION

Results are expressed as number of cells in vehicle and compound-treated cultures and are the mean of three separate experiments ± SE.

MODIFICATIONS OF THE METHOD

In addition to human umbilical veil endothelial cells, Pike et al. (1998) used fetal bovine heart endothelial cells and measured DNA synthesis by [^{3}H]thymidine deoxyribose uptake.

Oikawa et al. (1991) used vascular cells from bovine carotid arteries and tested cell proliferation in a collagen gel and cell migration in a Boyden chamber.

Bovine capillary endothelial cells were used by Folkman et al. (1979), Clapp et al. (1993), O'Reilly et al. (1997), Cao et al. (1999).

REFERENCES AND FURTHER READING

Benelli U, Lepri A, Nardi M, Danesi R, Del Tacca M (1995) Tradipil inhibits endothelial cell proliferation and angiogenesis in the chick chorioallantoic membrane and in the rat cornea. J Ocular Pharmacol 11:157–166
Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini R, Gaudino G, Tamagnone L, Coffer A, Comoglio PM (1992) Hepatocyte growth factor is a potent angiogenic growth factor which stimulates endothelial cell motility and growth. J Cell Biol 119:629–641
Cao R, Wu HL, Veitonmäki N, Linden P, Farnebo J, Shi G Y, Cao Y (1999) Suppression of angiogenesis and tumor growth by the inhibitor K1–5 generated by plasmin-mediated proteolysis. Proc Natl Acad Sci 96:5728–5733
Clapp C, Martial JA, Guzman RC, Rentier-Delrue F, Weiner RI (1993) The 16-kilodalton N-terminal fragment of human

prolactin is a potent inhibitor of angiogenesis. Endocrinology 133:1292–1299
Danesi R, Agen C, Benelli U, Di Paolo A, Nardini D, Bocci G, Basolo F, Camapgni A, Del Tacca M (1997) Inhibition of angiogenesis by the somatostatin analogue octreotide acetate (SMS 201–995). Clin Cancer Res 3:265–272
Folkman J, Haundenschild CC, Zetter BR (1979) Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA 76:5217–5221
Hu G-F (1998) Neomycin inhibits angiogenin-induced angiogenesis. Proc Natl Acad Sci USA 95:9791–9795
IurlaroM, Vacca A, Minischetti M, Ribatti D, Pellegrino A, Sardanelli A, Giacchetta F, Dammacco F (1998) Antiangiogenesis by cyclosporine. Exp Hematology 26:1215–1222
Oikawa T, Hasegawa M, Shimamura M, Ashino H, Murota SI, Morita I (1991) Eponemycin, a novel antibiotic, is a highly powerful angiogenesis inhibitor. Biochem Biophys Res Commun 181:1070–1076
O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–285
Pike SE, Yao L, Jones KD, Cherney B, Appella E, Sakaguchi K, Nakhasi H, Teruya-Feldstein J, Wirth P, Gupta G, Tosato G (1998) Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. J Exp Med 188:2349–2356
Vacca A, Iurlaro M, Ribatti D, Minischetti M, Nico B, Ria R, Pellegrino A, Dammacco F (1999) Antiangiogenesis is produced by nontoxic doses of vinblastine. Blood 94:4143–4155
Xin X, Yang S, Kowalski J, Gerritsen ME (1999) Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis *in vitro* and *in vivo*. J Biol Chem 274:9116–9121

A.8.4.3 Chorioallantoic Membrane Assay

PURPOSE AND RATIONALE

The chick chorioallantoic membrane assay, originally described by Auerbach et al. (1974), has been used with some modifications by several authors to test angiogenesis and inhibition of angiogenesis, e. g., by Taylor and Folkman (1982), Crum et al. (1985), Vu et al. (1985), McNatt et al. (1992, 1999), Barrie et al. (1993), Clapp et al. (1993), Gagliardi and Collins (1993), Benelli et al. (1995), Ribatti et al. (1995), Klauber et al. (1996), Oikawa and Shimamura (1996), Danesi et al. (1997), O'Reilly et al. (1997), Iurlaro et al. (1998), Cao et al. (1999), Vacca et al. (1999).

PROCEDURE

Fertilized White Leghorn chicken eggs are incubated at 37°C at constant humidity. On incubation day 3, a square window is opened in the shell and 2 to 3 ml of albumen is removed to allow detachment of the developing chorioallantoic membrane (CAM). The window is sealed with a glass and the eggs are returned to the incubator. On day 8, 1 mm^3 gelatin sponges loaded with 3 µl phosphate-buffered saline alone as the negative control or containing 3 µg (1 mg/ml) of the angiogenic recombinant basic fibroblast growth factor alone as positive control, or together with various doses of test compound, are implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAM are examined daily until day 12, when the angiogenic response peaks. On day 12, blood vessels entering the sponge within the focal plane of the CAM are recognized microscopically, counted by two observers in a double-blind fashion under a Zeiss SR stereomicroscope, and photographed in ovo with the MC63 Camera system (Zeiss, Oberkochen, Germany). To better highlight vessels, the CAM are injected into a large allantoic vein with India ink solution, fixed in Serra's fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate. On day 12, after microscopic counting, the embryos and their membranes are fixed in ovo in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions are removed, embedded in paraffin, sectioned at 8 µm along a plane parallel to the CAM surface, and stained with a 0.5% aqueous solution of toluidine blue.

EVALUATION

Angiogenesis is measured by a planimetric point count method (Ribatti et al. 1999): four to six 250× magnification fields covering almost the whole of every third section within 30 serial slides of each sponge per sample are analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm^2. Only transversely sectioned microvessels, ie, capillaries and venules with or without a 3 to 10 µm lumen occupying the intersection points, are counted and calculated as the mean ± 1 SD per section, per CAM, and groups of CAM. Statistical significance of differences is calculated by comparing the data from each experiment to their controls using Student's *t*-test.

MODIFICATIONS OF THE METHOD

Oh et al. (1997) studied the lymphatics of differentiated avian chorioallantoic membrane using microinjection of Mercox resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α-smooth muscle actin, and *in situ* hybridization with vascular endothelial growth factor VEGFR-2 and VEGFR-3 probes.

Using the chick chorioallantoic membrane assay, Giannopoulou et al. (2003) showed that amifostine, an inorganic thiophosphate-cytoprotective agent, inhibits angiogenesis *in vivo*.

REFERENCES AND FURTHER READING

Auerbach R, Kubai L, Knighton D, Folkman J (1974) A simple procedure for the long-term cultivation of chicken embryos. Dev Biol 41:391–394

Barrie R, Woltering EA, Hajarizadeh H, Mueller C, Ure T, Fletcher WS (1993) Inhibition of angiogenesis by somatostatin and somatostatin-like compounds is structurally dependent. J Surgical Res 55:446–450

Benelli U, Lepri A, Nardi M, Danesi R, Del Tacca M (1995) Tradipil inhibits endothelial cell proliferation and angiogenesis in the chick chorioallantoic membrane and in the rat cornea. J Ocular Pharmacol 11:157–166

Cao R, Wu HL, Veitonmäki N, Linden P, Farnebo J, Shi G Y, Cao Y (1999) Suppression of angiogenesis and tumor growth by the inhibitor K1–5 generated by plasmin-mediated proteolysis. Proc Natl Acad Sci 96:5728–5733

Clapp C, Martial JA, Guzman RC, Rentier-Delrue F, Weiner RI (1993) The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. Endocrinology 133:1292–1299

Crum R, Szabo S, Folkman J (1985) A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375–1378

Danesi R, Agen C, Benelli U, Di Paolo A, Nardini D, Bocci G, Basolo F, Camapgni A, Del Tacca M (1997) Inhibition of angiogenesis by the somatostatin analogue octreotide acetate (SMS 201–995). Clin Cancer Res 3:265–272

Gagliardi A, Collins DC (1993) Inhibition of angiogenesis by antiestrogens. Cancer Res 53:533–535

Giannopoulou E, Katsoris P, Kardamakin D, Papadimitiou E (2003) Amifostine inhibits angiogenesis in vivo. J Pharmacol Exp Ther 304:729–737

Klauber N, Browne F, Anand-Apte B, D'Amato RJ (1996) New activity of spironolactone. Inhibition of angiogenesis *in vitro* and *in vivo*. Circulation 94:2566–2571

IurlaroM, Vacca A, Minischetti M, Ribatti D, Pellegrino A, Sardanelli A, Giacchetta F, Dammacco F (1998) Antiangiogenesis by cyclosporine. Exp Hematology 26:1215–1222

McNatt LG, Lane D, Clark AF (1992) Angiostatic activity and metabolism of cortisol in the chorioallantoic membrane (CAM) of the chick embryo. J Steroid Biochem Molec Biol 42:687–693

McNatt LG, Weimer L, Yanni J, Clark AF (1999) Angiostatic activity of steroids in the chick embryo CAM and rabbit cornea models of neovascularization. J Ocular Pharmacol 15:413–423

Oh SJ, Jeltsch MM, Birkenhäger R, McCarthy JEG, Weich HA, Christ B, Alitalo K, Wilting J (1997) VEGF and VEGF-C: Specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 188:96–109

Oikawa T, Shimamura M (1996) Potent inhibition of angiogenesis by wortmannin, a fungal metabolite. Eur J Pharmacol 318:93–96

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–285

Ribatti D, Urbinati C, Nico B, Rusnati M,. Roncali L, Presta M (1995) Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick chorioallantoic membrane. Dev Biol 170:39–49

Ribatti D, Presta M, Vacca A, Ria R, Giulani R, Dell'Era P, Nico B, Roncali R, Damacco F (1999) Human erythropoietin induces a proangiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. Blood 93:2627–2636

Taylor S, Folkman J (1982) Protamine is an inhibitor of angiogenesis. Nature 297:307–312

Vacca A, Iurlaro M, Ribatti D, Minischetti M, Nico B, Ria R, Pellegrino A, Dammacco F (1999) Antiangiogenesis is produced by nontoxic doses of vinblastine. Blood 94:4143–4155

Vu MT, Smith CF, Burger PC, Klintworth GK (1985) An evaluation of methods to quantitate the chick chorioallantoic membrane assay in angiogenesis. Lab Invest 53:499–508

A.8.4.4 Cornea Neovascularization

PURPOSE AND RATIONALE

Neovascularization of the rabbit cornea has been used by several authors to study inhibition of angiogenesis (Gimbrone et al. 1974; Crum et al. 1985; BenEzra et al. 1987, 1997; Klauber et al. 1996; McNatt et al. 1999; Joussen et al. 1999).

PROCEDURE

New Zealand White rabbits are anesthetized for surgery and quantification of newly developed blood vessels with 5 mg/kg xylazine hydrochloride and 35 mg/kg ketamine hydrochloride i.m. Corneal blood vessels are induced by basic fibroblast growth factor which is applied in carrier pellets. These pellets are produced by dispersing 50 µl of 2% methylcellulose containing 500 ng human recombinant basic fibroblast growth factor diluted in 10 µl phosphate-buffered saline in plastic rods with a diameter of 4 mm. Dried pellets are folded twice and implanted intrastromally in the 12 o'clock position into a corneal tunnel. This tunnel is created by a central cut of approximately 50% depth and extended into the peripheral cornea to a point 2.0 mm away from the limbus. Following implantation, the central entrance of the tunnel is closed with a single 10–0 nylon suture in order to ensure that the tear film does not dissolve the pellet and uncontrolled liberation of the growth factor is prevented.

The test substance is dissolved in a viscous gel containing 0.002% polyacrylic acid, 0.04% sorbitol, and 0.001% cetrimide in a watery base. The eyes are treated once daily with 0.1 ml of this gel which is applied in the lower conjunctiva sac. The eyes are closed for several seconds to avoid loss of the substance. Each animal's contralateral eye receives gel without test substance and serves as control. Control animals receive the viscous gel without test substance.

Animals are observed daily under an operating microscope, and vascular growth is documented on days 6, 9, 12, and 16 after surgery. The number of blood vessels, their length and the dimension of the

vascularized area are quantified with a caliper under the operating microscope as well as on standardized photographs. On every observation day corneas are stained with fluorescein in order to show epithelial irregularities due to the topical treatment.

EVALUATION

Differences between treated eyes and controls are tested for significance using unpaired Student's *t*-test.

MODIFICATIONS OF THE METHOD

Damms et al. (1997) characterized the neovascularization that follows the intracorneal injection of bovine albumin in rabbits as a model of angiogenesis. New Zealand white rabbits received intracorneal injections of phosphate buffered saline with and without various amounts of bovine albumin. The rabbits were co-sensitized or pre-sensitized by intramuscular bovine albumin. The corneal response was quantified by ranking photographs taken periodically after the injection.

Babic et al. (1998) tested an angiogenesis promotor in the corneal pocket angiogenesis assay in **rats**.

Xin et al. (1999) studied inhibitors of angiogenesis in the corneal angiogenesis assay in rats. A 1.5 mm incision was made approximately 1 mm from the center of the cornea in anesthetized Sprague Dawley rats. Using a curved spatula, the incision was bluntly dissected through the stroma toward the outer canthus of the eye. A hydron pellet (2×20 mm) containing 200 ng vascular endothelial growth factor and 100 ng sucralfrate was inserted into the base of the pocket.

Foschi et al. (1994), Benelli et al. (1995), Danesi et al. (1997) studied neovascularization of rat cornea induced by **chemical injury**. Both eyes of ether-anesthetized rats were cauterized by applying a $AgNO_3$/KNO_3 (1:1, w/w) applicator to the surface of the cornea eccentrically at a point approximately 2 mm from the corneoscleral limbus. Rats were treated 4 times daily for 6 days with eye drops. The eyes were examined by slit-lamp microscopy daily for 6 days to the evaluate the growth of the vessels. On the 6th day after cauterization, the rats were anesthetized and the upper body perfused through a cannula inserted in the ascending aorta with Ringer's solution until the normal pink color of the fundi disappeared and then with a mixture of 10% India ink/6% gelatin in Ringer's solution. The eyes were enucleated and placed in 4% formaldehyde. The cornea and a 1 mm rim of adjacent scleral tissue were dissected from the rest of the globe and three full thickness peripheral radial cuts were made to allow flattening of the cornea. The corneas were then placed on a glass slide in mounting media, magnified, and photographed. The area occupied by blood vessels was calculated and the area vascularization of drug treated animals was compared to that of control rats.

Kenyon et al. (1996), Cao et al. (1999) performed the corneal micropocket assay in **mice**.

REFERENCES AND FURTHER READING

Babic AM, Kireeva ML, Kolesnikova TV, Lau LF (1998) CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 95:6355–6360

BenEzra D, Hemo I, Maftzir G (1987) The rabbit cornea. A model for the study of angiogenic factors. In: Ezra D, Ryan SJ, Glaser B, Murphy R (eds) Ocular Circulation and Neovascularization. Documenta Ophthalmologia Proceedings, Series 50, Martinus Nijhoff, Dordrecht, The Netherlands, pp 335–340

BenEzra D, Griffin BW, Maftzir G, Aharanov O, Sharif NA, Clark AF (1997) Topical formulations of novel angiostatic steroids inhibit rabbit corneal neovascularization. Invest Ophthalmol Vis Sci 38:1954–1962

Cao R, Wu HL, Veitonmäki N, Linden P, Farnebo J, Shi G Y, Cao Y (1999) Suppression of angiogenesis and tumor growth by the inhibitor K1–5 generated by plasmin-mediated proteolysis. Proc Natl Acad Sci 96:5728–5733

Crum R, Szabo S, Folkman J (1985) A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375–1378

Damms T, Ross JR, Duplessie MD, Klintwort GK (1997) Intracorneal bovine albumin: an immunologic model of corneal angiogenesis. Graefe's Arch Clin Exp Ophthalmol 235:662–666

Foschi D, Castoldi L, Corsi F, Radaelli E, Trabucchi E (1994) Inhibition of inflammatory angiogenesis in rats by locoregional administration of hydrocortisone and protamine. Agents Actions 42:40–43

Gimbrone MA, Cotran RS, Leapman SB, Folkman J (1974) Tumor growth and neo-vascularization: an experimental model using the rabbit cornea. J Nat Cancer Inst 52:413–427

Joussen AM, Kruse FE, Völcker HE, Kirchhof B (1999) Topical application of methotrexate for inhibition of corneal angiogenesis. Graefe's Arch Clin Exp Ophthalmol 237:920–927

Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ, (1996) A model of angiogenesis in the mouse cornea. Invest Ophthalmol Vis Sci 37:1625–1632

Klauber N, Browne F, Anand-Apte B, D'Amato RJ (1996) New activity of spironolactone. Inhibition of angiogenesis *in vitro* and *in vivo*. Circulation 94:2566–2571

McNatt LG, Weimer L, Yanni J, Clark AF (1999) Angiostatic activity of steroids in the chick embryo CAM and rabbit cornea models of neovascularization. J Ocular Pharmacol 15:413–423

Xin X, Yang S, Kowalski J, Gerritsen ME (1999) Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis *in vitro* and *in vivo*. J Biol Chem 274:9116–9121

A.8.4.5
Rat Subcutaneous Air Sac Model

PURPOSE AND RATIONALE

Lichtenberg et al. (1997, 1999) recommended the subcutaneous air sac model in rats as a simple method for *in vivo* screening of antiangiogenesis. Subcutaneous injection of air in the rat results in the formation of an air pouch. If air pouches are kept inflated by repeated injections of air the develop a structure of synovial lining (Edwards et al. 1981). The subcutaneous air pouch appears more like a bursal cavity than a synovial joint (Kowanko et al. 1986) and after 8–10 days the cells of the air sac appear as a transparent membrane on which the formation of new vessels can be studied.

PROCEDURE

Under anesthesia 10–15 ml of air is introduced dorsally to female Sprague Dawley rats weighing 150–180 g by subcutaneous injections using a 25 gauge needle to produce an air sac located approximately 4–5 cm behind the head of the animal. The air sacs are re-inflated every forth day. The wall of the air sac becomes progressively thicker with time and after approximately 10 days a sufficient lining of cells has been established. For sponge implantation, the animals are anesthetized again. A 1.5 cm incision is made through the clipped skin covering the air sac and blunt dissection is used to open a 2 cm deep cavity towards the cranial base of the air sac by careful separation of the skin from the membrane. A cellulose implant (Spontex sponge) with a diameter of 8 mm is carefully pressed into the cavity of the membrane away from the incision site and the incision closed by sutures. The animals are treated for 10 days with various doses of test compound in a volume of 10 ml/kg or vehicle. The subcutaneous injection is made under light CO_2/O_2 anesthesia into the hind leg 5–7 cm away from the air sac. This injection site is chosen to eliminate any risk of inducing irritative side-effects on the membrane. After 10 days treatment, the animals are sacrificed after having received 20 min before an injection of 1 μCi of ^{125}I-labelled immunoglobulin via the tail vein. The overlying skin of the air sac is removed to expose the transparent membrane. The extent of vascular proliferation is scored *in situ*:

1+: slight background vascularization;
2+: few new vessels reach the sponge;
3+: many new vessels reach and penetrate the implant;
4+: very intense formation of new vessels which reach and penetrate the implant.

Following *in situ* scoring, the implant and the membrane from each animal are placed in the same plastic vial containing 10% formalin and the radioactivity is measured in a γ-counter. The implants are examined microscopically after staining with haematoxylin and eosin.

EVALUATION

The extent of vascular proliferation scored *in situ* is compared between vehicle and treated animals by the Wilcoxon test. The angiogenic response measured by ^{125}I-activity in cpm is subjected to analysis of variance followed by Dunnett's *t*-test to compare each dose with the vehicle. The cpm's are log-transformed to obtain variance homogeneity. The correlation between *in situ* scores and cpm is estimated by Spearman's rank correlation coefficient after ranking cpm values.

MODIFICATIONS OF THE METHOD

In a further study, Lichtenberg et al. (1999) inoculated vascular endothelial growth factor producing tumor cells subcutaneously directly on the membrane, and the formation of vessels was measured 8 days later. Furthermore, slow-release pellets containing angiogenic factors, basic fibroblast growth factor or vascular endothelial growth factor, were implanted on the subcutaneous membrane.

Nakamura et al. (1999) studied suppression of angiogenesis induced by S-180 mouse tumor cells in the dorsal air sac assay in **mice**.

Funahashi et al. (1999) developed a mouse dorsal air sac model for quantifying *in vivo* tumor-induced angiogenesis which is determined by measuring the blood volume in an area of skin held in contact with a tumor cell-containing chamber, using ^{51}Cr-labeled red blood cells.

Schreiber et al. (1986) described the **hamster cheek pouch assay** for testing angiogenic/antiangiogenic activity.

REFERENCES AND FURTHER READING

Edwards JC, Sedgwick AD, Willoughby DA (1981) The formation of a structure with the features of synovial lining by subcutaneous injection of air. An *in vivo* tissue culture system. J Pathol 134:147–156

Funahashi Y, Wakabayashi T, Semba T, Sonoda J, Kitoh K, Yoshimatsu K (1999) Establishment of a quantitative mouse dorsal air sac model and its application to evaluate a new angiogenesis inhibitor. Oncol Res 11:319–329

Kowanko IC, Gordon TP, Rosenbilds AM, Brooks PM, Roberts-Thompson PJ (1986) The subcutaneous air pouch model of synovium and the inflammatory response to heat aggregated gammaglobulin. Agents Actions 18:421–428

Lichtenberg J, Hansen CA, Skak-Nilsen T, Bay C, Mortensen JT, Binderup L (1997) The rat subcutaneous air sac model:

a new and simple method for *in vivo* screening of antiangiogenesis. Pharmacol Toxicol 81:280–284
Lichtenberg J, Hjarnaa P JV, Kristjansen PEG, Hansen D, Binderup L (1999) The rat subcutaneous air sac model: A quantitative assay of antiangiogenesis in induced vessels. Pharmacol Toxicol 84:34–40
Nakamura M, Katsuki Y, Shibutani Y, Oikawa T (1999) Dienogest, a synthetic steroid, suppresses both embryonic and tumor-cell-induced angiogenesis. Eur J Pharmacol 386:33–40
Schreiber AB, Winkler ME, Derynk R (1986) Transforming growth factor-α: a more potent angiogenic factor than epidermal growth factor. Science 232:1250–1253

A.8.4.6 Mesenteric Window Angiogenesis Model

PURPOSE AND RATIONALE

Norrby et al. (1986, 1990, 1995) described the mesenteric window assay in rats for quantitative measurement of induction and inhibition of angiogenesis. The tissue being used is the membranous, 'window'-like parts of the mesentery which is normally vascularized and appears to lack significant physiologic angiogenesis. Since the mesenteric window natively measures only 5–10 µm in thickness, the vasculature is virtually two-dimensional. Due to the structural and metabolic simplicity of the test tissue, the mesenteric window microvasculature is regarded as an ideal test system for establishing the functional influences of defined factors (Zweifach 1973).

PROCEDURE

Angiogenesis is induced by i.p. injection of the mast-cell secretagogue compound 48/80 twice daily for 4.5 days to male Sprague Dawley rats weighing about 225 g. Test compounds or saline are injected s.c. 1 h before each injection of compound 48/80.

Angiogenesis is quantified by microscopically counting the number of vessel profiles per unit length of the mesenteric window in 4 microtome sections per specimen, cut perpendicularly to the surface, from the central part of the window. This reflects the degree of branching, the degree of tortuosity and the degree of spatial expansion of the vasculature. Four specimens per animal are analyzed.

Four mesenteric window specimens are spread, fixed on objective slides and stained with toluidine blue to measure the relative vascularized area. Three randomly selected vascular view fields per mesenteric-window spread are analyzed for microvascular length per unit area of vascularized tissue. The total microvascular length is computed from the vascularized area of each animal multiplied by the mean microvascular length for the corresponding treatment group.

EVALUATION

The non-parametric two-tailed Mann-Whitney U rank sum test for unpaired observations is used for statistical analysis.

REFERENCES AND FURTHER READING

Norrby K (1995) Evidence of a dual role of endogenous histamine in angiogenesis. Int J Exp Pathol 76:87–92
Norrby K, Jakobsson A, Sörbo J (1986) Mast cell-mediated angiogenesis. A novel experimental model using the rat mesentery. Virchow's Arch B. Cell Pathol 52:195–206
Norrby K, Jakobsson A, Sörbo J (1990) Quantitative angiogenesis in spreads of intact mesenteric windows. Microvasc Res 39:341–348
Zweifach BW (1973) The microcirculation in the intestinal mesentery. Microvasc Res 5:363–367

A.8.4.7 Quantification of Vascular Endothelial Growth Factor-C

PURPOSE AND RATIONALE

Vascular endothelial growth factor (VEGF) has achieved considerable therapeutic interest (Clauss 1998; Enholm et al. 1998; Ferrara et al. 2004).

Weich et al. (2004) described an ELISA for quantification of vascular endothelial growth factor-C.

PROCEDURE

Materials and Methods

The fully processed rat protein dNdC-VEGF-C and dNdC-VEGF-D were produced in insect cells and purified from supernatants (Kirkin et al. 2001). Human dNdC-VEGF-C and soluble VEGFR-3 production was achieved according to (Joukov et al. 1997; Hornig et al. 1999). A polyclonal antibody against rat VEGF-C was developed in rabbits (antibody 4080; BioGenes Berlin). A total amount of 1.2 mg rat VEGF-C (containing a C-terminal 6His-tag) was used for immunization of two New Zealand white rabbits. After immunization with 0.1 mg protein, each rabbit was boosted on days 7, 14, 28, 56 and 84 with the same amount. The dilution of the serum for half-maximal titer was 1:10,000. Total IgG from rabbit serum was isolated using HiTrap Protein A Sepharose columns (Amersham Bioscience, Freiburg).

Generations of Cell Lines, Serum-Free Cell Culture Supernatants, Lysates and Tissue Sample Preparation

Experiments were conducted using three human (PC-3 cells, 293 cells, COLO 800 cells) and four rat (10AS, ARIP, BRL3A, MT-450) tumour cell lines and 1640 medium (Gibco-BRL, Bethesda, Md., USA). Stable transfected clones were selected and tested for VEGF-

C expression and secretion using a polyclonal anti-VEGF-C antiserum.

The cells were grown to 80% confluence in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Conditioned media was collected under low-serum (2%) growth conditions. Tumor tissues were snap-frozen in liquid nitrogen and homogenized in RIPA buffer (0.1% SDS, 1% IGEPAL CA-630, 0.5% Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline).

VEGF-C Sandwich-ELISA

The VEGF-C antibody 4080 was isolated from serum using HiTrap Protein-A Sepharose columns. Then, depletion of the anti-his antibody fraction was done by antigen-affinity purification using 10 mg of immobilized 6H-tagged TxnTb protein [Tryparedoxin (Txn) from *Trypanosoma brucei* (Tb)]. Antigen-affinity purification for the antibody 4080 was performed by immobilizing 1 mg rat VEGF-C on an NHS-activated HiTrap column (Amersham Bioscience, Freiburg). The development of a highly sensitive and specific sandwich ELISA for VEGF-C was done using standard methods. Rabbit IgG 4080 (10 µg/ml) was used for coating and the antigen-affinity purified and biotinylated antibody 4080 at 1 µg/ml was used as a detector antibody. Biotinylation of antibody 4080 was done with 6 mg IgG in 100 mM carbonate buffer, pH 8.5 at 3 mg/ml with using biotin-amidohexanoic acid NHS (Sigma, St. Louis, Mo., USA). The molecular ratio biotin:protein was 30:1. As a standard, human and rat dNdC-VEGF-C was used over a concentration range between 0.1 and 6.25 ng/ml. For visualization of the detector, streptavidin-enzyme conjugate was used (Endogen, Woburn, Mass., USA) followed by the addition of TMB (tetra-methyl-benzidine; Roche Mannheim, Germany).

EVALUATION

After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 450 and 620 nm with an ELISA plate reader (Labsystems, Finland). Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicate. Samples were diluted at least 1:2 with sample diluent (BenderMedSystems, Vienna).

MODIFICATIONS OF THE METHOD

Rissanen et al. (2003) found that VEGF-D is the strongest angiogenesis and lymphangiogenetic factor among VEGFs delivered into skeletal muscle via adenoviruses.

REFERENCES AND FURTHER READING

Clauss M (1998) Functions of the VEGF receptor-1 (FLT-1) in the vasculature. Trends Cardiovasc Med 8:241–245

Enholm B, Jussila L, Karkkainen M, Alitalo K (1998) Vascular endothelial growth factor-C: A growth factor for lymphatic and blood vascular endothelial cell. Trends Cardiovasc Med 8:292–297

Ferrara N, LeCouter J, Lin R, Peale F (2004) EG-VEGF and Bv8: a novel family of tissue-restricted angiogenic factors. Biochim Biophys Acta 1654:69–78

Hamada K, Oike Y, Takakura N, Ito Y, Jussila L, Dumont DJ, Alitalo K, Suda T (2000) VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculoangiogenesis and hematopoiesis. Blood 96:3793–3800

Hornig C, Behn T, Bartsch W, Yayon A, Weich HA (1999) Detection and quantification of complexed and free soluble human vascular endothelial growth factor receptor-1 (sVEDFR-1) by ELISA. J Immunol Meth 226:169–177

Joukov V, Kaipainen A, Jeltsch M, Jajusola K, Olofsson B, Kumar V, Erikssan U, Alitalo K (1997) Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol 1732:211–215

Kirkin V, Mazitschek R, Krishnan J, Steffen A, Waltenberger J, Pepper MS, Giannis A, Sleeman J (2991) Characterization of indolines which preferentially inhibit VEGF-Cand VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. Eur J Biochem 286:5530–5540

Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, Kholová I, Keuppinen RAS, Achan MG, Stacker SA, Alitalo K, Ylä-Herttula S (2003) VeGF-D is the strongest angiogenesis and lymphangiogenetic factor among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 92:1098–1106

Weich HA, Bando H, Brokelmann M, Baumann P, Toi M, Barleon B, Alitalo K, Sipos B, Sleeman J (2004) Quantification of vascular endothelial growth factor-C (VEGF-C) by a novel ELISA: J Immunol Meth 285:145–155

A.8.4.8 Inhibitors of Vascular Endothelial Growth Factor

PURPOSE AND RATIONALE

Inhibitors of vascular endothelial growth factor have gained therapeutic interest (Whittles et al. 2002; Gingrich et al. 2003; Hamma-Kourbali et al. 2003; Verheul and Pinedo 2003;

Fernandez et al. 2004; Roberts et al. 2004; Baka et al. 2006). In particular, VEGF Trap, a soluble decoy receptor comprising portions of VEGF receptors 1 and 2, has been studied (Wulff et al. 2001; Holash et al. 2002; Kim et al. 2002; Hood and Cheresh 2003; Saishin et al. 2003; Fukasawa and Korc 2004; Fraser et al. 2005; Hu et al. 2005; Lau et al. 2005).

Byrne et al. (2003) found that vascular endothelial growth factor-Trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model.

PROCEDURE

Cell Lines

Ascites fluid from athymic mice previously inoculated with OVCAR-3 cells was used. Cells from the SKOV-3 human cystadenocarcinoma cell line were grown in McCoy's 5a medium with 1.5 mM L-glutamine, penicillin, and streptomycin, supplemented with 10% FCS. Cells were grown to confluence and harvested by trypsinization with 0.25 mg/ml trypsin/EDTA and suspended in PBS before inoculation into mice.

Animals

Athymic Balb c *nu/nu* mice were housed under pathogen-free conditions and fed autoclaved pellets and water.

Retroviral Constructs

The pLZR Phoenix vector was modified by the addition of a MCS followed by an internal ribosome entry sequence-GFP cassette (Rommel et al. 1999). Thus, genes subcloned into the MCS produce bicistronic constructs under the control of the viral 5′ long terminal repeat. The entire coding sequence of $mVEGF_{164}$ was inserted into the MCS for the construct used to transduce cells with VEGF, whereas the MCS was left empty for the GFP-only vector. Constructs were transfected into Amphotrophic packaging lines to produced infective virus using standard techniques (Grignani et al. 1998).

SKOV-3 MODEL

SKOV-3 cell lines were infected with Amphotrophic viruses encoding either $mVEGF_{164}$ and GFP or GFP only. Cells that were successfully transduced with the retroviruses were collected by FACS using a Cytomation MoFlo (Fort Collins, Colo., USA) with fluorescence emission from GFP measured with a 530/540 nm bandpass filter. More than 50% of the cells were GFP positive after infection, allowing $> 4.0 \times 10^5$ cells to be collected and used to establish cell lines. To verify viral transduction, cells were resorted several days later and found to be > 80% positive for GFP expression. Cells were then expanded, aliquoted, and frozen. All experiments were performed with an aliquot expanded by four to five passages and tested for viability before injection.

In Vivo Adenoviral and SKOV-3 Studies

Adenoviral constructs were achieved according to Thurston et al. (2000). Adenoviral plaque-forming units (5.0×10^8) or 1.0×10^7 SKOV-3 cells were suspended in a volume of 300–400 µl of PBS or serum-free cell culture medium and injected i.p. into female nude mice. VEGF-Trap or control buffer was delivered twice weekly at 25 mg/kg via s.c. injection in a volume of 50–200 µl. Mice were assessed daily for general health and development of ascites and weighed at least twice weekly. Animals were sacrificed if they had lost > 10% of body weight or had persistent ascites on three consecutive assessments. After sacrifice, ascites was removed with a sterile thin caliber plastic transfer pipette and quantified, and hematocrit was measured.

OVCAR-3 MODEL

OVCAR-3 cells obtained from ascites fluid were prepared (Hu et al. 2002). Briefly, 2×10^6 cells in 500 µl of RPMI 1640 were injected i.p. into athymic Balb/C nude (*nu/nu*) mice. Fourteen days after inoculation, blinded administration of VEGF-Trap or human Fc as control was initiated at a dose of 25 mg/kg. Injections were given s.c. in the nape of the neck using a 28.5-gauge needle and a 0.5-ml insulin syringe. Injections (0.05 ml) were administered twice weekly throughout the experimental period. Body weight and abdominal circumference were quantified twice weekly. In addition, animals were monitored daily for evidence of advanced disease (listlessness, extensive swelling of the abdominal cavity). At the end of the experiment, all remaining mice underwent euthanasia with CO_2 followed by cervical dislocation. The volume of ascites was measured, and tumors were excised and weighed. Immediately before sacrifice, mice received i.v. injection with FITC lycopersicon lectin (see below).

Tumor Vasculature

According to Holash et al. (2002) s.c. tumors were established. After small s.c. tumors became palpable (1 week after implantation), treatment with the VEGF-Trap was initiated. VEGF-Trap or an equivalent volume of vehicle was delivered twice weekly s.c. at the nape of the neck. Tumor vasculature was visualized by using antibodies to platelet-endothelial cell adhesion molecule for immunohistochemistry.

VEGF-Trap-treated OVCAR-3 tumor-bearing mice and control, untreated tumor-bearing mice were anesthetized by i.m. injection with ketamine (87 mg/kg) and xylazine (13 mg/kg), followed by i.v. injection with 100 µl of FITC lycopersicon lectin or 100 µg of Cy3 albumin (Jackson Immunology Research, West Grove, Pa., USA). Then 10 min later, mice were perfused through the ascending aorta with 4% paraformaldehyde in PBS for 2 min. Tumors and control organs were extracted and placed in fixative for 1–2 h followed by immersion in 30% sucrose/PBS

overnight, embedded in OCT, cryostat sectioned, and viewed by fluorescence microscopy.

REFERENCES AND FURTHER READING

Baka S, Clamp AR, Jayson GC (2006) A review on the latest clinical compounds that inhibit VEGF in pathological angiogenesis. Expert Opin Ther Targets 10.867–876

Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, Yancopoulos GD, Jaffe RB (2003) Vascular endothelial growth factor-Trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 9:5721–5728

Fernandez M, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J (2004) Anti-VEGF receptor-2 monoclonal antibody prevents portal-system collateral vessel formation in hypertensive mice. Gastrooenterology 126:886–894

Fraser HM, Wilson H, Morris KD, Swanston I, Wiegand SJ (2005) Vascular endothelial growth factor Trap suppresses ovarian function at all stages of the luteal phase in the macaque. J Clin Endocrinol Metab 90:5811–5818

Fukusawa M, Korc M (2004) Vascular endothelial growth factor-Trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. Clin Cancer Res 10:3327–3332

Gingrich DE, Reddy DR, Iqbal MA, Singh J, Almone LD, Angeles TS, Albom M, Yang S, Ator MA, Meyer SL, Robinson C, Ruggeri BA, Dionne CA, Vaught Jl, Mallamo JP, Hudkins RL (2003) A new class of potent vascular endothelial growth factor receptor tyrosine kinase inhibitors. Structure-activity relationships for a series of 9-alkoxymethyl-12-(3-hydroxypropyl)indeno[2,1-*a*]pyrrolo[3,4-c]carbazole-5-ones and the identification of CEP-52144 and its dímethylglycine ester prodrug clinical candidate CEP-7055. J Mol Chem 46:5375–5388

Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Grignani F, Lanfrancone L, Peschle C, Nolan GP, Pelicci PG (1998). High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res 58:14–19

Hamma-Kourbali Y, Di Benedetto M, Ledoux D, Oudar O, Leroux Y, Lecouvey M, Kraemer M (2003) A novel noncontaiining-nitrogen biphosphonate inhibits both in vitro and in vivo angiogenesis. Biochem Biophys Res Commun 310:816–823

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Buorova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yankopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398

Hood JD, Cheresh DA (2003) Building a better trap. Proc Natl Acad Sci USA 100:8624–8625

Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB (2002) Inhibition of phosphatidylinositol 3′-kinase increases efficacy of paclitaxel in *in vitro* and *in vivo* ovarian cancer models. Cancer Res 62:1087–1092

Hu L, Hofmann J, Holash J, Yancopoulos GD, Sood AK, Jaffe RB (2005) Vascular endothelial growth factor Trap combined with Paclitaxel strikingly inhibits tumor and ascites, prolonging survival in a human ovarian cancer model. Clin Cancer Res 11:6966–6971

Kim ES, Serur A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ (2002) Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 99:11399–11404

Lau SC, Rosa DD, Jayson G (2005) Technology evaluation: VEGF Trap (cancer). Regeneron/Sanofi-aventis.

Roberts DM, Anderson AL, Hidaka M, Swetenburg RL, Patterson C, Stanford WL, Bautch VL (2004) A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters. Mol Cell Biol 24:10515–10528

Rommel C, Clarke B, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ (1999) Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 286:1738–1741

Saishin Y, Saishin Y, Takahashi K, Lima-e-Silva R, Hylton D, Rudge JS, Wiegand SJ, Campochiaro PA (2003) VEGF-Trap (R1R2) suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. J Cell Physiol 195:241–248

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos G (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6:460–463

Verheul HMW, Pinedo HM (2003) Vascular endothelial growth factor and its inhibitors. Drug Today 39 [Suppl C]:81–93

Whittles CE, Pocock TM, Wedge SR, Kendrew J, Hennequin LF, Harper SJ, Bates DO (2002) ZM32881, a novel inhibitor of vascular endothelial growth factor-receptor-2 tyrosine kinase activity. Microcirculation 9:513–522

Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM (2001) Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor $Trap_{A40}$. J Lin Endocrinol Metab 86:3377–3386