J. Membrane Biol. 170, 67–77 (1999)

The Journal of
Membrane
Biology

β_1-Adrenergic Receptors but not β_2-Adrenergic or Vasopressin Receptors Regulate K^+ Secretion in Vestibular Dark Cells of the Inner Ear

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Received: 12 February 1997/Revised: 25 March 1999

Abstract. Receptors were identified pharmacologically in functional studies where K^+ secretion was monitored as transepithelial current (I_{sc}). Further, receptors were identified as transcripts by cloning and sequencing of reverse-transcriptase polymerase chain reaction (RT-PCR) products. I_{sc} under control conditions was 796 ± 15 $\mu A/cm^2$ ($n = 329$) in gerbilline VDC and 900 ± 75 $\mu A/cm^2$ ($n = 6$) in murine VDC. Forskolin (10^{-5} M) but not 1,9-dideoxy-forskolin increased I_{sc} by a factor of 1.42 ± 0.05 ($n = 7$). 10^{-9} M Arg^8-vasopressin and 10^{-9} M desmopressin had no significant effect in gerbilline and murine VDC. Isoproterenol, norepinephrine, epinephrine and prenalterol stimulated I_{sc} maximally by a factor of 1.38 ± 0.04 ($n = 7$), 1.59 ± 0.06 ($n = 6$), 1.64 ± 0.03 ($n = 8$) and 1.37 ± 0.03 ($n = 6$), respectively. The EC_{50} values were (1.4 ± 0.7) × 10^{-8} M ($n = 36$), (2.5 ± 1.0) × 10^{-8} M ($n = 31$), (1.7 ± 0.7) × 10^{-7} M ($n = 36$) and (5 ± 4) × 10^{-7} M ($n = 32$), respectively. Propanolol inhibited isoproterenol-induced stimulation of I_{sc}. Atenolol, ICI118551 and CGP20712A inhibited isoproterenol-induced stimulation of I_{sc} with a pK_{DB} of 5.0 × 10^{-8} M ($pK_{DB} = 7.30 \pm 0.07$, $n = 38$), 4.4 × 10^{-8} M ($pK_{DB} = 7.36 \pm 0.14$, $n = 37$) and 6.8 × 10^{-12} M ($pK_{DB} = 11.17 \pm 0.12$, $n = 37$), respectively. RT-PCR of total RNA isolated from microdissected vestibular labyrinth tissue using specific primers revealed products of the predicted sizes for β_1- and β_2-adrenergic receptors but not for β_3-adrenergic receptors. Sequence analysis of the amplified cDNA fragments from gerbilline tissues revealed a 96.4%, 91.5% and 89.6% identity compared to rat β_1-, β_2- and β_3-adrenergic receptors, respectively. These results demonstrate that K^+ secretion in VDC is under the control of β_1- but not β_2- or β_3-adrenergic receptors or vasopressin-receptors.

Key words: $Beta_1$-adrenergic receptors — Vestibular labyrinth — Ussing chamber — RT-PCR

Introduction

The vestibular labyrinth is a mechanosensitive organ, which provides the organism with information about the position and movements of the head in space. Its lumen contains about 145 mM K^+ which is the main charge carrier for the mechanosensory transduction process in the sensory hair cells. The sensory hair cells as well as K^+ secretory vestibular dark cells (VDC) are part of the epithelium, which encloses the luminal fluid space. Mechanical stimulation of the hair cells causes an efflux of K^+ from the lumen through the hair cells into the abluminal fluid space (Valli, Zucca & Botta, 1990). VDC take up this K^+ across their basolateral membrane and secrete it back into the luminal fluid across their apical membrane (Wangemann, 1995). In particular, VDC take up K^+ across the basolateral membrane via the $Na^+2Cl^-K^+$ cotransporter and the Na,K-ATPase and secrete K^+ across their apical membrane via the I_{sK} channel.

Homeostasis of the luminal fluid volume is necessary to provide a stable source of charge carrier for the transduction process as well as a stable mechanical environment for this mechanosensitive organ. In particular, shifts in the K^+ ion concentrations must be prevented to avoid fluid shifts and changes in the mechanical properties. Thus, it is most likely that the vestibular labyrinth employs homeostatic mechanisms, which monitor the luminal and abluminal K^+ concentrations and the fluid vol-

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umes and trigger corrective responses when necessary. One such mechanism monitors the abluminal K^+ concentration and adjusts the rate of K^+ secretion in VDC accordingly. Indeed, we have shown in an earlier study that a small elevation of the basolateral K^+ concentration by as little as 1 mM causes in VDC an increase in the rate of K^+ secretion by a factor of as much as 23% (Wangemann, Shen & Liu, 1996). Thus, we provided evidence for a local homeostatic mechanism in which K^+ is not only the ion to be transported but also the extracellular (first) messenger. In the present study, we hypothesized the presence of a systemic mechanism governing K^+ secretion. We hypothesized that an increase in the activity level of the organism, which is mostly associated with head movements and stimulation of the vestibular labyrinth, causes a stimulation of K^+ secretion in VDC. In the search of such a mechanism, we rationed that systemic hormones may be involved since VDC do not receive direct innervation. Hormones, which vary with the activity level of an organism, include norepinephrine and vasopressin. Plasma levels of norepinephrine are known to vary in humans between 0.7 nM during rest in supine position and 6 nM during extreme exercise (Lake et al., 1997) and plasma levels of vasopressin vary between 1.6 pM at rest and 4.8 pM during strenuous exercise (Baylis & Heath, 1977).

Several lines of evidence prompted us to focus our search on receptors, which are linked to the cAMP second messenger system. First, the enzyme generating cAMP, adenylate cyclase, has been demonstrated in the basolateral membrane of frog VDC (Oudar, Ferrary & Feldmann, 1990). Second, direct stimulation of adenylate cyclase with forskolin or indirect stimulation of adenylate cyclase by the β-adrenergic receptor against isoproterenol or the vasopressin receptor agonist vasopressin have been shown to cause an elevation in cAMP in a biochemical preparation of frog semicircular canals (Ferrary et al., 1991*a*,*b*). Third, direct addition of cAMP (as dbcAMP) or indirect accumulations of cAMP via inhibition of phosphodiesterases, enzymes of cAMP catabolism, have been shown to stimulate K^+ secretion in gerbilline VDC (Sunose et al., 1997).

Adrenergic and vasopressin receptors which mediate stimulation of adenylate cyclase include the β_1, β_2 and β_3-adrenergic receptor and the V_2-vasopressin receptor. The goal of the present study was to determine whether (i) K^+ secretion in VDC is controlled via β-adrenergic and/or vasopressin receptors and (ii) which receptor subtype is involved.

Pharmacologic tools are now well established to determine the presence of these receptors. V_2-vasopressin receptors can be distinguished from V_1-vasopressin receptors by the agonist desmopressin, which mimics the effects of the natural agonist vasopressin. β-adrenergic receptors can be distinguished from α-adrenergic receptors by the agonist isoproterenol which mimics the effects of the natural agonists norepinephrine (noradrenaline) and epinephrine (adrenaline) and by the antagonist propanolol which prevents agonist-induced effects. Further, the β_1-, β_2- and β_3-adrenergic receptor subtypes can be distinguished by the affinities of the antagonists atenolol, ICI118551 and CGP20712A (*for references, see* Table 4). In addition, evidence for the presence or absence of β_1-, β_2- and β_3-adrenergic receptors can be obtained with molecular techniques based on the known nucleotide sequences of the rat and mouse β_1-adrenergic receptors (Machida et al., 1990; Shimomura & Terada, 1990; Jasper et al., 1993), the rat and hamster β_2-adrenergic receptor (Dixon et al., 1986; Buckland et al., 1990; Jiang & Kunos, 1995) and the rat β_3-adrenergic receptor (Nahmias et al., 1991).

In this study, we demonstrate that K^+ secretion in VDC is stimulated via β_1-adrenergic receptors but not via vasopressin receptors since physiologically relevant concentrations of norepinephrine but not vasopressin caused a significant stimulation of K^+ secretion. These findings support our hypothesis that an increase in the activity level of the animal causes stimulation of K^+ secretion in the vestibular labyrinth to accommodate an anticipated elevated level of vestibular stimulation. Parts of this study have been presented at recent meetings (Wangemann & Liu, 1996; Liu & Wangemann, 1997).

Materials and Methods

Preparations

The method of dissection of VDC epithelium has been described earlier (*for references, see* (Wangemann, 1995)). Briefly, gerbils and mice were anesthetized with pentobarbital (100 mg/kg i.p.) and decapitated under deep anesthesia. The procedures concerning animals reported in this study were approved by the Institutional Animal Care and Use Committee at Boys Town National Research Hospital and Kansas State University. The temporal bones housing the inner ear were removed and quickly transferred for microdissection into cold (4°C) solution containing (in mM) 150 Na-gluconate, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 4.0 Ca-gluconate, 1.0 $MgSO_4$, 5.0 glucose, pH 7.4. Under microscopic observation the ampullae was dissected free and a patch of epithelium including dark cell epithelium was carefully trimmed. For measurement of I_{sc} the tissue was used as a flat sheet. For the molecular biologic studies, whole vestibular labyrinths were isolated from gerbils and for the development of the primers whole brains and subcutaneous fat tissues were isolated from rats and gerbils.

Measurement of the Equivalent Short Circuit Current (I_{sc})

The methods were described previously (*for references, see* Wangemann, 1995). Briefly, tissue was mounted in a micro-Ussing chamber by sealing the apical membrane of VDC to the aperture (80 μm diameter). The apical and basolateral sides of the tissue were perfused independently. Solution changes on each side were complete within 1

sec. The transepithelial voltage (V_t) was measured with calomel electrodes connected to the chambers via 1 M KCl bridges. The transepithelial resistance (R_t) was obtained from the voltage changes induced by current pulses (50 nA for 34 msec at 0.3 Hz). Sample and hold circuitry was used to obtain a signal proportional to R_t. The current (I_{sc}), which under many conditions has been shown to be proportional to K^+ secretion, was obtained according to Ohm's law ($I_{sc} = V_t/R_t$) from measurements of V_t and R_t.

Determination of the EC_{50} of Agonists and the K_{DB} of Antagonists

Dose-response curves were obtained by testing in one tissue the effect of only one concentration of agonist either in the absence or in the presence of an antagonist. It was not possible to obtain cumulative dose-response curves since preliminary experiments revealed that the effect of a second dose was significantly smaller compared to experiments in which this dose had been given first. The agonist concentration, which induced a half-maximal effect (EC_{50}), was obtained by fitting the data to the equation

$$E = E_{max} \times C^h/(EC_{50}{}^h + C^h) \quad (1)$$

where E is the change in I_{sc} relative to the change in I_{sc} caused by 2×10^{-6} M isoproterenol, E_{max} is the maximal effect, C is the concentration of the agonist and h is the slope.

The affinity of antagonists to the receptor (K_{DB}) was obtained through competitive inhibition using isoproterenol as the agonist. K_{DB} was obtained from the Schild-equation

$$p(K_{DB}) = log(B) - log(DR - 1) \quad (2)$$

where DR is the dose-ratio and B is the concentration of the antagonist. The DR was obtained according to $DR = EC_{50Antagonist}/EC_{50Isoproterenol}$ where $EC_{50Antagonist}$ is the EC_{50} of isoproterenol in the presence of the antagonist and $EC_{50Isoproterenol}$ is the EC_{50} of isoproterenol in the absence of antagonists. $EC_{50Antagonist}$ was obtained according to Eq. 1 in which E_{max} and h were fixed to the values obtained in the absence of antagonists. $p(K_{DB})$ values were averaged and converted to K_{DB} values ($K_{DB} = 10\hat{}p(K_{DB})$) for more common appeal.

All nonlinear curve fits were obtained by a least-squares algorithm using the programmable spreadsheet and plotting software Origin 4.1 (Microcal).

Extraction of Total RNA

Total RNA was extracted from microdissected vestibular labyrinths and blood obtained from gerbils as well as from whole brains and subcutaneous fat obtained from rats and gerbils, as described earlier (Shimozono et al., 1998). Methods employed for the extraction of RNA from vestibular labyrinths and blood were different from those employed for brains and subcutaneous fat due to the lower amount of RNA available. Brains were sliced with razor blades and frozen in liquid nitrogen within 5 min of sacrifice. The frozen tissue was pulverized in liquid nitrogen and immediately transferred into TRIzol Regent (GIBCO BRL, Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate. Total RNA was extracted using the TRIzol Reagent according to manufacturer's procedure. Total RNA was precipitated by isopropanol, and dissolved in RNase-free water (diethylpyrocarbonate-treated water). The nucleic acid concentration was determined spectrophotometrically and adjusted to be between 1.0 and 2.0 μg/μl. RNA samples were stored at −70°C. Before analysis of the RNA samples by reverse-transcription polymerase chain reaction (RT-PCR), DNA contamination of the RNA samples from brain was removed by treatment with DNase I (GIBCO BRL, Life Technologies) in the presence of RNasin (Promega) for 30 min at 37°C. Total RNA was then precipitated with ethanol in the presence of 2 M NH_4OAC.

Vestibular labyrinths from gerbils was isolated by microdissection and directly transferred from the dissection medium into the TRIzol Reagent within 7 min (first ear) and 15 min (second ear) of sacrifice of each animal. Tissues from 8 ears were pooled in the TRIzol Reagent and disrupted by sheer stress induced by several passes of the RNA extracts through a 25-gauge hypodermic syringe needle. Total RNA from the vestibular labyrinth was extracted using the TRIzol Reagent according to the manufacturer's procedure. The final concentration of RNA from the vestibular labyrinth samples was about 0.3 μg/μl. RNA samples were stored at −70°C. Residual genomic DNA in RNA samples from the vestibular labyrinth was removed prior to RT-PCR by treatment with amplification-grade RNase-free DNase I (GIBCO BRL, Life Technologies) for 30 min at room temperature followed by heat inactivation in the presence of EDTA, according to the protocol specified by the manufacturers.

cDNA Synthesis and PCR Amplification

Total RNA was reverse transcribed into cDNA in a 20 μl reaction. The reaction contained 0.2 or 0.75 μg total RNA, 20 units RNasin (Promega), 1 mM dNTP (GIBCO BRL, Life Technologies), 50 units Moloney Murine Leukemia Virus Reverse Transcriptase (Perkin-Elmer), 2.5 mM $MgCl_2$ (GIBCO BRL, Life Technologies), 25 pmol oligo d(T), 20 mM Tris-HCl and 50 mM KCl. Tris-HCl (pH 8.4) and KCl were added as a 10× PCR buffer (GIBCO BRL, Life Technologies). The RT reaction was incubated at 42°C for 50 min, 5 min at 99°C, and 5 min at 5°C.

The 100 μl PCR reaction contained the 20 μl RT reaction mix, 2.5 units Taq DNA polymerase (GIBCO BRL, Life Technologies) and 25 pmol of the antisense and the sense primer for the β_1-, β_2 or β_3-adrenergic receptor, respectively. The final concentrations of $MgCl_2$, KCl and Tris-HCl were adjusted to 2.5, 50 and 20 mM, respectively. The PCR reaction mix was incubated as follows: denaturation for 3 min at 95°C; 45 amplification cycles consisting of 1 min denaturation at 95°C, 1 min annealing at 50°C and 1 min extension at 72°C; and extension cycle for 5 min at 72°C.

The primers used are shown in Table 1. Sense specific primers for β_1-, β_2- or β_3-adrenergic receptors were designed based on the published sequences (GenBank/EMBL database) using the software Prime (Wisconsin Package, Genetics Computer Group). In addition, the sense and antisense β_1-adrenergic receptor primers contained 12 extra nucleotides at the 5′ end of the sequence that was recognized by the restriction enzymes *Bam*HI and *Hind*III. The specificity of the primers was validated by RT-PCR of RNA obtained from gerbil brain and subcutaneous fat tissue known to contain β_1-, β_2- and β_3-adrenergic receptors, respectively (*data not shown*). PCR products were analyzed by horizontal electrophoresis in 2% agarose gels and visualized by ethidium bromide.

Cloning and Sequencing of Amplified cDNA Fragments

Amplified cDNA fragments were extracted from the agarose gels using the QIA quick gel extraction kit (Qiagen) and cloned into a pCR® 2.1 vector with a TA cloning® kit (Invitrogen). Recombinant plasmids were isolated from positive colonies using the standard alkaline lysis procedure, purified by phenol/chloroform extraction, and precipitated

Table 1. Primers

		Primer	Location[1]	Size
β_1	Sense[2]	5′-GGATCCAAGCTTTCGTGTGCACCGTGTGGGCC-3′	H620-639	289 bp
	Antisense[2]	5′-GGATCCAAGCTTAGGAAACGGCGCTCGCAGCTGTCG-3′	H861-884	
β_2	Sense	5′-CACCAACTACTTCATAACCTC-3′	R2452-2472	805 bp
	Antisense	5′-GGCAATCCTGAAATCTGGGCTCCGGCAG-3′	H2593-2565	
β_3	Sense	5′-TGCGCCCATCATGAGCCAGTGGTG-3′	R552-575	550 bp
	Antisense	5′-GCGAAAGTCCGGGCTGCGGCAGTA-3′	R1078-1101	

[1] According to known sequences in the rat (R) and human (H): β_1 accession #J03019, β_2 rat accession #X17607 and human accession #M15169, β_3 accession #S73473.
[2] Primers for the β_1-adrenergic receptors contained at the 5′ end restriction enzyme-specific sequences (underlined).

and washed with ethanol. Insertion of the PCR product into the plasmid was confirmed by restriction endonuclease digestion with *Eco*RI and subsequent horizontal gel electrophoresis. The recombinant double stranded plasmid served as a template for cycle sequencing using M13 forward and reverse primers or β-adrenergic receptor subtype specific primers using fluorescence-labeled dideoxy nucleotides (PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit, Perkin Elmer). The sequence was then determined using the ABI Model 373 DNA Sequencer (Applied Biosystems) and confirmed by the cloning and sequencing of RT-PCR products from at least three separate RT-PCR reactions. The identity of the gerbil sequences was determined by a FastA (Wisconsin Package, Genetics Computer Group) comparison to known GenBank/EMBL sequences.

Statistics

Data of functional studies are presented as the mean ± SEM. The number of observations (*n*) is number of tissue samples. Comparisons were made using Student's *t*-test for paired and unpaired samples as appropriate. A level of $P < 0.05$ was taken as statistically significant.

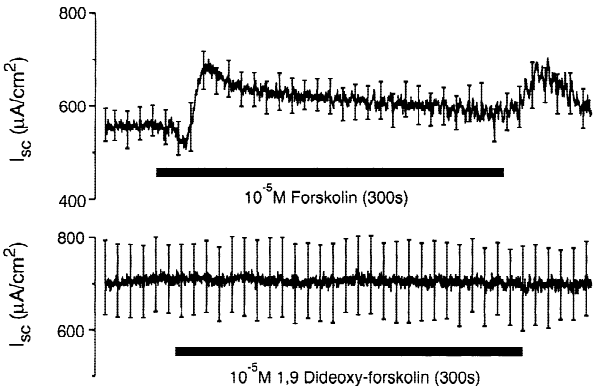

Fig. 1. Effect of forskolin on I_{sc} in vestibular dark cells (VDC). Forskolin (*top*) increased I_{sc} whereas the inactive forskolin-analogue 1,9-dideoxy forskolin (*bottom*) had no significant effect on I_{sc}. The average of 7 (*top*) and 6 experiments (*bottom*) are shown. For clarity, not all error bars are shown. The initial transient decrease in I_{sc} and the transient increase in response to the removal of forskolin remained unexplained.

Results

Adenylate cyclase in frog VDC has been shown to be associated with the basolateral membrane and to generate cAMP upon stimulation (Oudar et al., 1990). It was expected that direct stimulation of adenylate cyclase with forskolin would cause stimulation of K^+ secretion and an increase of I_{sc} since dbcAMP had been shown to cause an increase in I_{sc} consistent with an increase in K^+ secretion (Sunose et al., 1997). Thus, in a first series of paired experiments the effect of 10^{-5} M forskolin on I_{sc} was determined (Fig. 1). Forskolin increased I_{sc} from 482 ± 28 to 598 ± 35 μA/cm^2 ($n = 7$) whereas the inactive forskolin isoform, 10^{-5} M 1,9-dideoxy forskolin, had no significant effect on I_{sc} (603 ± 52 *vs.* 588 ± 56 μA/cm^2, $n = 7$). These observations are consistent with the interpretation that forskolin-induced stimulation of adenylate cyclase led to an increased cytosolic cAMP concentration and thereby to a stimulation of K^+ secretion.

Adenylate cyclase in frog VDC is stimulated by vasopressin suggesting the presence of vasopressin receptors (Oudar et al., 1990; Ferrary et al., 1991*b*). This observation led to the hypothesis that vasopressin stimulates adenylate cyclase in gerbilline VDC and that the resulting increase in cAMP would stimulate K^+ secretion. Thus, it was expected that vasopressin and desmopressin would cause an increase in I_{sc}. Therefore, in a second series of experiments the effects of arginine vasopressin (AVP) and desmopressin on I_{sc} were determined in paired experiments. Contrary to the hypothesis, neither 10^{-9} M AVP nor 10^{-9} M desmopressin had a significant effect on I_{sc} in gerbilline VDC (657 ± 67 *vs.* 670 ± 66 μA/cm^2, $n = 7$; 818 ± 115 *vs.* 819 ± 109 μA/cm^2, $n = 7$) although 10^{-8} M AVP and 10^{-8} M desmopressin caused a small but significant increased I_{sc} (from 2137 ± 400 to 2181 ± 408 μA/cm^2, $n = 5$) and from 625 ± 46 to 640 ± 47 μA/cm^2, $n = 6$), respectively. This series of experiments was repeated in murine VDC to eliminate the possibility that a low sensitivity to vasopressin is specific to the gerbil which, as a desert animal, has a higher circulating vasopressin concentration than non-desert animals (Baddouri & Quyou, 1991). In-

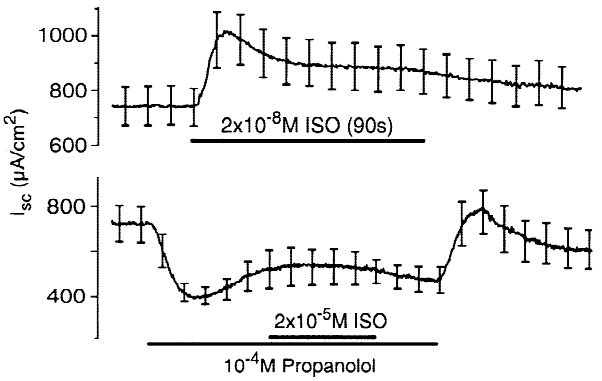

Fig. 2. Effect of isoproterenol (ISO) on I_{sc} in the absence (*top*) and presence (*bottom*) of propanolol. The average of 7 (*top*) and 8 experiments (*bottom*) are shown. For clarity, not all error bars are shown.

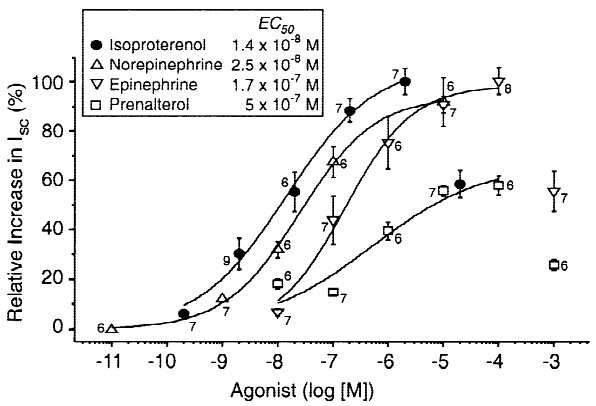

Fig. 3. Dose-response curves for the effects of isoproterenol, norepinephrine, epinephrine and prenalterol on I_{sc}. Data were normalized to factor (= 1.64) by which 2×10^{-6} M isoproterenol increased I_{sc}. Curves are the result of a nonlinear fit to Eq. 1 (*see* Materials and Methods). Some of the dose-response curves appeared to be bell-shaped. Data right of the peak were not included in the fit. EC_{50}, E_{max}, and h values resulting from the fit are detailed in Table 2. EC_{50} are also given in the legend. The numbers next to the symbols depict the number of experiments.

deed, plasma concentrations in well-hydrated mice can be assumed to be near 2×10^{-13} M based on available data in rats (Saito et al., 1997) whereas plasma concentration in well-hydrated gerbils have been found to be in the order of 1.5×10^{-10} M (Baddouri & Quyou, 1991). No significant increase of I_{sc}, however, was observed in murine VDC in response to 10^{-8} M AVP (900 ± 75 *vs.* 926 ± 78 μA/cm^2, $n = 6$). These observations suggest that K^+ secretion in gerbilline and murine VDC is not regulated via vasopressin receptors since effective concentrations of the agonist vasopressin (10^{-8} M) are unlikely to be reached under physiologic conditions.

Stimulation of adenylate cyclase by the β-adrenergic receptor agonist isoproterenol has been shown to cause an elevation in cAMP in a biochemical preparation of frog semicircular canals (Ferrary et al., 1991*a*,*b*). This observation led to the hypothesis that β-adrenergic receptor agonists stimulate K^+ secretion and cause an increase in I_{sc} in gerbilline VDC. Thus, in a third series of experiments the effects of the β-adrenergic receptor agonists norepinephrine, epinephrine, isoproterenol and prenalterol on I_{sc} were determined. All of these agonists caused a dose-dependent stimulation of I_{sc}. An example is shown in Fig. 2 (*top*). Dose-response curves are summarized in Fig. 3 and EC_{50}, E_{max} and h values are summarized in Table 2. The agonist potency order, isoproterenol <= norepinephrine < epinephrine <= prenalterol, suggest that K^+ secretion in VDC is stimulated via β_1-adrenergic receptors. The observation that h ranged between 0.4 and 0.7 remains unexplained. The observation that E_{max} of isoproterenol, norepinephrine and epinephrine was not significantly different from 100 suggests that these agonists are full agonists whereas the observation that E_{max} of prenalterol was 67 suggests that prenalterol is a partial agonist in VDC. Interestingly, it has been shown that the effectiveness of prenalterol depends on the amount of available β_1-adrenergic receptors (Kenakin, 1997). The pronounced dose-dependency of the effect of prenalterol can thus be taken as evidence for a high density of β_1-adrenergic receptors in VDC.

Table 2. EC_{50}, E_{max} and h for β-adrenergic agonists

Agonist	EC_{50}	E_{max}	h
Isoproterenol	$(1.4 \pm 0.7) \times 10^{-8}$ M, n = 36	107 ± 8	0.5 ± 0.1
Norepinephrine	$(2.5 \pm 1.0) \times 10^{-8}$ M, n = 31	94 ± 6	0.6 ± 0.1
Epinephrine	$(1.7 \pm 0.7) \times 10^{-7}$ M, n = 36	99 ± 6	0.7 ± 0.2
Prenalterol	$(5.2 \pm 4.0) \times 10^{-7}$ M, n = 32	67 ± 8	0.4 ± 0.1

To verify the conclusion that β-adrenergic receptors mediated the effects by isoproterenol, norepinephrine and epinephrine, it was necessary to demonstrate that the agonist-induced effects were antagonized by a β-adrenergic antagonist such as propanolol. Thus, in a fourth series of experiments the effect of propanolol on isoproterenol-induced stimulation of I_{sc} was determined in unpaired experiments (Fig. 2). Addition of 10^{-4} M propanolol caused a significant decrease of I_{sc} from 722 ± 80 to 393 ± 37 μA/cm^2 ($n = 8$). Isoproterenol (2×10^{-5} M) in the presence of propanolol caused an increase in I_{sc} by a factor of 1.11 ± 0.04 ($n = 8$) which is significantly smaller than the increase by a factor of 1.38 ± 0.04 ($n = 7$) which was observed in the absence of propanolol. These observations support the conclusion that β-adrenergic receptors mediate regulation of K^+ secretion in VDC.

Three different β-adrenergic receptor subtypes (β_1, β_2, β_3) are pharmacologically well characterized and a fourth subtype (β_4) has most recently been described. β_1, β_2, and β_3-adrenergic receptors are known to stimu-

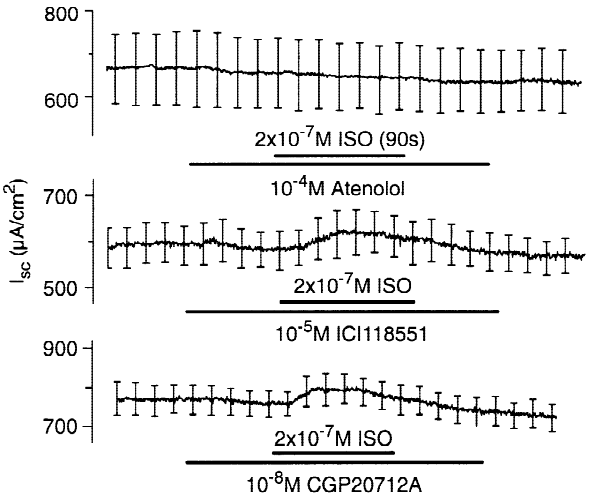

Fig. 4. Effect of isoproterenol (ISO) on I_{sc} in the presence of the antagonists atenolol (*top*), ICI118551 (*middle*) and CGP20712A (*bottom*). The average of 7 (*top*), 6 (*middle*) and 8 (*bottom*) experiments are shown. For clarity, not all error bars are shown. Note, that the addition of the antagonists atenolol, ICI118551 and CGP20712A had no effect on I_{sc} in contrast to the antagonist propanolol (*see* Fig. 2, *bottom*). Further, note that the antagonist at the concentrations indicated blocked isoproterenol induced stimulation of I_{sc}. The effect is only evident when compared to the effect of isoproterenol in the absence of antagonists (Fig. 2, *top*).

late adenylate cyclase but differ in their sensitivity to the antagonists atenolol, ICI118551 and CGP20712A. Thus, the subtype dominating the observed stimulation of K^+ secretion can be determined pharmacologically. In a fifth series of experiments the effects of atenolol, ICI118551 and CGP20712A on isoproterenol-induced stimulation of I_{sc} were determined in unpaired experiments. In contrast to propanolol, which decreased I_{sc} in the absence of agonist, neither addition of 10^{-4} M atenolol, 10^{-5} M ICI118551 nor of 10^{-8} M CGP20712A had a significant effect on I_{sc} (714 ± 35 *vs.* 709 ± 35 $\mu A/cm^2$, $n = 22$; 641 ± 35 *vs.* 637 ± 33 $\mu A/cm^2$, $n = 20$; 761 ± 23 *vs.* 765 ± 21 $\mu A/cm^2$, $n = 23$, respectively; Fig. 4). Dose-response curves for isoproterenol, however, were shifted to the right in the presence of 10^{-6} and 10^{-4} M atenolol, 10^{-6} and 10^{-5} M ICI118551 and 10^{-10} and 10^{-8} M CGP20712A (Fig. 5*A–C*). The $p(K_{DB})$ values for atenolol, ICI118551 and CGP20712A were calculated according to Eq. 2 (*see* Materials and Methods) to be 7.30 ± 0.07, $n = 38$; 7.36 ± 0.14, $n = 37$ and 11.17 ± 0.12, $n = 37$, respectively, corresponding to K_{DB} values of 5.0×10^{-8} M, 4.4×10^{-8} M and 6.8×10^{-12} M.

If the effects elicited by these antagonists were competitive in nature, it would be expected that the slopes of Schild-plots (*log* (*DR*-1) plotted *vs. log(Antagonist)*) were 1 (Kenakin, 1997). Indeed, slopes of Schild-plots for atenolol, ICI118551 and CGP20712A were 1.14, 1.4 and 1.005 (Fig. 5*D*), all of which are reasonably close to unity, thus supporting competitive action of the antagonists. The intersections of the linear regression curves of the Schild-plots with the x-axis at $log(DR\text{-}1) = 0$ gives estimates of the K_{DB} values (Kenakin, 1997). These estimates differed by no more than a factor of 3 from the calculated K_{DB} values, thus verifying the calculations. The determination of the K_{DB} values provides the basis to conclude that K^+ secretion in VDC is predominantly stimulated via β_1-adrenergic receptors (*see* Discussion).

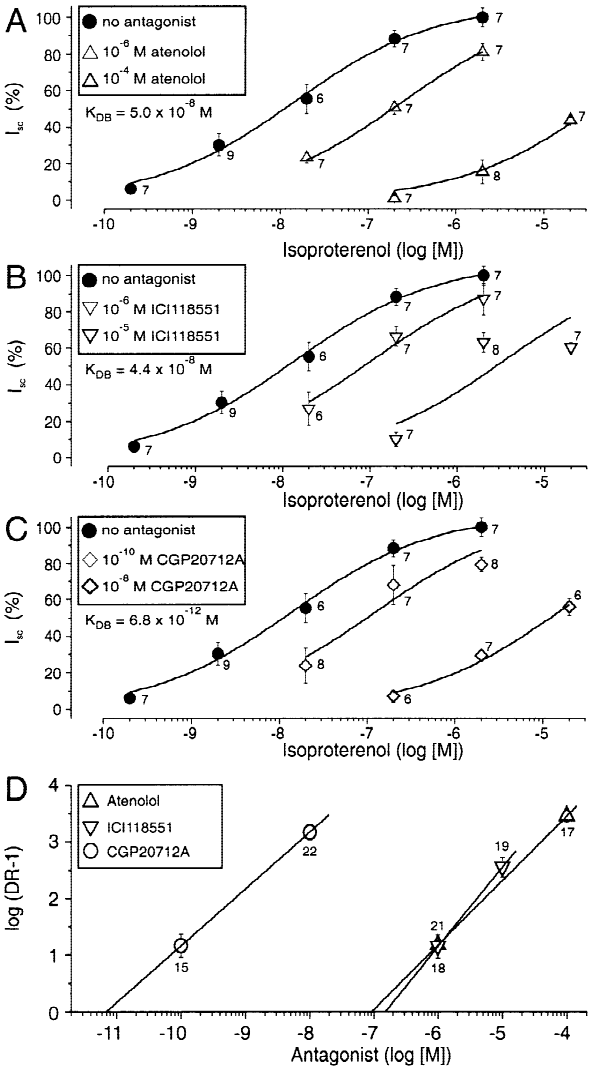

Fig. 5. Dose-response curves for the effect of isoproterenol on I_{sc} in the absence and presence of the antagonists (*A*) 10^{-6} and 10^{-4} M atenolol, (*B*) 10^{-6} and 10^{-5} M ICI118551 and (*C*) 10^{-10} and 10^{-8} M CGP20712A. Data were normalized to the factor (= 1.64) by which 2×10^{-6} M isoproterenol increased I_{sc} in the absence of antagonists. The dose-response curve for isoproterenol in the absence of antagonists has been added to *A*, *B* and *C* to allow comparison. Curves are the result of a nonlinear fit to Eq. 1 (*see* Materials and Methods). (*D*) Schild-plots for atenolol, ICI118551 and CGP20712A. The numbers next to the symbols depict the number of experiments.

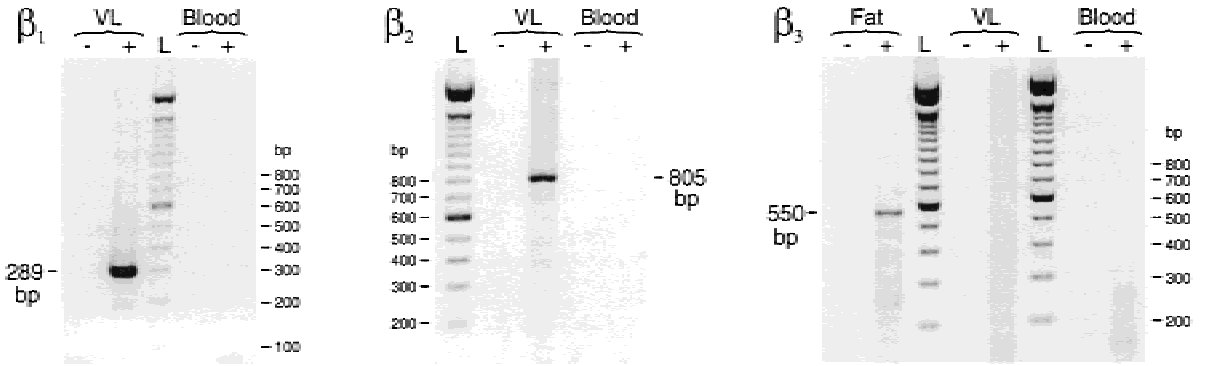

Fig. 6. Agarose gel electrophoresis of reverse-transcriptase polymerase chain reaction (RT-PCR) products. *Left:* RT-PCR was performed with gene-specific primers for β_1-adrenergic receptors on 0.2 μg of total RNA obtained from microdissected vestibular labyrinth (VL) or 0.2 μg of total RNA obtained from blood in the presence (+) and absence (−) of reverse-transcriptase. The DNA markers (L) consisted of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp. Note that a band of the expected size was found in VL but not in Blood. *Middle:* RT-PCR was performed with gene-specific primers for β_2-adrenergic receptors on 0.2 μg total RNA obtained from microdissected VL and 0.2 μg total RNA obtained from blood. Note that a band of the expected size was found in VL but not in blood. *Right:* RT-PCR was performed with gene-specific primers for β_3-adrenergic receptors on 0.75 μg of total RNA obtained from microdissected VL, 0.75 μg of total RNA obtained subcutaneous fat tissue and 0.75 μg of total RNA obtained from blood. Note that a band of the expected size was found in fat tissue but not in VL or blood. The observation that no products were observed when reactions were performed in the absence of reverse transcriptase (−) demonstrates that samples of RNA were free of contamination by DNA. The observation that no product of the expected size was obtained from blood rules out the possibility that blood, which was a contaminant of the microdissected VL and fat tissue, provided a source of transcripts for the β-adrenergic receptors. RT-PCR products were cloned and sequenced. Sequences for the β_1-, β_2- and β_3-adrenergic receptor fragments are available in Genbank under accession numbers AF055349, AF055350 and AF055351, respectively.

In addition to the pharmacologic determination of β-adrenergic receptors in VDC, the presence of β_1-, β_2- and β_3-adrenergic receptor transcripts was determined by RT-PCR of total RNA isolated from the whole vestibular labyrinth. The presence of β-adrenergic receptor transcripts was determined in RNA from vestibular labyrinth rather than from VDC because RNA cannot readily be obtained from VDC. In a sixth series of experiments RT-PCR was performed on total RNA extracted from vestibular labyrinth with primer pairs specific for the β_1-, β_2- and β_3-adrenergic receptor transcripts. Reactions with primers for β_1- and β_2-adrenergic receptors revealed RT-PCR products of the expected sizes of 289 and 805 bp, respectively (Fig. 6, lanes marked 'VL +'). This observation suggests that the vestibular labyrinth contains transcripts for β_1- and β_2-adrenergic receptors. In contrast, no appropriate products were found in reactions performed with primers specific for the β_3-adrenergic receptor. The specificity of the β_3 primers was verified through RT-PCR performed with total RNA extracted from gerbil subcutaneous fat tissue (Fig. 6, lanes marked 'Fat +'). These reactions revealed a PCR product of the expected size of 550 bp. The observation that no product was found in reaction performed with total RNA from the vestibular labyrinth suggests that the vestibular labyrinth does not contain β_3-adrenergic receptors.

It is conceivable that blood present inside the capillaries of vestibular labyrinth or subcutaneous fat tissue provided a source of mRNA for the β-adrenergic receptors. To evaluate this possibility, we determined whether transcripts for β_1-, β_2- and β_3-adrenergic receptors could be amplified from total RNA extracted from gerbil blood samples. No appropriate products were obtained using primers specific for β_1-, β_2- or β_3-adrenergic receptor transcript (Fig. 6, lanes marked 'Blood +'). These observations demonstrate that blood present in the samples of vestibular labyrinth and subcutaneous fat tissue did not provide a source of message for either β-adrenergic receptor subtype. Further, RNA samples were tested to be free of DNA contamination by showing that no PCR products resulted from reactions in which RNA was not reverse transcribed into cDNA (Fig. 6, lanes marked '−').

The identity of the PCR products was confirmed by sequencing the products and comparing the sequences to known sequences (Table 3). The nucleotide sequences of the amplified fragments of β_1-, β_2- and β_3-adrenergic receptors from the gerbil vestibular labyrinth and subcutaneous fat tissue have been deposited in Genbank under the accession numbers AF055349, AF055350 and AF055351, respectively. The sequences given exclude the primer sequences.

Discussion

Adenylate Cyclase and the Regulation of K^+ Secretion

The observation that basolateral application of forskolin but not 1,9-dideoxy forskolin caused an increase in I_{sc} is

Table 3. Accession # and % identity of sequenced PCR products

	Gerbil accession #	Rat accession # % identity to gerbil	Mouse accession # % identity to gerbil	Human accession # % identity to gerbil
β_1	AF055349	E03837	L10084	J03019
		96.4% in 221 nt	95.5% in 221 nt	98.2% in 221 nt
β_2	AF055350	X17607	X15643	M16106
		91.9% in 756 nt	91.5% in 756 nt	87.5% in 754 nt
β_3	AF055351	S73473	X72862	X72861
		90.4% in 502 nt	92.4% in 502 nt	84.7% in 502 nt

consistent with the interpretation that a functional adenylate cyclase is present near the basolateral membrane and that this adenylate cyclase is involved in the regulation of K^+ secretion. This interpretation is consistent with a number of observations made in frog VDC, biochemical preparations of frog semicircular canals and gerbilline VDC (*see* Introduction). The mechanism for the cAMP-induced stimulation of K^+ secretion may be twofold. On the other hand, it has been shown that cAMP stimulates the apical I_{sK} channel and thereby stimulates K^+ secretion across the apical membrane (Sunose et al., 1997). On the other hand, it is likely that cAMP stimulates K^+ uptake via the basolateral $Na^+/2Cl^-/K^+$ cotransporter as it has been shown in other tissues, which are known to contain this transporter (Whisenant et al., 1991).

Vasopressin Receptors are not Involved in the Regulation of K^+ Secretion

The observation that neither the nonspecific vasopressin receptor agonist AVP nor the V_2-specific receptor agonist desmopressin at a concentration of 10^{-9} M stimulated I_{sc} in gerbilline VDC and that 10^{-8} M AVP had no significant effect in murine VDC suggests that vasopressin-receptors are not involved in the regulation of K^+ secretion in these species. It is presently unclear, whether our observations in gerbils and mice point to a species difference between gerbils and mice on the one hand and frogs on the other hand. Indeed, it has not yet been shown in the frog whether direct or AVP-mediated stimulation of adenylate cyclase leads to stimulation of K^+ secretion. It is conceivable that vasopressin-receptors in the frog VDC have a different task than in gerbilline and murine VDC and that these tasks of cAMP are separated by compartmentalization. Such a compartmentalization of cAMP has recently been demonstrated in cardiac myocytes (Jurevicius & Fischmeister, 1996).

β_1-Adrenergic Receptors Mediate Regulation of K^+ Secretion

Two lines of evidence support the conclusion that β-adrenergic receptors are involved in the regulation of K^+ secretion. On the one hand, the agonist potency order was isoproterenol < norepinephrine < epinephrine < prenalterol (Table 2). This potency order has been found in a variety of tissues containing β_1-adrenergic receptors (Nahmias et al., 1991; Van Ermen et al., 1992; Elalouf et al., 1993; Zhong & Minneman, 1993). On the other hand, isoproterenol-induced stimulation of I_{sc} was sensitive to the β-adrenergic receptor antagonists propanolol, atenolol, ICI118551 and CGP20712A (Figs. 2 and 5).

Four different subtypes of β-adrenergic receptors have so far been described, β_1-, β_2-, β_3- and most recently β_4-adrenergic receptors. A pharmacologic differentiation between β_1-, β_2- and β_3-adrenergic receptors has been attempted in the present study. The antagonists atenolol, ICI118551 and CGP20712A differ in their affinity to β_1-, β_2- and β_3-receptor subtypes. Although ICI118551 is commonly referred to as a selective β_2-adrenergic receptor antagonist and atenolol and CGP20712A are commonly referred to as selective β_1-adrenergic receptor antagonists, their selectivities are not absolute. Further, affinity constants vary greatly between different preparations (Table 4). Less variation, however, occurs for the ratios of these affinity constants. These reduced variations may be due to the elimination of differences in experimental conditions. Indeed, experimental conditions such as the NaCl concentration present in a ligand-binding study have been shown to affect affinity constants (Mauriege et al., 1988). The ratio of affinity constants for CGP20712A and ICI118551 averaged 0.02 for β_1-adrenergic receptors, 5300 for β_2-adrenergic receptors and 3.3 for β_3-adrenergic receptors (Table 4). The ratio of affinity constants for atenolol and ICI118551 averaged 0.9 for β_1-adrenergic receptors and 2256 for β_2-adrenergic receptors and the ratio of affinity constants for atenolol and CGP20712A averaged 40 for β_1-adrenergic receptors. To determine which receptor subtype dominates regulation of K^+ secretion in VDC, the ratios of the K_{DB} values obtained in VDC were graphically compared to those obtained in tissues in which the receptor subtype is known (Fig. 7). The apparently best correlation was found using data from tissues, which contain β_1-adrenergic receptors. This observation suggests that K^+ secretion in VDC is chiefly, if not solely, under the control of β_1-adrenergic receptors.

Consistent with the functional demonstration of β_1-adrenergic receptors in VDC is the finding that vestibular labyrinth tissue, which encompasses VDC, contains transcripts for β_1- and β_2-adrenergic receptors (Fig. 6). However, there are questions, which remain unanswered from this study: (i) Where are the β_2-adrenergic receptors located? (ii) What is their function? (iii) Is the distribution of the β_1-adrenergic receptors restricted to VDC in the vestibular labyrinth? Future studies are indicated to determine the presence and function of β_2-adrenergic receptor in the vestibular labyrinth. Further,

Table 4. Affinity constants of Atenolol (A), ICI118551 (ICI) and CGP20712A (CGP) for β_1-, β_2- and β_3-adrenergic receptors

β_1 Receptors	Affinity constants			Ratio			Reference
Tissue	Atenolol	CGP20712A	ICI118551	A/ICI	A/CGP	CGP/ICI	
Heart (F)	4.1×10^{-6}	–	2.2×10^{-6}	1.9	–	–	Nathanson, 1985
Capillary endothelial cells (B)	8×10^{-13}	–	2.5×10^{-13}	3.2	–	–	Das et al., 1994
Cultured adrenal cells (F)	4.4×10^{-7}	–	2.7×10^{-7}	1.6	–	–	De Lean et al., 1984
Cultured cardiac cells (F)	5.54×10^{-8}	8.49×10^{-10}	3.69×10^{-7}	0.2	65	0.002	Pauwels et al., 1989
Cultured cardiac cells (F)	1.32×10^{-7}	1.08×10^{-7}	–	–	12	–	Pauwels et al., 1989
Human fat cells (B)	–	4×10^{-9} 6×10^{-10}	7.1×10^{-7} 7.1×10^{-7}	–	–	0.006 0.0009	Mauriege et al., 1988
C_6 glioma cells (B)	–	1.5×10^{-9}	5×10^{-8}	–	–	0.03	Zhong & Minneman, 1993
Thick ascend. limb of kidney (F)	–	1×10^{-8}	1×10^{-7}	–	–	0.1	Elalouf et al., 1993
Mouse trachea (B)	–	2.8×10^{-10}	1.5×10^{-7}	–	–	0.002	Henry et al., 1990
Mouse lung (B)		1.8×10^{-9}	3.47×10^{-7}			0.005	Henry et al., 1990
Rat liver (B)	–	2.3×10^{-8}	9.3×10^{-7}			0.025	Van Ermen et al., 1992
Gerbil vestibular dark cells (F)	5.0×10^{-8}	6.8×10^{-12}	4.4×10^{-8}	1.1	7349	0.00015	Present study
β_2 Receptors	Affinity constants			Ratio			Reference
Tissue	Atenolol	CGP20712A	ICI118551	A/ICI	A/CGP	CGP/ICI	
Gerbil cultured strial marginal cell (F)	–	1×10^{-5}	1×10^{-8}	–	–	1000	Martin et al., 1994
Human fat cells (B)	–	1.02×10^{-6} 1.5×10^{-6}	4.6×10^{-9} 4.6×10^{-9}	–	–	222 326	Mauriege et al., 1988
Rat liver (F)	–	$>2 \times 10^{-5}$	2×10^{-9}	–	–	>10,000	Van Ermen et al., 1992
Rat liver (B)	–	1.3×10^{-5}	7.1×10^{-9}			1830	Van Ermen et al., 1992
Mouse trachea (B)	–	2.6×10^{-6}	4.2×10^{-10}	–	–	6190	Henry et al., 1990
Mouse lung (B)		1.29 ± 10^{-5}	9.33×10^{-10}			13,826	Henry et al., 1990
Ciliary processes (F)	1.1×10^{-5}	–	4.3×10^{-9}	2558	–	–	Nathanson, 1985
Leukemia cells (B)	1.7×10^{-6}	–	1.5×10^{-9}	1133	–	–	Sager et al., 1988
Cultured cardiac cells (B)	$>6 \times 10^{-6}$	–	1.2×10^{-8}	>500	–	–	Pauwels et al., 1989
Eosinophils (B)	1.4×10^{-6}	–	2.8×10^{-10}	5000	–	–	Yukawa et al., 1990
β_3 Receptors	Affinity constants			Ratio			Reference
Tissue	Atenolol	CGP20712A	ICI118551	A/ICI	A/CGP	CGP/ICI	
CHO-Moβ_3 (F)	–	6.4×10^{-6}	5.0×10^{-6}	–	–	1.3	Nahmias et al., 1991
CHO-Huβ_3 (F)	–	6.7×10^{-6}	7.7×10^{-7}	–	–	8.7	Nahmias et al., 1991
Adipocyte (B)	–	4.6×10^{-6}	5.4×10^{-6}	–	–	0.8	Feve et al., 1991
CHO-β_3 (B)	–	5.8×10^{-6}	2.4×10^{-6}	–	–	2.4	Feve et al., 1991

Affinity constants given here were obtained either in functional studies (F) or binding studies (B). Values were calculated in most cases according to the Cheng and Prusoff equation ($K_i = IC_{50}/(1 + C/EC_{50})$) where IC_{50} is the half maximal concentration of antagonist inhibiting function elicited by one concentration of agonist, C is this concentration of agonist and EC_{50} is the half-maximal concentration of agonist causing function.

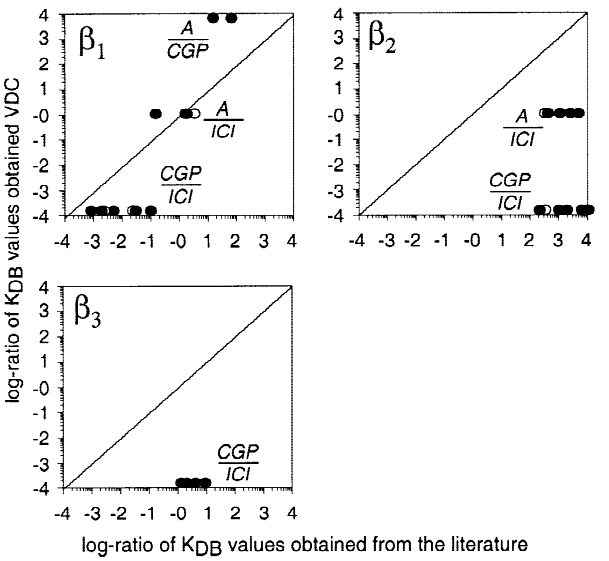

Fig. 7. Analysis of K_{DB} values for atenolol, ICI118551 and CGP20712A. Ratios of the K_{DB} values obtained in vestibular dark cells (VDC) were formed (K_{DB} for atenolol/K_{DB} for CGP20712A, A/CGP; K_{DB} for atenolol/K_{DB} for ICI118551, A/ICI and K_{DB} for CGP20712A/K_{DB} for ICI118551, CGP/ICI) and plotted on the *y*-axes of unity-plots against ratios for K_{DB} values obtained in cells known to contain β_1-, β_2- or β_3-adrenergic receptors. K_{DB} values and ratios are detailed and referenced in Table 4. Lines of identity are given for orientation. It is apparent that β_1-adrenergic receptors provide the closest fit. A predominant contribution of β_1- and β_2-adrenergic receptors can be excluded in both cases by the mismatch of the ratio CGP/ICI. From this analysis, we conclude that β_1-adrenergic receptor are responsible for stimulation of K^+ secretion in VDC.

future studies are indicated to determine whether the cAMP second messenger system is solely responsible for the observed β_1-adrenergic receptor mediated stimulation of K^+ secretion or whether the cAMP second messenger system works in synergism with other second messenger systems.

Two potential sources of agonist need to be considered for the β_1-adrenergic receptor in VDC, norepinephrine provided by sympathetic innervation and via the blood plasma. Sympathetic nerve fibers have been demonstrated near the sensory parts of the vestibular labyrinth nerve fibers (Hozawa & Kimura, 1989). VDC themselves, however, do not receive direct innervation and thus, it is unclear whether the innervation of the sensory parts provides a source of agonist for the β_1-adrenergic receptors in VDC. Nevertheless, this innervation may be the source for the norepinephrine, which can be demonstrated with high-pressure liquid chromatography in biochemical preparations of the vestibular labyrinth (Gil-Loyzaga et al., 1997).

The other potential source of agonist for the β_1-adrenergic receptors in VDC is blood plasma. VDCs are supplied by a dense capillary network, which comes in close contact with their basolateral membrane. Plasma may be a source of agonist since the plasma norepinephrine concentrations in humans vary between 0.7 and 6 nM (Lake et al., 1997) and capillary walls in the inner ear have been shown to be permeable to organic compounds of similar molecular weight (Jung, Gattaz & Schön, 1989). Interestingly, the norepinephrine concentration in plasma varies in humans with vestibular stimulation. Indeed, the plasma norepinephrine concentration has been found to be 0.7 nM during rest in supine position, to double to 1.4 nM in standing position, to increase further to 2.4 nM during light exercise and to reach a measurable maximum of 6.0 nM during strenuous exercise (Lake et al., 1997).

The finding that the natural agonist norepinephrine stimulated K^+ secretion in VDC at physiologically relevant concentrations supports our hypothesis that the rate of K^+ secretion in VDC is under systemic control. Clearly, a high frequency of head movements involving an elevated level of vestibular stimulation is more likely to occur when the organism is alert and standing than when the organism is resting in the supine position. Stimulation of the vestibular labyrinth results in an efflux of K^+ across the sensory hair cells (Valli et al., 1990). A major shift of K^+ ions from the lumen to the abluminal side, however, must be prevented to avoid accompanying water fluxes and concurrent deformations of the membranous labyrinth, which would alter the mechanical properties of this mechanoreceptive organ. According to our hypothesis, an elevated plasma norepinephrine concentration, which coincides with an elevated activity level of the animal, would reach the β_1-adrenergic receptor in the basolateral side of VDC and stimulate K^+ secretion into the lumen of the vestibular labyrinth. This mechanism would prevent potentially deleterious effects associated with unimpeded shifts in K^+ from the lumen to the abluminal side.

The authors wish to thank Novartis Pharmaceutical, Summit, NJ for the much-appreciated gift of CGP20712A and Astra Hässle, Mölndal, Sweden for the much-appreciated gift of prenalterol. Further, the authors wish to acknowledge Mr. Michael Bruchas who helped to develop primers for the gerbil β_1-adrenergic receptor during his undergraduate research experience. The support by Research Grant R01-DC-00212 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health is gratefully acknowledged.

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