α- and β-Adrenoreceptor Binding

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Contents

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F.J. Hock (ed.), *Drug Discovery and Evaluation: Pharmacological Assays*,
DOI 10.1007/978-3-319-05392-9_1

α1-Adrenoceptor Binding

Purpose and Rationale

α1-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 the smooth muscle, cardiac stimulation, cellular proliferation, and activation of hepatic gluconeogenesis and glycolysis. In the CNS, the activation of α1-adrenoceptors results in depolarization and increased neuronal firing rate. α1-Adrenergic receptors are members of the G protein-coupled receptor superfamily with three α1-adrenoceptor subtypes – α1A, α1B, and α1D.

The α-adrenoceptor population of plasma membranes from rat heart ventricles consists only of the α1-adrenoceptor 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 to 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 sites. 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 concentration binding characteristics of drugs at the α1-adrenoceptor (Morrow and Creese 1986; Minneman and Esbenshade 1994).

Procedure

Solutions

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

(*continued*)

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, the 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 2,000 *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

Nonspecific binding:

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

Competition Experiments

- 50 µl ^{3}H-prazosin (1 constant concentration, 2–3 $\times 10^{-10}$ 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
- Nonspecific binding
- Specific binding = total binding − nonspecific 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.

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

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.

$K_D\,{}^{3}\mathrm{H}$ = 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 α1-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 α1-adrenoceptor similar to that found in the vas deferens. To overcome some limitations of conventional binding methods such as a low yield of receptors and changes in receptor environment, the tissue segment binding method has been developed (Muramatsu et al. 2005; Tanaka et al. 2004). Both groups discuss extensively advantages and limitations of the method.

Subtypes of the α1-Adrenoceptor

The first attempt to distinguish α1-adrenoceptors using radioligand binding method revealed existence of α1A and α1B subtypes. The decision to discriminate was based on the affinities of different agonists and antagonists, especially WB4101 and prazosin, and also on the ability of chlorethylclonidine to inactivate the α1B but not α1A subtype (Ahlquist 1948; Morrow and Creese 1986; Han et al. 1987, 1990; Johnson and Minneman 1987; Minneman et al. 1988; Bylund et al. 1994; Sayet et al. 1993; Endoh et al. 1992; García-Sáinz et al. 1992; García-Sáinz 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).

Later, with the advance of molecular biology and cloning techniques, the three subtypes of α1-adrenoceptors have been cloned – α1B (cloned α1b), α1A (cloned α1c and previously designated by some authors α1a/c), and novel subtype with unique pharmacological properties α1D adrenoceptor (cloned α1d and previously designated by some authors α1a/d) (Cotecchia et al. 1988; Esbenshade et al. 1995; Hieble and Ruffolo 1997; Schwinn et al. 1990, 1995; Perez et al. 1991; Hirasawa et al. 1993; Forray et al. 1994).

Initial confusion in nomenclature was resolved with acceptance of three subtypes of α1-adrenoceptors – α1A, α1B, and α1D (Ford et al. 1994; Calzada and de Artiñano 2001; Muramatsu et al. 2008; Zhong and Minneman 1999; Bylund et al. 1994; 1998; Hieble and Ruffolo 1997; Alexander et al. 2001).

Binding of the radioligand [3H]-prazosin to the α1A-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat submaxillary glands (Michel et al. 1989).

Binding of the radioligand [3H]-prazosin to the α1B-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 α1A and α1B adrenoceptor subtypes in the 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 "α1A-adrenoceptors in smooth muscle contraction."

Stam et al. (1998) found that (+)-cyclazocine, which behaves as a selective, high-affinity α1B-adrenoceptor ligand in binding experiments, did not show the profile of a α1B-adrenoceptor antagonist in functional tissues.

Decreased blood pressure response in mice deficient of the α1b-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 α1-adrenoceptor antagonists for characterization of a α1D-adrenoceptor.

Understanding the role of each α1-adrenoceptor subtypes and linking it to particular physiological function is clearly important. The main obstacles on this path are the partially overlapped tissue distribution of the subtypes α1-adrenoceptors and the lack of sufficiently subtype-selective agonists and antagonists (Docherty 2010; Perez 2007; Chen and Minneman 2005).

The animal models with genetically manipulated α1-adrenoceptors have been successfully used to identify receptor subtypes, their tissue distribution, subcellular localization, and involvement in a specific physiological function or drug effect (Philipp and Hein 2004; Koshimizu et al. 2002; Link et al. 1995; Rokosh and Simpson 2002; Tanoue et al. 2002a, b; O'Connell et al. 2003; Koch et al. 2000; Cavalli et al. 1997; Muramatsu et al. 2008; Hague et al. 2003).

Multiple studies demonstrate that the levels of α1-adrenoceptors in the heart determined by ligand binding are relatively constant across the most species, including mice, guinea pig, rabbit, pig, and cow (Steinfath et al. 1992a; Yang et al. 1998; Lin et al. 2001; O'Connell et al. 2003; Rokosh and Simpson 2002). The exception is rat's heart tissue, in which the α1-adrenoceptors are approximately tenfold higher (Steinfath et al. 1992a; Michel et al. 1994; Noguchi et al. 1995; Stewart et al. 1994).

α1-Adrenoceptor expression levels in the human heart (by ligand binding methods) are similar to mouse and other species but rat (Bristow et al. 1988; Steinfath et al. 1992b; Hwang et al. 1996; Jensen et al. 2009), suggesting that careful interpretation of the results from studies in rats is required and also that the mouse could be more appropriate model to investigate cardiac α1-adrenoceptor function than the rat (O'Connell et al. 2014).

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*α*2-Adrenoceptor 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 the smooth muscle contraction, platelet aggregation, and inhibition of insulin secretion. Activation of postsynaptic *α*2-adrenoceptors in the brain stem results in an inhibition of sympathetic outflow in the periphery.

Clonidine is a nonselective α2-adrenoceptor agonist and 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 also weakly activate α1-adrenoceptors; therefore, the evaluation of the effects of this compound in complex systems may be difficult (Minneman 1988; Bylund 1992).

The purpose of this assay is to assess the interaction of hypotensive agents with central

$\alpha 2$-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 $\alpha 2$-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 is 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 nonspecific 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 Brinkmann Polytron and centrifuged at 40,000 *g* for 15 min. The supernatant is discarded and 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
1,000 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 three 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

Motulsky et al. (1980), along with Perry and U'Prichard (1981), described [^{3}H]yohimbine and its isomer [^{3}H]rauwolscine as a specific radioligand for brain α2-adrenergic receptors, with [^{3}H]yohimbine showing slightly greater α2A-receptor selectivity (Bylund 1988; Boyajian et al. 1987).

Cheung et al. (1982) and Broadhurst et al. (1988) used [^{3}H]rauwolscine and [^{3}H] yohimbine radioligand binding to rats cerebral and human platelet membranes to propose heterogeneity of α2-adrenoceptors.

Goldberg and Robertson (1983) reviewed yohimbine as a pharmacological probe for the study of the α2-adrenoceptor.

Bylund et al. (1988) used [^{3}H]-yohimbine and [^{3}H]-rauwolscine in five different tissues and cell lines containing only one subtype of α2-receptors to demonstrate distinct pharmacological profile for α-2A and α-2B adrenoceptors.

Blaxall et al. (1991) and Murphy and Bylund (1988) confirmed that OK (opossum kidney) cells and membranes from opossum kidney express a novel subtype of α2-adrenergic receptors, which they termed α2C subtype.

The compound UK 14,304 appears to activate all known α2-adrenoceptors and has little action on α1- and β-adrenoceptors, thus could be considered a selective full α2-adrenoceptor agonist (Beckeringh et al. 1984).

Marjamäki et al. (1993) recommended the use of recombinant human α2-adrenoceptors to characterize subtype selectivity of antagonist binding.

Uhlén et al. (1994) found that the α2-adrenergic radioligand [3H]-MK912 is α2C selective among human α2A-, α2B-, and α2C-adrenoceptors.

Uhlén et al. (1998) tested the binding of the radioligand [3H]RS79948–197 to human, guinea pig, and pig α2A-, α2B-, and α2C-adrenoceptors and compared the values with MK912, RX821002, rauwolscine, and yohimbine. [3H] RS79948–197 was nonselective for the α2-adrenoceptor subtypes, showing high affinity for all three.

Bücheler et al. (2002) used atipamezole on transgenic mouse lines lacking different α2-receptors subtypes to determine the localization and density of brain α2-receptors (Bücheler et al. 2002).

The advantages and obstacles in using different α2-adrenoceptor agonists/antagonists for radioligand binding studies have been discussed in the following reviews: Piascik et al. (1996), Starke (2001), and Calzada and de Artiñano (2001).

The binding of the radioligand [^{3}H]-rauwolscine to the α2A-adrenoceptor subtype can be measured in membranes prepared from rabbit spleens (Michel et al. 1989).

Binding of the radioligand [^{3}H]-yohimbine to the α2B-adrenoceptor subtype can be measured in membranes prepared from male Wistar rat kidney cortices (Connaughton and Docherty 1989).

Subtypes of the α2-Adrenoceptor

α2-Adrenoceptors are one of the three types of adrenoceptors that belong to the same superfamily of G protein-coupled receptors. Three distinct α2-adrenoceptor subtypes – α2A, αB, and α2C – have been characterized and cloned (Cheung et al. 1982; Feller and Bylund 1984; Neylon and Summers 1985; Bylund 1992; Bylund et al. 1994; Kobilka et al. 1987; Regan et al. 1988; Lomasney et al. 1990; Murphy and Bylund 1988; Blaxall et al. 1991; Ruffolo et al. 1993; Lorenz et al. 1990; Ruffolo 1990; Uhlén and Wikberg 1990; Satoh and Takayanagi 1992; Takano et al. 1992).

For the extensive reviews on the subtypes of α2-adrenoceptors, see the following publications: Docherty (1998), Starke (2001), Fairbanks et al. (2009), Philipp and Hein (2004), Gilsbach and Hein (2012), and Knaus et al. (2007).

All three subtypes differ in tissue distribution and pharmacological properties and show different radioligand biding characteristics. However, without specific subtype-selective agonists/antagonists, it has been difficult to assign physiological responses to individual α2-adrenergic receptor subtypes.

As an alternative to using pharmacological ligands, genetically modified mice with inactivated or modified genes of α2-adrenoceptor subtypes have been used (Philipp et al. 2002; Bücheler et al. 2002; Starke 2001; Kable et al. 2000; MacMillan et al. 1996; Link et al. 1995, 1996; Altman et al. 1999; Hein et al. 1999; MacDonald and Scheinin 1995; MacDonald et al. 1997; Hein 2001; Trendelenburg et al. 2001, 2003; Knaus et al. 2007).

α2A-adrenoceptor is the main presynaptic inhibitory autoreceptor regulating norepinephrine release from central and peripheral sympathetic nerves. Mice with mutated or deleted α2A-receptor subtype do not exhibit hypotensive, sedative, antinociceptive, or hypothermic effects in response to α2-adrenergic agonists and have higher resting systemic blood pressure and heart rate (Altman et al. 1999; Guyenet 1997; Lakhlani et al. 1997; Flordellis et al. 2004; Bücheler et al. 2002; Stone et al. 1997). α2A-Adrenoceptor is alternatively called α2A-/D-adrenoceptor because the rodent version of this receptor (α2D) differs pharmacologically from the human version (α2A).

α2B Subtype plays a major role in peripheral vasoconstriction in response to α2-adrenergic agonist (Altman et al. 1999; Link et al. 1996; MacDonald et al. 1997) development of salt-induced hypertension (Makaritsis et al. 1999) and the development of placental vascular system (Philipp et al. 2002).

The blood pressure response to intravenous injection of α2 agonist consists of two phases – short hypertensive phase, which is a result of activation peripheral of α2B-receptors, and long-lasting hypotensive phase, mediated by α2A-receptors (Altman et al. 1999; Hein et al. 1999; Philipp et al. 2002).

Adrenoceptors of α2C subtype also participate to presynaptic inhibition of norepinephrine release; however, they are particularly efficient at low stimulation frequencies, comparing with high stimulation frequency of α2A-adrenoceptors. (Hein et al. 1999; Scheibner et al. 2001; Trendenelburg et al. 2001; Bucheler et al. 2002; Blaxall et al. 1991).

Sallinen et al. (1999) demonstrated that α2C-adrenoceptors affect a number of behavioral functions (for the reviews, see Philipp et al. 2002; Scheinin et al. 2001).

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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, 2001; Raiteri et al. 1984; Miller 1998; Philipp et al. 2002; Docherty 1998). Presynaptic adrenergic α2-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 α2-adrenergic agonists and antagonists. The method was described in details in the following publications: Reynolds et al. (2005), Dooley et al. (2002), Vizi et al. (2004), Trendelenburg et al. (2003), Scheibner et al. (2001), Trendelenburg et al. (2001), and Bücheler et al. (2002).

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).

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 and 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.

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.)

Tissue Preparation

Male Wistar rats (100–150 g) are decapitated and 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.

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, and 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.

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Imidazoline Receptor Binding

Purpose and Rationale

Imidazoline receptors constitute a family of nonadrenergic high-affinity binding sites for clonidine, idazoxan, and allied drugs. Existence of specific imidazoline binding sites was propose by Bousquet and colleagues (Bousquet et al. 1984; 1981; Bousquet 1995, 1998) to explain central hypotensive effect of clonidine without involving α2-adrenoceptors. Later, the imidazoline binding sites were described to fulfill all the essential criteria of true receptor (binding, physiological function, location, and correlation of affinity with function) (Ernsberger 1999).

The importance of imidazoline receptors in cardiovascular action of centrally acting antihypertensive agent was discussed in the following publication: Head (1995), Head (1999), Head et al. (1998), Bock et al. (1999), and Szabo et al. (1999).

Three distinct subclasses of imidazoline receptors have been identified – I1, I2, and I3; none of them belongs to the family of G protein-coupled receptors and should be correctly called imidazoline binding sites.

The I1 sites are located in rostroventrolateral medulla, have high affinity to clonidine, and partly mediate antihypertensive effect of clonidine-like substance.

I2-Receptor is characterized by high affinity for idazoxan and low affinity for clonidine. Most I2 binding sites are located on the monoamine oxidase A and B enzymes in the outer membrane of mitochondria in the CNS and peripheral tissues, and binding to these sites allosterically modulates the enzymes (Coupry et al. 1987; Tesson et al. 1991, 1995).

The binding site I3 was identified in pancreatic β-cells; the activation of these sites increase insulin secretion (Morgan et al. 1999; Head and Mayorov 2006; Nikolic and Agbaba 2012; Khan et al. 1999; Szabo 2002; Eglen et al. 1998).

A several endogenous clonidine-displacing substance have been identified (Atlas and Burstein 1984; Prell et al. 2004; Li et al. 1994; Parker et al. 1999; Hudson et al. 1999; Robinson et al. 2003; Musgrave and Badoer 2000; Raasch et al. 2001, 2002; Reis et al. 1995; Chan et al. 1997).

From the first suggestion of the existence of imidazoline receptors, there has been the continuing debate about their contribution to antihypertensive actions of clonidine-like substance or ever existence of imidazoline receptors at all (Berdeu et al. 1996; Eglen et al. 1998; Guyenet 1997; Head et al. 1998; Szabo 2002).

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. The 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 1,000 *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 1,000 *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 the determination of specific binding to I_1-imidazoline sites and α2-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 α2-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 (Mc Pherson 1985). Then, several experiments are analyzed simultaneously with the LIGAND program for nonlinear curve fitting (Munson and Rodbard 1980). IC_{50} values are estimated from inhibition curves by nonlinear curve fitting (Mutolsky and Ransnas 1987). Protein assay data are also analyzed by nonlinear curve fitting (Mc Pherson 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]iodophenoxy]methyl imidazoline.

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

Molderings and Göthert (1995) determined electrically or K^+-evoked tritium overflow from superfused rabbit aortic strips preincubated 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-imidazoline 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 analogs (Jordan et al. 1996; Lione et al. 1996; Alemany et al. 1997; Hosseini et al. 1997; Wiest and Steinberg 1997; Hudson et al. 1997)

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β-Adrenoceptor Binding

Purpose and Rationale

Subtypes of β-adrenoceptor *β*-adrenergic receptors were differentiated from *α*-receptors (Ahlquist 1948) and subsequently divided into two distinct subtypes, *β*1 and *β*2 (Lands et al. 1967), based on differing pharmacology in different tissues. Emorine et al. (1992) cloned human β3-adrenoceptors.

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 the liver and skeletal muscle; and stimulation of lipolysis in the adipocyte (Daly and McGrath 2011).

β-Adrenoceptors belong to the family of G protein-coupled receptors and considered one of the most powerful regulators of cardiac function among the estimated 200 G protein-coupled receptors in the heart (for reviews see Brodde et al. 2006; Rockman et al. 2002; Ferguson 2001; Rajagopal et al. 2010; Vasudevan et al. 2011; Wachter and Gilbert 2012; Woo and Xiao 2012).

In addition to the heart, they are also expressed in the kidney, central nervous system, adipocytes, bronchial and vascular smooth muscle cells, lymphocytes, endothelial cells, and hepatocytes (Daly and McGrath 2011; Brodde 2008). The mammalian heart expresses all three β-adrenoceptor subtypes, with the β1- and β2-adrenoceptors as the main mediators of cardiac response to adrenergic stimulation. The population of β-adrenoceptors in the non-failing human heart is composed of β1- and β2-adrenocepors in a ratio of 8:2; however, in both ageing and failing heart, the proportion of β1 subtypes decreases due to mRNA downregulation, while levels of β2-adrenoceptors remain stable, thus achieving a 1:1 ratio of β1 and β2 subtypes (Bristow et al. 1986, 1990, 1991; Steinberg 1999; Brodde et al. 2006; Wachter and Gilbert 2012; White et al. 1994; Lohse et al. 2003).

Acute stimulation of cardiac β-adrenergic receptors results in positive ionotropic and chronotropic responses, whereas chronic β-adrenoceptor stimulation leads to pathological cardiac remodeling (Hasenfuss et al. 1996; Teerlink et al. 1994; Bristow et al. 1982, 1986, 1989; Buxton et al. 1987; Calderone et al. 1991; Cartagena et al. 1993; Pelá et al. 1990; Steinfath et al. 1991; Pérez-Schindler et al. 2013; Wang and Dhalla 2000).

The use of gene-targeted mouse and isolated cell line models has advanced identification of distinct molecular signaling mechanisms of β-adrenoceptors (for reviews, see Brodde et al. 2006; Woo and Xiao 2012; Wang and Dhalla 2000; Vasudevan et al. 2011; Philipp and Hein 2004; Wachter and Gilbert 2012; Shore and Moore 2003).

Three *β*-adrenoceptor proteins have been cloned, and the characteristics of these recombinant receptors correspond with those of the three well-characterized *β*-adrenoceptors in native tissue, designated as *β*1, *β*2, and *β*3

(Frielle et al. 1987; Kobilka et al. 1987; Emorine et al. 1989; Tate et al. 1991; Machida et al. 1990). The possible roles of β3-adrenoceptors in the cardiovascular system were discussed by Gauthier et al. (1996). An additional β-adrenoceptor modulating cardiac contractility has been designated as the β_4-adrenoceptor (Kaumann 1997; Kaumann et al. 1998; however, recent evidence suggest that putative β4 receptor is more likely a novel functional state of β1 receptor (Granneman 2001; Strosberg 1998; Kaumann et al. 2001; Konkar et al. 2000). It is now established that β-adrenoceptor polymorphism exists (see reviews by Small et al. 2003; Kirstein and Insel 2004; Leineweber et al. 2004; Brodde and Leineweber 2005; Brodde 2008).

Bronchodilation appears to be mediated by the β2-adrenoceptor. The β3-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 β1-adrenoceptor (Waitling 2006).

The β-adrenoceptor population of plasma membranes from bovine heart ventricles consists of 75–80 % β1- and 20–25 % β2-adrenoceptors. The use of this tissue allows a parallel investigation of the binding characteristics of drugs at both the β1- and β2-adrenoceptors. Both the β1- and β2-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).

β-Receptors have been labeled in a number of tissues including the 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; Rugg et al. 1978), and (^{125}I)-iodohydroxypindolol (Weiland et al. 1980). DHA is a potent β-antagonist (Mukherjee et al. 1975), which labels both β1- and β2-adrenergic receptors.

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
$MgCl_2 \times 6H_2O$	1 mM
D(+)-sucrose	250 mM
pH 7.4	
310 mOsm sodium phosphate buffer	
pH 7.4	Rinse buffer
Tris–HCl	50 mM
$MgCl_2 \times 6H_2O$	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-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 nonspecific binding experiments:

(−)Isoprenaline(+)bitartrate salt (Sigma)

Bovine hearts are obtained fresh from the local slaughter house. The lower part of the left ventricle from five 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

Nonspecific 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 $\times$ 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
- Nonspecific binding
- Specific binding = total binding–nonspecific 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-supported analysis of the binding data.

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

IC_{50} = concentration of the test drug, which competes with 50 % of specifically bound ^{3}HDHA 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 β1- and β2-adrenoceptor affinity of atenolol and metoprolol in tissues from the rat, the guinea pig, and man with various radioligands, such as $[^{125}I](\pm)$hydroxybenzylpindolol, $[^{125}I](-)$pindolol, $[^3H](-)$dihydroalprenolol, and $[^3H](-)$CGP 12177.

Rugg et al. (1978) proposed coexistence of β1- and β2-adrenoceptors in mammalian lung based on the binding characteristics [^{3}H]DHA and selective agonists in rat and rabbit lung tissue.

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

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β1-Adrenoceptor Binding

Purpose and Rationale

Lesioning studies (Wolfe et al. 1982; Dooley et al. 1986), combined with nonlinear regression analysis of data, have shown that while β-receptors in rat cerebellum are primarily of the β2 subtype, the β1 occurring in rat cerebral cortex are physiologically more significant. The assay can be used to evaluate the direct interaction of drugs with β-receptors labeled 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 three 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 three 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 20712A as a useful tool for quantitating β1- and β2-adrenoceptors.

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β2-Adrenoceptor Binding

Purpose and Rationale

Since [3H]-dihydroalprenolol is a nonspecific ligand, it is necessary to select a tissue which is enriched in β2-receptors in order to convey specificity to this assay. Tissues with predominantly β2-receptors include the 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 the forebrain, heart, and avian erythrocytes are relatively enriched in the β1 subtype (Lefkowitz et al. 1983). Due to poor binding characteristics in cerebellum, the rat lung is chosen as the tissue for β2-adrenergic receptors.

A compound with β2 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 β2-adrenergic receptor subtype. A measure of receptor subtype selectivity can be determined when data are compared with those obtained in the β1-adrenergic assay in rat cerebral cortex.

The present nomenclature of β1, β2, and β3 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).

2. (±)-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 three tubes to determine nonspecific binding (yielding a final concentration of 1 µM in a 1 ml assay).

3. 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 three 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 β1- and β2-adrenoceptors.

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

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 β2-adrenergic receptors. The β2-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 GenBank entries. The translated amino acid sequences corresponded to the published sequences for the β1-adrenergic receptor (Frielle et al. 1987), β2-adrenergic receptor (Schofield et al. 1987), and β3-adrenergic receptor (Emorine et al. 1989). With respect to polymorphisms, the β-adrenergic receptors used in this study corresponded to the following variants: β1-receptor 49-Ser and 389-Gly; β2-receptor 16-Arg, 27-Gln, and 164-Thr; and β3-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 two or three 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 β1- and β2-receptors, up to 400 pM ^{125}I-CYP and for β3-receptors up to 1,500 pM ^{125}I-CYP were used. Nonspecific binding was determined in the presence of 10 µM alprenolol. For competition binding, 50 pM ^{125}I-CYP in the case of β1- and β2-receptors or 80 pM ^{125}I-CYP for β3-receptors were used. For most of the competition binding experiments, membranes with intermediate receptor expression (β1, 367 ± 75 fmol/mg protein; β2, 282 ± 19 fmol/mg protein; β3, 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 three 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 nonlinear 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 300U/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 was 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 β1- and β2-adrenoceptors (about 40 pM), [^{125}I]-cyanopindolol and [^{3}H]-dihydroalprenolol had four- to sixfold higher affinity for β2- as compared to β1-adrenoceptors (10 vs. 45 and 187 vs. 1,021 pM, respectively). The affinity of [^{125}I]-cyanopindolol at β3-adrenoceptors was considerably lower (440 pM) than at the other two subtypes. The β3-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 and 30,000 pM for [^{3}H]-CGP 12,177 and [^{3}H]-dihydroalprenolol, respectively). All three radioligands were ill-suited to labeling β3-adrenoceptors, particularly in preparations co-expressing multiple subtypes. In the absence of alternatives, [^{125}I]-cyanopindolol appears the least unsuitable for labeling β3-adrenoceptors. At present, there is still a need for high-affinity radioligands that are selective for β3-adrenoceptors.

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