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ORIGINAL ARTICLE

Alpha-adrenoceptor agonistic activity of oxymetazoline and xylometazoline

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ABSTRACT

Oxymetazoline and xylometazoline are both used as nasal mucosa decongesting α-adrenoceptor agonists during a common cold. However, it is largely unknown which of the six α-adrenoceptor subtypes are actually present in human nasal mucosa, which are activated by the two alpha-adrenoceptor agonists and to what extent. Therefore, mRNA expression in human nasal mucosa of the six α-adrenoceptor subtypes was studied. Furthermore, the affinity and potency of the imidazolines oxymetazoline and xylometazoline at these α-adrenoceptor subtypes were examined in transfected HEK293 cells. The rank order of mRNA levels of α-adrenoceptor subtypes in human nasal mucosa was: $\alpha_{2A} > \alpha_{1A} \geq \alpha_{2B} > \alpha_{1D} \geq \alpha_{2C} >> \alpha_{1B}$. Oxymetazoline and xylometazoline exhibited in radioligand competition studies higher affinities than the catecholamines adrenaline and noradrenaline at most α-adrenoceptor subtypes. Compared to xylometazoline, oxymetazoline exhibited a significantly higher affinity at α_{1A}- but a lower affinity at α_{2B}-adrenoceptors. In functional studies in which adrenoceptor-mediated Ca^{2+} signals were measured, both, oxymetazoline and xylometazoline behaved at α_{2B}-adrenoceptors as full agonists but oxymetazoline was significantly more potent than xylometazoline. Furthermore, oxymetazoline was also a partial agonist at α_{1A}-adrenoceptors; however, its potency was relatively low and it was much lower than its affinity. The higher potency at α_{2B}-adrenoceptors, i.e. at receptors highly expressed at the mRNA level in human nasal mucosa, could eventually explain why in nasal decongestants oxymetazoline can be used in lower concentrations than xylometazoline.

INTRODUCTION

Alpha-adrenoceptors are cell membrane receptors which belong to the seven transmembrane spanning G-protein-linked family of receptors. Six genes for α-adrenoceptors (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, and α_{2C}) have been identified and sequenced [1]. The α_1-adrenoceptors couple via $G_{q/11}$ to phospholipase C, and activation of the receptors results in the production of inositol triphosphate (IP3) and diacyl glycerol and thereby in an increase of intracellular Ca^{2+} and activation of protein kinases such as PKC [2]. The α_2-adrenoceptors negatively couple via $G_{i/o}$ to adenylate cyclase to decrease cAMP. Activation of α_{2A}-adrenoceptors causes inhibition of neurotransmitter release. Nasal obstruction associated with acute or allergic rhinitis is probably due to an increase in nasal microvascular permeability as a consequence of the dilatation of plexus cavernosum and mucosal congestion. The mucosal tissue located on nasal septum and lateral as well as medial turbinate is highly vascularized. In the superficial nasal mucosa, nasal blood flows typically from arteries to capillaries and into veins at a rate of about 40 mL/100 g of tissue per minute [3]. Mucosal tissue contracts when exposed to sympathomimetic vasoconstrictors such as the standard topical decongestant oxymetazoline, and the smooth muscles of

vasculature in nasal mucosa are believed to be the only tissue that possesses contractility [4].

Previous studies have shown that α_2-adrenoceptors are distributed in dog [5], pig [6] and human [7,8] nasal mucosa and that α_2-adrenoceptors mediate vasoconstriction in pig [6] and human [8] nasal mucosa. Radioligand binding studies using the α_1-adrenoceptor antagonist [^{3}H]prazosin and the α_2-adrenoceptor antagonist [^{3}H]rauwolscine have demonstrated the expression in the human nasal mucosa of both α_2- and α_1-adrenoceptor protein [9]. However, these radioligands cannot discriminate between adrenoceptor subtypes. In addition, the involvement of the receptor subtypes in vasoconstriction by oxymetazoline and xylometazoline is largely unknown. Like the catecholamines noradrenaline and adrenaline both imidazolines are regarded as nonselective α-adrenoceptor agonists [8]. Only oxymetazoline has been shown to induce Ca^{2+} responses in cells heterologously expressing human α_{1A}-adrenoceptors [10]. Therefore, in the present study we examined the mRNA expression of α-adrenoceptor subtypes in human nasal mucosa and we investigated the receptor affinity, potency and efficacy of the α-adrenoceptor agonists oxymetazoline and xylometazoline at the six human α-adrenoceptor subtypes.

METHODS

mRNA expression of human alpha-adrenoceptors in nasal mucosa

Tissue collection

Nasal mucosa was obtained from ten healthy persons, i.e. patients that underwent turbinoplasty to improve their nasal ventilation (six women; mean age of 39.83 ± 10.09 and four men; mean age of 34.00 ± 10.80). All tissue samples were collected in the Department of Otolaryngology at the University Clinics of Bonn. Samples were stored at −80 °C in 5 mL RNAlater (Qiagen, Hilden, Germany) until use.

RNA isolation and reverse transcription

RNA from nasal mucosa was isolated using the RNeasy Tissue Mini Kit (Qiagen) with DNAse treatment according to the manufacturer's instructions. RNA quantity was determined by spectrophotometry (UV-mini, Shimadzu, Kyoto, Japan). Total RNA (2 μg per sample) was reverse transcribed according to the manufacturer's instructions (RevertAid cDNA Synthesis Kit; Fermentas, St Leon-Rot, Germany) using random hexamer primers. The same procedure was used to determine the mRNAs of the six α-adrenoceptors expressed in native HEK293 cells as well as in cells transfected with the corresponding cDNA.

qPCR

For quantitative real-time polymerase chain reaction (qPCR) 35 μL of amplification mixture (QuantitectSYBR-Green Kit; Qiagen) was used containing 20 ng reverse transcribed RNA and 300 nM of the following primers (sense/antisense):

ADRA1A (α_{1A}) TGCATCATCTCCATCGACCG/CGTTG ATCTGGCAGATGGTC;

ADRA1B (α_{1B}) TCATCGCTCTACCGCTTGGC/GAACTC CTTGCTGGAGCATGG;

ADRA1D (α_{1D}) AGCCTGTCGCACAAGATCC/CTCGTG TGGGACGCCTAG;

ADRA2A (α_{2A}) TGCTGCTCACCGTGTTCG/GAGAAAG GGATGACGAGCG

ADRA2B (α_{2B}) TGCTCTTCTGCACCTCGTCC/GGTCGC CCTTGTAGATGAGG

ADRA2C (α_{2C}) GCCTCAACGACGAGACCTG/GTTTTC GGTAGTCGGGGACG

Reactions were run on an Mx 3000P real-time cycler (Stratagene, Amsterdam, the Netherlands) using the following cycling conditions: 15 min polymerase activation at 95 °C and 45 cycles at 95 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s. Each assay included negative controls and a standard curve for each gene. The results were analysed using the Stratagene software (Version Mx 3000 Pro) and the data were exported to Excel 2003 (Microsoft) and Prism 5.0 (GraphPad, San Diego, CA, USA) for further analysis.

The identity of the PCR products was confirmed by melt point analysis and by dideoxy chain termination sequencing.

The relative mRNA expression of each α-adrenoceptor was calculated from the ratio α-receptor/ADRA2A (α_{2A}) within one sample of nasal mucosa. Results are displayed as $\log_2$- and fold-values.

cDNA of human alpha-adrenoceptor subtypes

The cDNAs of the human α-adrenoceptor genes ADRA1A (α_{1A}), ADRA1B (α_{1B}), ADRA2A (α_{2A}), ADRA2B (α_{2B}) and ADRA2C (α_{2C}) were obtained from UMR cDNA Resource Center (University of Missouri-Rolla, MO, USA). All above mentioned cDNAs were cloned into the vector pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany). The enzymes used for cloning into the multiple cloning site of the vector were: *Eco*RI (5′) and *Xho*I (3′) for ADRA1A, *Eco*RI (5′) and *Xba*I (3′) for ADRA1B, *Eco*RI (5′) and *Xho*I

(3′) for ADRA2A, *Kpn*I (5′) and *Xho*I (3′) for ADRA2B and *Bam*HI (5′) and *Xho*I (3′) for ADRA2C. The cDNA of ADRA1D (α_{1D}) was obtained from OriGene (Rockville, MD, USA). ADRA1D was cloned into the vector pCMV6 at *Eco*RI (5′)- and *Sal*I (3′) sites.

All human α-adrenoceptor cDNAs were analyzed for correct nucleotide sequence (according to the reference sequence given in the GenBank at NCBI) by dideoxy chain termination sequencing. In case of false nucleotides or deletions, the Quik Change Mutagenesis Kit (Stratagene) or insertion PCR were used to obtain the correct sequence. Site-directed mutagenesis was carried out for ADRA1B, ADRA1D, ADRA2A and ADRA2C; insertion PCR reactions were used for ADRA2C. The final nucleotide sequences matched with the reference gene sequences of the GenBank at NCBI. The GenBank accession numbers are: AY389505 for ADRA1A, NM_000679 for ADRA1B, NM_000678 for ADRA1D, NM_000681 for ADRA2A, NM_000682 for ADRA2B and NM_000683 for ADRA2C.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) were seeded in either 75 cm^2 (Ca^{2+} measurements) or 175 cm^2 (radioligand binding) culture flasks in DMEM/Ham's F12 (1/1) + 10% dialysed fetal calf serum (FCS) in order to obtain a cell density of 40–70% for transient transfection the following day. Transfection was performed by lipofection with TransIT-293 Transfection Reagent (Mirus Bio, Madison, WI, USA). For radioligand binding assays cells were exclusively transfected with cDNAs coding for the α-adrenoceptors. For Ca^{2+} measurements using chemiluminescence resonance energy transfer (CRET) the pG5A vector containing the cDNAs for apoaequorin and enhanced green fluorescent protein (EGFP) [11] was used. The cDNA coding for the promiscuous human $G_{\alpha 16}$, which resembles the G_q class of alpha subunits [12–14], was cloned into the expression vector pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany). Cells were transfected with the pG5A vector, the α-adrenoceptor cDNA and the $G_{\alpha 16}$ cDNA at a ratio of 2 : 1 : 1. Cells were used 48 h post transfection.

Plasma membrane preparation and radioligand binding assays

For radioligand binding experiments, plasma membranes were prepared from transiently transfected HEK293 cells. Cells were harvested 48 h after transfection and washed once with binding assay buffer (150 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5.4 mM KOH, pH 7.4). All steps were carried out on ice. After resuspension of the cells in 2 mL binding assay buffer, they were homogenized using an ultraturrax homogenizer, and cell debris were pelleted. The supernatant was centrifuged in an ultracentrifuge. The resulting pellet was washed once with binding assay buffer and centrifuged. The final pellet was resuspended in binding assay buffer and homogenized by pipetting through a gauche needle. Protein measurement was carried out by the method of Lowry et al. [15] using bovine serum albumin as standard. For determination of K_d and B_{max} values in radioligand saturation binding studies, plasma membranes from HEK293 cells transiently transfected with α-adrenoceptor cDNAs were incubated with increasing concentrations of [^{3}H]prazosin (in case of α_1-adrenoceptors) or [^{3}H]RX821002 (in case of α_2-adrenoceptors). Experiments were carried-out in triplicates. Non-specific binding was determined in parallel experiments at cell membranes of native (not transfected) HEK293 cells.

For competition binding experiments, plasma membranes were incubated with [^{3}H]prazosin or [^{3}H]RX821002 with or without the drug under study (unlabeled adrenaline, noradrenaline, oxymetazoline or xylometazoline) for 60 min at room temperature. In case of adrenaline and noradrenaline 1 mM ascorbic acid was added to the incubation solution to prevent oxidation. All reactions were carried out in triplicates. Specific binding was determined as the fraction of [^{3}H]prazosin or [^{3}H]RX821002 which could be displaced by 10 μM phentolamine or 10 μM rauwolscine, respectively.

Incubation mixes were filtered through polyethylene imine (0.3%)-coated GF/B filters (Whatman, Kent, UK) using a Brandel cell harvester. Radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Measurement of intracellular Ca^{2+} responses

The measurement of intracellular Ca^{2+} responses was performed by using an aequorin-EGFP fusion protein according to the method of CRET. The assay was done as previously described [16]. In brief, harvested cells were loaded with 5 μM coelenterazine *h* for 2.5 h at room temperature. Suspensions of cells in assay buffer containing (in mM) NaCl 150, $CaCl_2$ 1.8, KCl 5.4, Hepes 10, D-glucose 20 (pH 7.4) were used for luminometric determination of intracellular Ca^{2+} transients in 96-well plates in a Berthold Centro LB 960 luminometer. In the case of recording Ca^{2+} responses induced by adrenaline and noradrenaline, 1 mM ascorbic acid was added. Light

emission was recorded 5 s prior and 25–60 s upon autoinjection of the agonist dissolved in assay buffer at a sampling rate of 2 Hz.

Data analysis

Peak values for the concentration-response curves in the Ca^{2+} assay were obtained by subtraction of baseline light emission from the agonist-induced peak signal. Responses were normalized to the noradrenaline (300 μM)-induced light emission. Concentration-response curves, inhibition curves and saturation binding curves as well as the corresponding constants pEC_{50}, pIC_{50}, B_{max} and K_d were calculated by means of GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed with one-way ANOVA followed by Dunnett's post test. Differences were considered significant at $P < 0.05$.

Substances

Phentolamine hydrochloride, rauwolscine hydrochloride and the (−) enantiomers of noradrenaline ((−)-norepinephrine (+)-bitartrate) and adrenaline ((−)-epinephrine (+)-bitartrate) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Oxymetazoline hydrochloride and xylometazoline hydrochloride were gifts from Merck (Darmstadt, Germany). [^{3}H]Prazosin (specific activity: 85 Ci/mmol) and [^{3}H]RX821002 (specific activity: 70 Ci/mmol) were from GE Healthcare Life Sciences (Freiburg, Germany). All other substances and chemicals were from Sigma-Aldrich (Taufkirchen, Germany), if not stated otherwise.

RESULTS

Alpha-adrenoceptor expression in human nasal mucosa

By means of qPCR of reverse transcribed mRNAs we examined for the first time the mRNA expression of the diverse α-adrenoceptor subtypes in human nasal mucosa from healthy subjects of central European origin. From the six receptor subtypes, the highest expression was observed for the mRNA of the ADRA2A gene (α_{2A}-adrenoceptor) whereas the mRNA of the ADRA1B gene (α_{1B}-adrenoceptor) showed the lowest expression (*Figure 1*). The rank order of mRNA expression of the human α-adrenoceptors was: $\alpha_{2A} > \alpha_{1A} \geq \alpha_{2B} > \alpha_{1D} \geq \alpha_{2C} >> \alpha_{1B}$. This result may indicate that the highly expressed α-adrenoceptors (α_{1A}, α_{2A}, and α_{2B}) could be the primary targets for the vasoconstricting nasal decongestants oxymetazoline and xylometazoline.

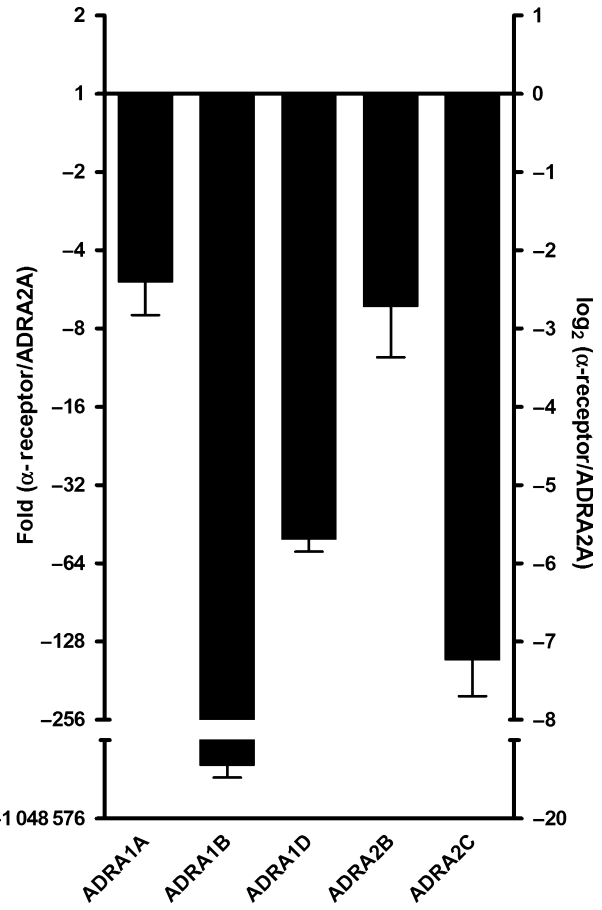

Figure 1 Relative mRNA expression of alpha-adrenoceptor subtypes in human nasal mucosa related to ADRA2A (α_{2A}), the alpha-adrenoceptor with the highest expression in human nasal mucosa. Shown are means (±SEM) of nine to 10 samples. Results are presented as fold- and log_2-values.

Heterologous expression of human alpha-adrenoceptors in HEK293 cells

Since translation of mRNA into protein and especially processing and trafficking of a receptor protein to the cell surface might differ from gene to gene, the mRNA expression must not necessarily mirror the protein expression of the corresponding receptor gene. Therefore, we determined mRNA expression (by means of qPCR) as well as receptor protein expression (by means of radioligand saturation binding) of the diverse adrenoceptors after transfection of HEK293 cells with the corresponding receptor cDNAs. Native HEK293 cells expressed extremely low levels of the six adrenoceptors which were on the limit of being measurable. After transfection with the corresponding adrenoceptor cDNA the mRNA content was about 40.000-fold above the endogenous levels.

Four of the six adrenoceptor mRNAs showed similar high expression levels whereas the mRNA of the α_{1B}- and α_{2C}-adrenoceptor was expressed at a clearly (about 50-fold) lower level (data not shown).The B_{max} values of radioligand binding to isolated membranes of transiently transfected cells are a measure of the receptor density at the cell surface. As shown in *Figure 2*, the receptor densities (B_{max} in fmol/mg protein) ranged from about 121 000 (α_{2A}) to about 2400 (α_{1D}) with the following ranking order: $\alpha_{2A} > \alpha_{2B} > \alpha_{1A} \geq \alpha_{1B} \geq \alpha_{2C} > \alpha_{1D}$ (*Table I*). These results indicate that receptor protein processing and trafficking to the cell membrane must be different between the six α-adrenoceptors. However, it should be noted that B_{max} values in membrane preparations do not necessarily reflect receptor expression at the cell surface and that the radioligand binding data measured in HEK293 cell membranes may not mirror the situation in the nasal mucosa.

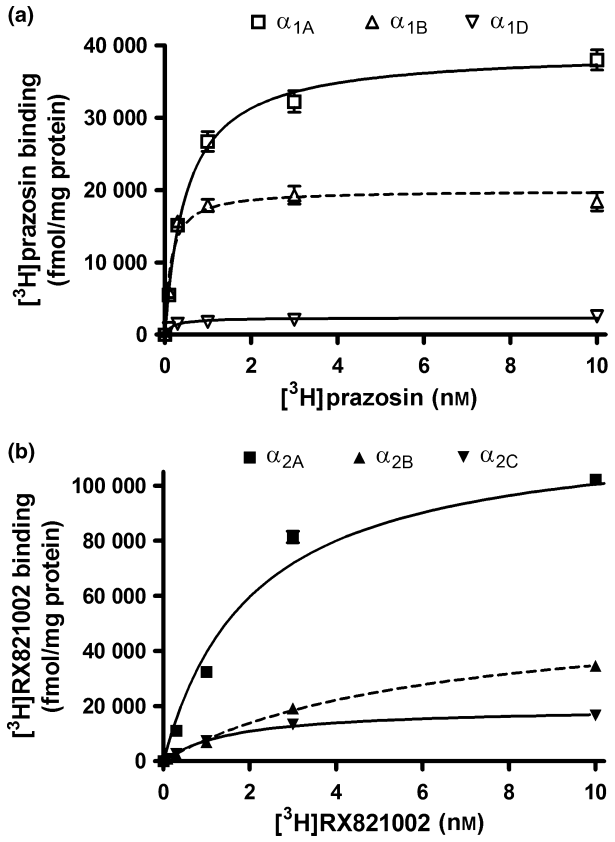

Figure 2 Radioligand saturation binding to human (a) α_1- and (b) α_2-adrenoceptor subtypes expressed in transiently transfected HEK293 cells. Specific saturation binding to α_1- and α_2-adrenoceptors was measured at isolated cell membranes using the radioligands [^{3}H]prazosin and [^{3}H]RX821002 for α_1- and α_2-adrenoceptors, respectively. Non-specific binding was determined in parallel experiments at cell membranes of native HEK293 cells. Shown is one typical experiment (with means of triplicates ± SEM) at each of the adrenoceptor subtypes.

The affinity constants (K_d values) of prazosin for the three α_1-adrenoceptors were relatively similar ranging from 0.15 nM (at α_{1D}-adrenoceptors) to 0.64 nM (at α_{1A}-adrenoceptors) (*Table I*). The affinity of the α-adrenoceptor antagonist RX821002 to the three human α_2-adrenoceptors was between 2 and 5 nM (*Table I*), indicating that this ligand does not differentiate between the α_2-adrenoceptor subtypes.

Affinities of alpha-adrenoceptor agonists for human alpha-adrenoceptors

The imidazolines oxymetazoline and xylometazoline and the catecholamines adrenaline and noradrenaline were examined for their affinities at human α-adrenoceptor subtypes expressed in transiently transfected HEK293 cells. Affinities were determined by drug-induced inhibition of specific binding of [^{3}H]prazosin to α_1-adrenoceptors and [^{3}H]RX821002 to α_2-adrenoceptors. As shown in *Figure 3*, all four examined α-adrenoceptor agonists caused concentration-dependent inhibition of radioligand binding to all six α-adrenoceptor subtypes. The affinities (K_i values) of the four substances at the α-adrenoceptor subtypes, however, were markedly different (*Figure 3*, *Table II*); as a rule, the two catecholamines exhibited lower affinities compared to the two imidazolines (*Table II*). At α_{1A}-adrenoceptors oxymetazoline exhibited a significantly higher affinity than xylometazoline (*Figure 3*, *Table II*), at α_{1B}-, α_{1D}-, α_{2A}- and α_{2C}-adrenoceptors both imidazolines showed comparable affinities (*Figure 3*, *Table II*), whereas at α_{2B}-adrenoceptors xylometazoline exhibited a significantly higher affinity than oxymetazoline (*Table II*).

Potencies and efficacies of alpha-adrenoceptor agonists at human alpha-adrenoceptors

HEK293 cells loaded with coelenterazine *h* and transiently transfected with the cDNAs encoding apo-aequorin together with EGFP as well as a human α-adrenoceptor and the promiscuous G-protein $G_{\alpha 16}$ were used to measure α-adrenoceptor agonist-mediated increase in cytosolic Ca^{2+} ions and thereby a light signal through aequorin-EGFP-CRET. In these cells, adrenaline and noradrenaline induced concentration-dependent Ca^{2+} signals with about identical maximum values (*Figure 4*, *Table III*). Only at α_{1B}-adrenoceptors the maximum response was about 30% higher for adrenaline

Table I K_d and B_{max} values for binding of [^{3}H]prazosin and [^{3}H]RX821002 to human α_1- and α_2- adrenoceptor subtypes, respectively. Binding was measured in isolated cell membranes of HEK293 cells transiently transfected with the respective adrenoceptor cDNA

Radioligand [^{3}H]	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
Prazosin	α_{1A}		α_{1B}		α_{1D}	
Mean ± SEM (*n*)	0.64 ± 0.16 (5)	36.9 ± 4.9 (5)	0.35 ± 0.12 (5)	27.3 ± 8.1 (5)	0.15 ± 0.05 (4)	2.4 ± 0.5 (4)
RX821002	α_{2A}		α_{2B}		α_{2C}	
Mean ± SEM (*n*)	2.61 ± 0.84 (3)	121 ± 34 (3)	5.34 ± 0.52 (5)	60.7 ± 15.3 (5)	1.77 ± 0.19 (5)	16.5 ± 2.3 (5)

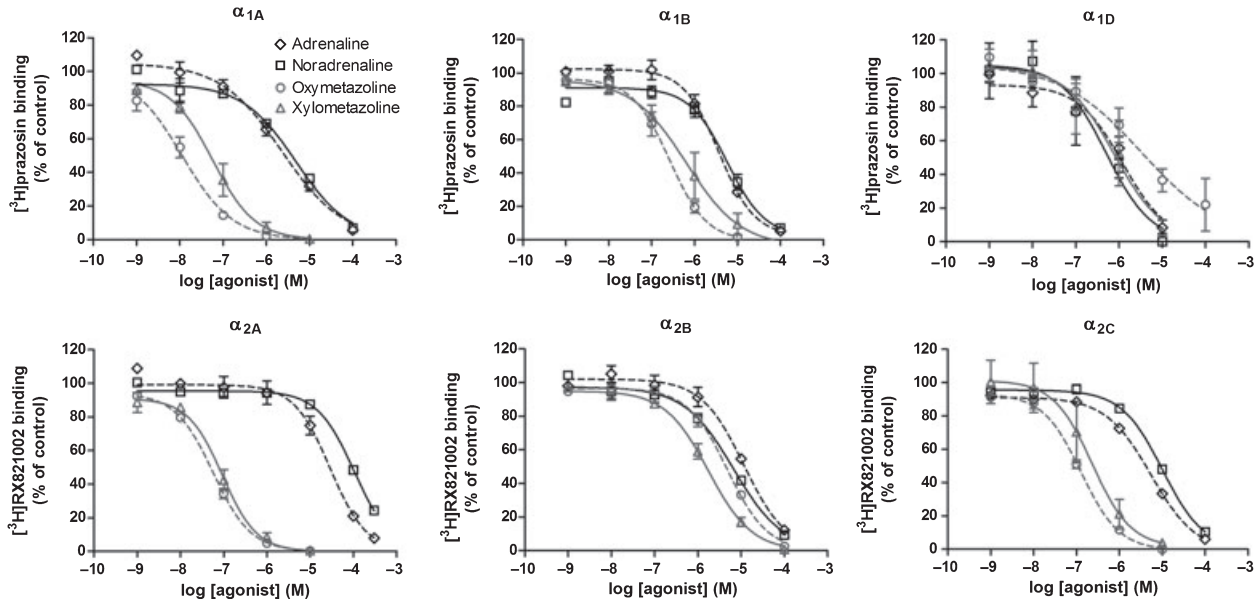

Figure 3 Displacement by adrenaline, noradrenaline, oxymetazoline and xylometazoline of specific binding of [^{3}H]prazosin and [^{3}H]RX821002 to α_1- and α_2-adrenoceptor subtypes, respectively, expressed in isolated cell membranes of transiently transfected HEK293 cells. Shown are means (±SEM) of three to five experiments carried out in triplicates.

than for noradrenaline. At α_1-adrenoceptors, the EC_{50} values for adrenaline were lower than those for noradrenaline whereas the contrary was observed at α_2-adrenoceptors (*Table III*), indicating higher potency of adrenaline and noradrenaline at α_1- and α_2-adrenoceptors, respectively. All EC_{50} values were in the low micromolar range (*Table III*).

The imidazolines behaved different from the catecholamines since at most α-adrenoceptors (except α_{1A}- and α_{2B}-adrenoceptors) increasing agonist concentration caused no increased Ca^{2+} signal (*Figure 4*). At α_{2B}-adrenoceptors, xylometazoline and oxymetazoline produced pronounced and concentration-dependent Ca^{2+} signals, and the maximum response was even 30% above that of the catecholamines (*Table III*). At α_{1A}-adrenoceptors xylometazoline showed no measurable activity whereas oxymetazoline behaved as partial agonist with about 50% efficacy compared to noradrenaline (*Table III*). The EC_{50} values of the imidazolines were between 10 and 100 μM (*Table III*).

DISCUSSION

One aim of the present study was to identify the α-adrenoceptor subtypes expressed in human nasal mucosa. We could show for the first time that among the α_1-adrenoceptor mRNAs the α_{1A}-subtype and among the α_2-adrenoceptor mRNAs the α_{2A}- and the α_{2B}-adrenoceptor mRNA are most abundantly expressed. These three adrenoceptors are also expressed as mRNA and as plasma membrane protein of HEK293 cells at a much higher density compared to e.g. α_{2C}-adrenoceptors

Table II IC_{50}- (and K_i-) values of noradrenaline, adrenaline, oxymetazoline and xylometazoline for the displacement of binding of the α-adrenoceptor antagonists [^{3}H]prazosin (0.3 nM) and [^{3}H]RX821002 (0.3 nM) from α_1- and α_2-adrenoceptor subtypes, respectively. The receptors were expressed in plasma membranes of transiently transfected HEK293 cells. Shown are means ± SEM of three to four experiments

	Noradrenaline pIC_{50} ± SEM IC_{50} [K_i][a] μM	Adrenaline pIC_{50} ± SEM IC_{50} [K_i][a] μM	Oxymetazoline pIC_{50} ± SEM IC_{50} [K_i][a] μM	Xylometazoline pIC_{50} ± SEM IC_{50} [K_i][a] μM
α_{1A}	5.28 ± 0.06 5.2 [3.6]	5.61 ± 0.24 2.4 [1.7]	7.79 ± 0.07* 0.02 [0.01]	7.12 ± 0.17 0.08 [0.05]
α_{1B}	5.25 ± 0.04 5.6 [3.0]	5.45 ± 0.04 3.6 [1.9]	6.59 ± 0.07 0.25 [0.14]	6.25 ± 0.27 0.56 [0.30]
α_{1D}	6.27 ± 0.15 0.62 [0.21]	6.07 ± 0.19 0.86 [0.29]	5.39 ± 0.26 4.1 [1.4]	6.34 ± 0.51 0.45 [0.15]
α_{2A}	3.98 ± 0.04 104 [93]	4.35 ± 0.17 44 [40]	7.24 ± 0.05 0.58 [0.52]	7.01 ± 0.13 0.98 [0.88]
α_{2B}	5.16 ± 0.07 6.9 [6.6]	4.97 ± 0.03 11 [10]	5.24 ± 0.05* 5.8 [5.5]	5.76 ± 0.11 1.8 [1.7]
α_{2C}	5.01 ± 0.03 9.7 [8.3]	5.28 ± 0.03 5.2 [4.5]	6.90 ± 0.03 0.13 [0.11]	6.65 ± 0.21 0.22 [0.19]

[a]K_i values were calculated from the mean K_d value for [^{3}H]prazosin and [^{3}H]RX821002 given in *Table I*.
*$P < 0.05$ (compared with xylometazoline; Student's *t*-test).

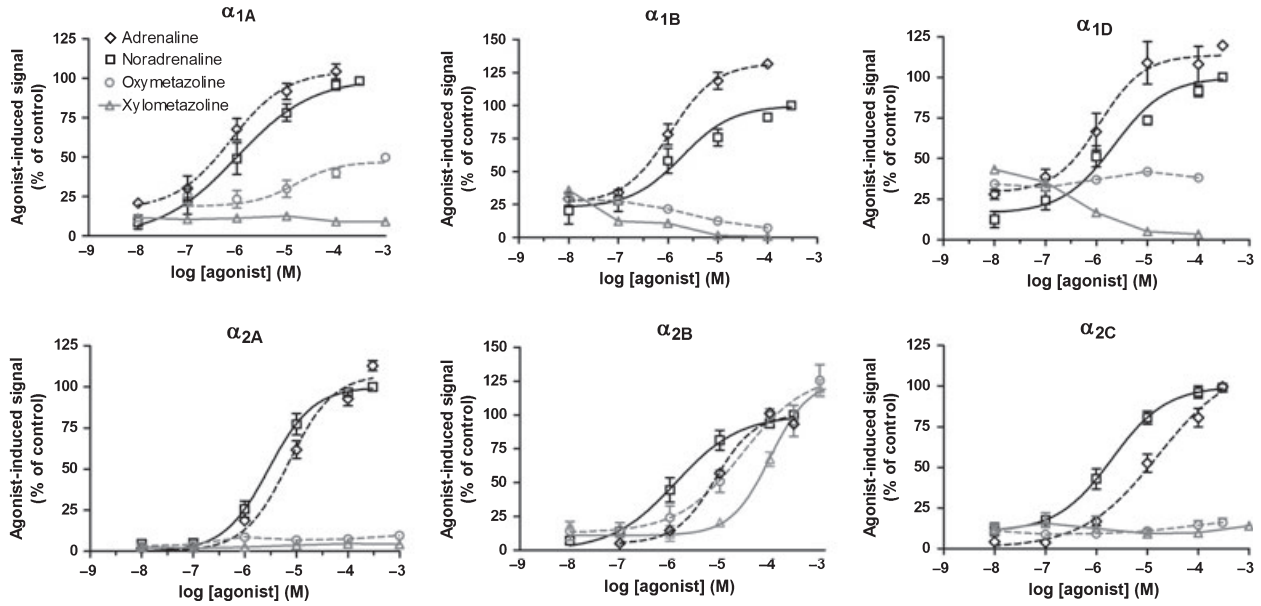

Figure 4 Concentration-dependent increases in intracellular Ca^{2+} concentration induced by the α-adrenoceptor agonists adrenaline, noradrenaline, oxymetazoline and xylometazoline at HEK293 cells transiently expressing one of the six human α-adrenoceptor subtypes together with the G-protein $G_{\alpha 16}$. Ca^{2+} transients were measured using an aequorin-EGFP fusion protein as described in Methods. Shown are means (±SEM) of three to five experiments carried out in triplicates.

(see B_{max} values in *Table I*) although the cells were transiently transfected with equal amounts of the corresponding receptor cDNAs. These results may indicate that the α_{1A}-, α_{2A}- and/or α_{2B}-adrenoceptors may play a major role in vasoconstriction of the human nasal mucosa, and that these receptors could be the primary targets for the vasoconstricting nasal decongestants oxymetazoline and xylometazoline. However, α_{2A}-adrenoceptors are typically expressed as presynaptic autoreceptors on sympathetic nerve endings where their activation induces inhibition of noradrenaline release through e.g. inhibition of voltage-gated Ca^{2+} channels and/or activation of Ca^{2+} -dependent K^+ channels [17–19]. The observed targeting of α_{2A}-adrenoceptors to nerve terminals in differentiated neuronal cells (PC12) is in line with the putative physiological role of this

	Noradrenaline pEC_{50} ± SEM (EC_{50} μM) [E_{max}][a]	Adrenaline pEC_{50} ± SEM (EC_{50} μM) [E_{max}][a]	Oxymetazoline pEC_{50} ± SEM (EC_{50} μM) [E_{max}][a]	Xylometazoline pEC_{50} ± SEM (EC_{50} μM) [E_{max}][a]
α_{1A}	5.77 ± 0.198 (1.7) [100]	6.25 ± 0.30 (0.6) [103 ± 6]	4.96 ± 0.23 (11) [48 ± 1]	nm
α_{1B}	5.78 ± 0.26 (1.7) [100]	5.89 ± 0.18 (1.3) [134 ± 2]	nm	nm
α_{1D}	5.68 ± 0.12 (2.1) [100]	5.97 ± 0.15 (1.1) [111 ± 9]	nm	nm
α_{2A}	5.53 ± 0.10 (2.9) [100]	5.14 ± 0.07 (7.2) [108 ± 3]	nm	nm
α_{2B}	5.87 ± 0.20 (1.4) [100]	5.09 ± 0.04 (8.2) [101 ± 5]	4.83 ± 0.23 (15) [121 ± 12]*	4.01 ± 0.12 (99) [130 ± 6]
α_{2C}	5.73 ± 0.12 (1.9) [100]	5.07 ± 0.08 (8.6) [95 ± 11]	nm	nm

Table III Potencies (pEC_{50} values) and efficacies (E_{max} values) of noradrenaline, adrenaline, oxymetazoline and xylometazoline at human α-adrenoceptors expressed in transiently transfected HEK293 cells. Agonist effects were measured using a calcium assay as described in Methods. Shown are means ± SEM of three to five experiments

[a]E_{max} is given as mean % (±SEM) of the E_{max} of noradrenaline which was set as 100%; nm, not measurable
*$P < 0.05$ (compared with xylometazoline; Student's *t*-test).

receptor subtype as a presynaptic autoreceptor [20]. In addition, a predominant presynaptic localization of α_{2A}-adrenoceptors has also been demonstrated in α-adrenoceptor knock-out mice (for review see [21]). This seems to indicate that among the more strongly expressed receptors (respectively their mRNAs) presumably α_{1A}- and α_{2B}-adrenoceptors may play the most important role in vasoconstriction of human nasal mucosa. Although α_{2A}-adrenoceptor mRNA shows the highest expression in human nasal mucosa, nothing is known about the physiological role of this receptor in this tissue; according to our results this receptor is not involved in imidazoline-induced Ca^{2+} responses. Interestingly, in an overview on adrenoceptor pharmacology, the α_{2B}-adrenoceptor was reported to represent the only α_2-adrenoceptor which physiologically is involved in vasoconstriction [22]. However, we cannot exclude that an involvement of α_{2A}-adrenoceptors in imidazoline-mediated vasoconstriction in nasal mucosa could be indirect. Both imidazolines could be high potency inhibitors for these receptors and so prevent autoinhibition of presynaptic noradrenaline release.

The level of mRNA expression must not necessarily mirror the level of protein expression of a membrane receptor since processing and trafficking of the translation product may vary between different receptors. Although the amount of adrenoceptor cDNA used for transient transfection of HEK293 cells was identical for all adrenoceptors, the density (B_{max}) of the α_{2C}- and α_{1D}-receptor protein expressed in the plasma membrane was by a factor of 7.3 and 51, respectively, lower than that of the α_{2A}-adrenoceptor which was expressed with the highest density (see *Table I*). However, measurement of α-adrenoceptor mRNA expression of transfected cells showed that the mRNA levels were of similar height for the α_{1A}-, α_{2A}-, α_{2B} and α_{1D}-adrenoceptors but they were much lower for the mRNA of α_{1B}- and α_{2C}-adrenoceptors. These results indicate that there were discrepancies between mRNA and receptor protein expression and the highest discrepancy was observed for the α_{1D}-adrenoceptor. Thus, there are obviously clear differences in translation and/or trafficking and processing of adrenoceptor subtypes, indicating that regulation of these receptors may not only occur at the transcriptional level but also post-transcriptionally and/or post-translationally. A very low membrane expression and predominant intracellular localization of α_{1D}-receptors in transfected HEK293 cells has already been observed in a previous study [23]; the non properly processing of this receptor is obviously a consequence of the lack of receptor glycosylation in this cell line. Furthermore, in transfected neuronal cells (PC12) α_{2C}-adrenoceptor protein has also been observed to be mostly located in an intracellular compartment [20]. However, since this receptor also showed low mRNA expression in transfected cells, less efficient transcription may also contribute to its low membrane expression. Functionally, there are further components which contribute to diversities between adrenoceptors, since even if α-adrenoceptor subtypes are expressed at comparable densities in the cell membrane of transfected cells, they can cause different efficacies of the natural α-adrenoceptor agonist noradrenaline due to

differences in receptor coupling [24]. Taken together, these potential diversities as well as the above mentioned differences between mRNA and receptor protein expression in HEK293 cells which may be different in the human nasal mucosa indicate that the α-adrenoceptor mRNA densities measured in the human nasal mucosa must not necessarily mirrow their height of receptor protein expression nor their physiological importance.

A further aim of this study was to explore the interaction of two catecholamine agonists (noradrenaline and adrenaline) and two imidazoline agonists (oxymetazoline and xylometazoline) at human α-adrenoceptor subtypes heterologously expressed in HEK293 cells. These agonists have not yet been examined before within one study and at all human α-adrenoceptor subtypes. The affinities (K_i values) were determined from the IC_{50} values of agonist-induced inhibition of [^{3}H]prazosin binding to α_1-adrenoceptors and [^{3}H]RX821002 binding to α_2-adrenoceptors expressed in the plasma membrane of the transiently transfected cells. The affinities of the four α-adrenoceptor agonists at the six adrenoceptor subtypes measured in radioligand binding experiments were different between catecholamines and imidazolines. As a rule, the two catecholamines, noradrenaline and adrenaline, exhibited lower affinities (K_i values between 0.2 and 93 μM) compared to the two imidazolines, oxymetazoline and xylometazoline (K_i values between 0.01 and 5.5 μM). The affinities for the two catecholamines were nearly identical and the rank order of affinities was: $\alpha_{1D} > \alpha_{1B} = \alpha_{1A} \geq \alpha_{2B} \geq \alpha_{2C} > \alpha_{2A}$. The rank order for the two imidazolines also was almost identical; for oxymetazoline it was: $\alpha_{1A} > \alpha_{2A} > \alpha_{2C} > \alpha_{1B} \geq \alpha_{1D} > \alpha_{2B}$. At α_{2B}-adrenoceptors xylometazoline exhibited a nearly three-fold higher affinity than oxymetazoline, whereas at α_{1A}-adrenoceptors oxymetazoline showed an about five-fold higher affinity than xylometazoline. These differences between the affinities of the two imidazolines to the respective receptor subtypes were significant and most pronounced at the α_{1A}-adrenoceptors. Presently, we have no explanation for this result.

Since affinity data give no information whether a substance is an agonist (full or partial) or an antagonist, we examined the four α-adrenoceptor agonists for their agonistic potency at the six α-adrenoceptor subtypes transiently expressed in HEK293 cells. To enable the measurement of receptor-induced activation of phospholipase C (PLC) and the subsequent IP3-induced increases in intracellular Ca^{2+} ion concentration, HEK293 cells were transfected with the α-adrenoceptor subtype cDNA together with the $G_{\alpha16}$ cDNA. It is known that, unlike other members of the G_q family of G-proteins, the $G_{\alpha16}$ protein can promiscuously couple G-protein coupled receptors (GPCRs) and mediate agonist-induced stimulation of PLC [12–14,25]. Thus, this co-transfection allowed the measurement of receptor-mediated calcium signals independent from eventually preferred coupling to other endogenously expressed G-proteins. Since HEK293 cells do not express endogenous voltage-sensitive calcium channels [16], the agonist-induced calcium signal should be exclusively due to Ca^{2+} released from intracellular stores.

The two catecholamine agonists noradrenaline and adrenaline led to concentration-dependent increases in intracellular Ca^{2+} at all six α-adrenoceptor subtypes. The EC_{50} values (potencies) were in the low micromolar range. The low potency of the catecholamines at α-adrenoceptors agrees with their contractile potency in the human nasal mucosa [8,26]. The EC_{50} values at HEK293 cells, however, are higher than those reported in the literature for, e.g. noradrenaline at α_1- and α_2-adrenoceptor subtypes heterologously expressed in either stably transfected Chinese hamster ovary (CHO) cells [10,27,28] or in transiently transfected Sf9 insect cells [29]. However, the potency of noradrenaline (EC_{50} of 1.6 μM) at the embryonic rat aorta smooth muscle cell line A7r5 stably transfected with the cDNA of the human α_{2B}-adrenoceptor [30] agrees with our data.

Only in α_{2B}-adrenoceptor expressing HEK293 cells the imidazoline xylometazoline caused a concentration-dependent Ca^{2+} response; this response was characterized by a very low potency (EC_{50} about 100 μM) but a high maximum. This result cannot be compared with data in the literature since almost nothing is known about the pharmacology of xylometazoline at native or heterologously expressed α-adrenoceptors. Oxymetazoline behaved as a full agonist at α_{2B}-adrenoceptors, too, which are (at the mRNA level) the third highest expressed α-receptors in the human nasal mucosa. At this receptor, oxymetazoline exhibited the same high efficacy (about 120%) as xylometazoline, but it was about seven-fold more potent (EC_{50} about 15 μM) than xylometazoline. It should be noted that there was a discrepancy between the potency and affinity values for these two agonists at this receptor. While oxymetazoline showed a 2.7-fold lower potency than affinity, the potency of xylometazoline was by a factor of 59 lower than its affinity. Nevertheless, the IC_{50} and EC_{50} values of oxymetazoline for the α_{2B}-adrenoceptor are quite

close. This fact indicates the possibility of a faster binding process and onset of action compared to xylometazoline. The latter point could be examined in future kinetic studies.

Oxymetazoline (but not xylometazoline) showed partial agonistic activity (with EC_{50} values of about 11 μM and E_{max} values of about 50%) at human α_{1A}-adrenoceptors which are the second highest expressed α-adrenoceptors (at the mRNA level) in the human nasal mucosa. However, at this receptor oxymetazoline's potency was nearly 1000-fold lower than its affinity. There are several potential reasons for differences between binding and functional results such as differences in buffer composition, absence of GTP in binding studies, duration of the experiment (long lasting equilibrium binding versus very short and transient Ca^{2+} signaling). However, presently we have no clear-cut explanation for the observed pronounced discrepancies which remain to be examined in future studies.

Partial agonistic activity of oxymetazoline at α_{1A}-adrenoceptors has also been shown in other cell systems such as stably tranfected CHO cells [10,27], and this was also observed at α_{2C}-adrenoceptors [28]. Very low or not correctly measurable agonistic activity of oxymetazoline has been observed by others in transfected CHO cells expressing α_{1D}- [10,27] or α_{2A}-adrenoceptors [31]. In the present study at HEK293 cells agonistic activities of the two imidazolines were very low and not correctly measurable at α_{1B}-, α_{1D}-, α_{2A}- and α_{1C}-adrenoceptors. To our knowledge, this is the first study in which affinities and potencies of the two catecholamines, noradrenaline and adrenaline, and of the two imidazolines, xylometazoline and oxymetazoline, simultaneously have been studied in transiently transfected HEK293 cells. The fact that the imidazolines showed no agonist activitiy at some alpha-adrenoceptors together with the observed discrepancies between potencies and affinities at some α-adrenoceptors may eventually indicate that Ca^{2+} signaling through α-adrenoceptors heterologously expressed in HEK293 cells may in some aspects differ from α-adrenoceptor-mediated signal transduction in smooth muscle cells.

In conclusion, we could show that human nasal mucosa expresses at mRNA level all six α-adrenoceptor subtypes but only α_{1A}-, α_{2A}- and α_{2B}-adrenoceptors are expressed at high levels. Among the imidazolines, xylometazoline exhibited full agonist properties only at α_{2B}-adrenoceptors whereas oxymetazoline was a more potent and full agonist at α_{2B}- and additionally a weak partial agonist at α_{1A}-adrenoceptors.

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REFERENCES

1 Bylund D.B., Bond R.A., Clarke D.E., et al. Adrenoceptors. The IUPHAR Compendium of Receptor Characterization and Classification, IUPHAR Media, London, UK, 1998, pp. 58–74.
2 Docherty J.R. Subtypes of functional alpha$_1$- and alpha$_2$-adrenoceptors. Eur. J. Pharmacol. (1998) **361** 1–15.
3 Druce H.M., Bonner R.F., Patow C., Choo P., Kaliner M.A. Response of nasal blood flow to neurohormones as measured by laser-Doppler velocimetry. J. Appl. Physiol. (1984) **57** 1276–1283.
4 Ichimura K., Jackson R.T. Evidence of alpha2-adrenoceptors in the nasal blood vessels of the dog. Arch. Otolaryngol. (1984) **110** 647–651.
5 Berridge T.L., Roach A.G. Characterization of the α-adrenoceptors in the vasculature of the canine nasal mucosa. Br. J. Pharmacol. (1986) **88** 345–354.
6 Corboz M.R., Varty L.M., Rizzo C.A., et al. Pharmacological characterization of α_2-adrenoceptor-mediated responses in pig nasal mucosa. Auton. Autacoid Pharmacol. (2003) **23** 208–219.
7 Andersson K.-E., Bende M. Adrenoceptors in the control of human nasal mucosal blood flow. Ann. Otol. Rhino Laryngol. (1984) **93** 179–182.
8 Corboz M.R., Rivelli M.A., Varty L.M., et al. Pharmacological characterization of postjunctional α-adrenoceptor in human nasal mucosa. Am. J. Rhinol. (2005) **19** 495–502.
9 Van Megen Y.J.B., Klaassen A.B.M., Rodrigues de Miranda J.F., van Ginneken C.A.M., Wentges B.T.R. Alterations of adrenoceptors in the nasal mucosa of allergic patients in comparison with nonallergic individuals. J. Allergy Clin. Immunol. (1991) **87** 530–540.
10 Horie K., Obika K., Foglar R., Tsuimoto G. Selectivity of the imidazoline α-adrenoceptors agoniosts (oxymetazoline and cirazoline) for human cloned α1-adrenoceptor subtypes. Br. J. Pharmacol. (1995) **116** 1611–1618.
11 Baubet V., Le Mouellic H., Campbell A.K., Lucas-Meunier E., Fossier P., Brulet P. Chimeric green fluorescent protein-aequorin as bioluminescent Ca^{2+} reporters at the single-cell level. Proc. Natl. Acad. Sci. USA (2000) **97** 7260–7265.
12 Offermanns S., Simon M.I. G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. J. Biol. Chem. (1995) **270** 15175–15180.
13 Zhu X., Birnbaumer L. G protein subunits and the stimulation of phospholipase C by Gs-and Gi-coupled receptors: Lack of receptor selectivity of Galpha(16) and evidence for a synergic interaction between Gbeta gamma and the alpha subunit of a receptor activated G protein. Proc. Natl. Acad. Sci. USA (1996) **93** 2827–2831.
14 Lee J.W.M., Joshi S., Chan J.S.C., Wong Y.H. Differential coupling of mu-, delta-, and kappa-opioid receptors to G

alpha16-mediated stimulation of phospholipase C. J. Neurochem. (1998) **70** 2203–2211.
15 Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. (1951) **193** 265–275.
16 Walstab J., Combrink S., Brüss M., Göthert M., Niesler B., Bönisch H. Aequorin luminescence-based assay for 5-hydroxytryptamine (serotonin) type 3 receptor characterization. Anal. Biochem. (2007) **368** 185–192.
17 Altman J.D., Trendelenburg A.U., MacMillan L., et al. Abnormal regulation of the sympathetic nervous system in a_{2A}-adrenergic receptor knockout mice. Mol. Pharmacol. (1999) **56** 154–161.
18 Starke K. Presynaptic autoreceptors in the third decade: focus on alpha2-adrenoceptors. J. Neurochem. (2001) **78** 685–693.
19 Kubista H., Boehm S. Molecular mechanisms underlying the modulation of exocytotic noradrenaline release via presynaptic receptors. Pharmacol. Ther. (2006) **112** 213–242.
20 Olli-Lähdesmäki T., Kallio J., Scheinin M. Receptor sybtype-induced targeting and subtype-specific internalization of human α_2-adrenoceptors in PC12 cells. J. Neurosci. (1999) **19** 9281–9288.
21 Philipp M., Hein L. Adrenergic receptor knockout mice: distinct functions of 9 receptor subtypes. Pharmacol. Ther. (2004) **101** 65–74.
22 Robinson E., Hudson A. Adrenoceptor pharmacology. Tocris Review No 8 (1998) (http://www.tocris.com).
23 Khattar S.K., Bora R.S., Privadartsiny P., et al. Molecular cloning, stable expression and cellular localization of human $alpha_1$-adrenergic receptor subtypes: effect of charcoal/dextran treated serum on expression and localization of $alpha_{1D}$-adrenergic receptor. Biotechnol. Lett. (2006) **28** 1731–1739.
24 Theroux T.L., Esbenshade T.A., Peavy R.D., Minnemann K.P. Coupling efficiencies of human α1-adrenergic receptor subtypes: titration of receptor density and responsiveness with inducible and repressive expression vectors. Mol. Pharmacol. (1996) **50** 1376–1387.
25 Kostenis E. Is $G\alpha_{16}$ the optimal tool for fishing ligands of orphan G-protein-coupled receptors? Trends Pharmacol. Sci. (2001) **22** 560–564.
26 Chiba Y., Matsuo K., Sakai H., Abe K., Misawa M. Regional differences in vascular responsiveness of nasal mucosae isolated from naive guinea pigs. Auris Nasus Larynx (2007) **34** 197–201.
27 Obika K., Shibata K., Horie K., Foglar R., Kimura K., Tsujimoto G. NS-49, a novel α1a-adrenoceptor-selective agonist characterization using recombinant human α1-adrenoceptors. Eur. J. Pharmacol. (1995) **291** 327–334.
28 Kukkonen J.P., Renvaktar A., Shariatmadari R., Akerman K.E. Ligand- and subtype-selective coupling of human alpha-2 adrenoceptors to Ca^{++} elevation in Chinese hamster ovary cells. J. Pharmacol. Exp. Ther. (1998) **287** 667–671.
29 Holmberg C.I., Kukkonen J.P., Bischoff A., et al. α_{2B}-adrenoceptors couple to Ca^{2+} increase in both endogenous and recombinant expression systems. Eur. J. Pharmacol. (1998) **363** 65–74.
30 Huhtinen A., Scheinin M. Expression and characterization of the human α_{2B}-adrenoceptor in a vascular smooth muscle cell line. Eur. J. Pharmacol. (2008) **587** 48–56.
31 Pauwels P.J., Finana F., Tardif S., Colpaert F.C., Wurch T. Agonist efficacy at the α_{2A}-adrenoceptor:$G_{\alpha 15}$ fusion protein: an analysis based on Ca^{2+} responses. Naunyn Schmiedebergs Arch. Pharmacol. (2000) **361** 672–679.