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Noradrenaline release-inhibiting receptors on PC12 cells devoid of α_{2-} and CB_1 receptors: similarities to presynaptic imidazoline and edg receptors

G.J. Molderings *, H. Bönisch, R. Hammermann, M. Göthert, M. Brüss

Institute of Pharmacology and Toxicology, University of Bonn, Reuterstraße 2b, 53113 Bonn, Germany

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Abstract

The aim of the present study was to classify release-inhibiting receptors on rat pheochromocytoma PC12 cells. Veratridine-evoked [^{3}H]noradrenaline release from PC12 cells was inhibited by micromolar concentrations of the imidazoline and guanidine derivatives cirazoline, clonidine, aganodine, 1,3-di(2-tolyl)guanidine, BDF6143 and agmatine, and of the cannabinoid receptor agonist WIN55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-yl](1-naphthalenyl)-methanone mesylate), but not by noradrenaline. The inhibitory effect of clonidine was antagonized by micromolar concentrations of rauwolscine and SR141716A (*N*-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1*H*-pyrazole-3-carboxamide). The potencies of the agonists and antagonists were compatible with an action at previously characterized presynaptic imidazoline receptors. 1-Oleoyl-lysophosphatidic acid, but not sphingosine-1-phosphate, produced an inhibition of release that was antagonized by 30 μM rauwolscine, 1 μM SR141716A and 10 μM LY320135 as well as by pretreatment of the cells with 100 μM clonidine for 72 h. Polymerase chain reaction (PCR) experiments on cDNA from PC12 mRNA suggest mRNA expression of lysophospholipid receptors encoded by the genes edg2, edg3, edg5 and edg7, but not of receptors encoded by edg1, edg4, edg6 and edg8, and not of α_{2A}-nd CB_1 receptors. In conclusion, PC12 cells are not endowed with α_2-adrenoceptors and CB_1 cannabinoid receptors, but with an inhibitory receptor recognizing imidazolines, guanidines and WIN55,212-2 similar to that on sympathetic nerves. The PCR results and the ability of 1-oleoyl-LPA to mimic these drugs (also with respect to their susceptibility to antagonists) suggest that the release-inhibiting receptor may be an edg-encoded lysophospholipid receptor.

Keywords: PC12 cells-imidazoline receptor; α_2-Adrenoceptor; Cannabinoid receptor; Noradrenaline release; Lysophospholipid receptor

1. Introduction

In addition to the 'classical' presynaptic modulatory receptors on the axon terminals of postganglionic sympathetic nerves (Starke, 1981), a non-I_1/non-I_2 imidazoline receptor mediating inhibition of noradrenaline release has been identified as a further presynaptic receptor in animal and human blood vessels and heart (for a review, see Molderings and Göthert, 1999). This presynaptic imidazoline receptor is activated by both imidazoline and guanidine derivatives, and differs pharmacologically from the presynaptic α_2-adrenoceptor. Among other criteria that differentiate between both receptors, the presynaptic imidazoline receptor is blocked with low potency by the α_2-adrenoceptor antagonist rauwolscine and with moderate potency by the CB_1 cannabinoid receptor antagonists SR141716A and LY320135 (Molderings and Göthert, 1998, 1999; Molderings et al., 1999).

To prove that the presynaptic imidazoline receptor is an entity independent of α_2-adrenoceptors and cannabinoid receptors, a cell line that expresses such release-inhibiting imidazoline receptors but not α_2 and CB_1 cannabinoid receptors would be helpful. The rat pheochromocytoma cell line PC12 was hypothesized to be suitable for this purpose because it possesses many

* Corresponding author. Tel.: +49-228-735-421; Fax: +49-228-735-404.

E-mail address: molderings@uni-bonn.de (G.J. Molderings).

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properties of sympathetic neurones and adrenal chromaffin cells (for a review, see Greene and Tischler, 1976). In these cells, the α_2-adrenoceptor has been reported to be absent (Schwegler and Bönisch, 1986; Duzic and Lanier, 1992); however, this is not generally accepted since other groups proposed its existence (Gatti et al., 1988; Gollasch et al., 1992; Kim et al., 1993). Therefore, the first aim of the present study was to establish the absence or presence of α_2-adrenoceptors and to examine whether PC12 cells lack CB_1 cannabinoid receptors, using radioligand binding and molecular biological techniques. The second aim was to investigate whether PC12 cells express imidazoline receptors with pharmacological properties comparable with those of the presynaptic imidazoline receptors on the sympathetic neurones of man, rat and rabbit. If so, the third aim finally was to investigate whether the release-inhibiting imidazoline receptor can be classified as a member of an already known receptor family defined by its pharmacological and/or molecular biological properties.

2. Experimental procedures

2.1. Cell culture

PC12 cells were cultured in suspension culture as described by Harder and Bönisch (1984). The culture medium was composed of 85% RPMI 1640 medium (Gibco, Karlsruhe, Germany), 10% heat-inactivated horse serum (Gibco), 5% fetal calf serum (Gibco) and was buffered with 24 mM $NaHCO_3$. For the experiments on intact cells, PC12 cells were cultured in a humidified CO_2-incubator (at 37 °C and in the presence of 9% CO_2) on dishes (60 mm; Nunc) coated with polyornithine 0.1 g/l (in 0.15 M boric acid, and 67 mM NaOH, adjusted with HCl to pH 8.4). Each culture dish contained about 10^7 cells, corresponding to about 1 mg cell protein.

2.2. Membrane preparation

Undifferentiated PC12 cells were harvested and homogenized in HEPES buffer (HEPES-Na^+ 5 mM; EGTA, 0.5 mM; $MgCl_2$, 0.5 mM; ascorbic acid, 0.1 mM; pH 7.4, 4 °C) with a polytron homogenizer for 1 min and then centrifuged for 10 min ($1000 \times g$, 4 °C) to pellet cellular debris. The supernatant was centrifuged for 30 min ($40{,}000 \times g$, 4 °C) and the pellet resuspended in fresh HEPES buffer and washed twice. Finally, the washed pellet was stored at −80 °C until use. Before the membranes were added to the incubation assay, they were centrifuged (20 min, $40{,}000 \times g$, 4 °C), resuspended in the buffer, homogenized by ultrasonication and diluted to a final protein concentration of about 0.6 mg/ml.

2.3. Radioligand binding

A 400-μl aliquot of the membranes was incubated for 30 min with 16.8 nM [^{3}H]rauwolscine or for 60 min with 10 nM [^{3}H]SR141716A at room temperature in a final volume of 0.5 ml. The reaction was stopped by rapid vacuum filtration with a Brandel cell harvester through Whatman GF/C glass-fiber filters presoaked with polyethylenimine 0.5 M followed by rapid washing of the incubation tubes and filters with 10 ml ice-cold buffer. Filters were placed in 6 ml scintillation fluid and shaken overnight, and the radioactivity was determined by liquid scintillation counting at 44% efficiency. Non-specific binding was defined as [^{3}H]rauwolscine binding in the presence of 10 μM rauwolscine and as [^{3}H]SR141716A binding in the presence of 3 μM CP55,940. Results are expressed as mean values ± S.E.M. All experiments were carried out in triplicate. Data were analyzed using the least-squares fitting program PRISM (GraphPad Software Inc., San Diego, CA, USA).

2.4. PCR amplification of α_{2A-}, CB_1 and lysophospholipid receptor DNA

Template DNAs were first-strand cDNA from undifferentiated PC12 cells and genomic DNA prepared from rat whole blood. To prepare cDNA, cells were chemically dissected; total RNA from PC12 cells was isolated with the Quiagen RNeasy kit according to the manufacturer's protocol. Then oligo dT primed first-strand cDNA synthesis was performed with AMV Reverse Transcriptase (Promega), followed by RNase digestion, phenol/chloroform extraction and cDNA purification with Chroma Spin 30 columns (Clontech). Genomic DNA was purified from rat whole blood with the Quiagen QIAamp Blood Midi kit according to the manufacturer's protocol. The purified DNA was used as a template for subsequent polymerase chain reaction (PCR) amplification of the receptor DNA under the following conditions. Primer sequences for the rat α_{2A}-adrenoceptor (sense primer, 5′-catctccttcccgccactcatc-3′; antisense primer, 5′-atacgcacgtagaccaggatc-3′) and for the rat CB_1 cannabinoid receptor (sense primer, 5′-ctg-gc(ac)(gt)(ag)gc(agct)gac(agct)tcctg-3′; antisense primer, 5-a(gt)(ag)g(ct)(ag)tagat(agct)a(agct)(agct)gggttc-3′) were chosen according to the sequences of Gene Bank accession numbers M62372 and X81120, respectively. Primer sequences for the lysophospholipid receptors were chosen according to the following sequences: edg1, accession number U10303 (sense primer, 5′-cttcagcctc-cttgctatcg-3′; antisense primer, 5′-gcaggcaatgaaga-cactca-3′); edg2, accession number AF014418 (sense primer, 5′-ccaaactacagcactctcatg-3′; antisense primer, 5′-gcttccttctaaaccacagag-3′); edg3, accession number NM005226 (sense primer, 5′-tcagggagggcagtatgttc-3′;

antisense primer, 5′-ctgactttcgaagaggatgg-3′); edg4, accession number AW477544 (sense primer, 5′-cactcctggcactgcctctg-3′; antisense primer, 5′-cttgcagcccagaccatccag-3′); edg5, accession number U10699 (sense primer, 5′-ttctggtgctaatcgcagtg-3′; antisense primer, 5′-gagcagagagttgagggtgg-3′); edg6, accession number AW141943 (sense primer, 5′-ggagtacctgcgcggcatg-3′; antisense primer, 5′-catggcctcggacatggacac-3′); edg7, accession number AW107032 (sense primer, 5′-atgaatgagtgtcactatgac-3′; antisense primer, 5′-catacatgtagatgcgtacgt-3′); edg8, accession number AF233649 (sense primer, 5′-catgcacccatgttcctgctc-3′; antisense primer, 5′-gatcggcttgcagaagcacag-3′). PCR was performed in a total volume of 100 μl containing 15 nM primer (each), 5 U Taq DNA Polymerase (Gibco), 2 mM $MgCl_2$, 200 μM dNTPs (each), 10 μl 10 × Taq-Buffer (Gibco) and 3–5 μl template DNA. PCR was performed for 37–40 cycles. PCR products were separated by agarose gel electrophoresis, and the band of interest was cut off the gel, purified with 'GeneElute' columns (Supelco), ligated into the 'TA-cloning' vector pCR2.1 (Invitrogen) and transformed into '*Escherichia coli* InVα' (Invitrogen). The subcloned DNA fragments were sequenced with an automated sequencer (Li-COR 4200; MWG-Biotech, Ebersberg, Germany) and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Freiburg, Germany).

2.5. Release experiments

To load the cells with labelled noradrenaline, 10 nM [^{3}H]noradrenaline was added to the culture medium (3 ml/dish) and the cells were incubated for 3 h in a CO_2 incubator (37 °C). To minimize oxidation of the catecholamine, L(+)-ascorbic acid (1 mM) was present in all solutions. The cells were then washed twice with culture medium and incubated for 60 min with [^{3}H]noradrenaline-free culture medium (with a change of the medium after 30 min). Subsequently, the cell dishes were transferred to a water bath of 37 °C (onset of the experiment) and the cells were then incubated until the end of the experiment with prewarmed (37 °C) [^{3}H]noradrenaline-free HEPES-buffered salt solution (composition (mM unless stated otherwise): NaCl, 125; KCl, 15; KH_2PO_4, 1.2; $CaCl_2$, 2.6; $MgSO_4$, 1.2; HEPES-NaOH (pH 7.4), 25; D(+)-glucose, 5.6; L(+)-ascorbic acid, 1.0) with a change of the medium every 5 min (total incubation time in the HEPES buffer, 85 min). To minimize loss of cells during washing and to inhibit the neuronal noradrenaline transport system, the solution contained 1 g/l bovine serum albumin and 1 μM desipramine, respectively (Friedrich and Bönisch, 1986). The agonist and/or antagonist under study was added to the HEPES buffer at the onset of the exposure to this buffer. Sixty minutes after onset of incubation with the buffer, 1 mM veratridine was added for 5 min. In a few experiments, the HEPES-buffered salt solution contained 4.8 mM instead of 15 mM KCl and stimulation was carried out either by 1 mM veratridine or by increasing the KCl concentration to 15 mM (time schedule identical to the standard procedure). In one series of experiments, culture medium was changed daily (instead of changing it every 3 days as standard condition) and 100 μM clonidine was added to the medium for 72 h. The cells were then washed twice with clonidine-free culture medium (with a change of the medium after 30 min). Subsequently, the cell dishes were transferred to a water bath of 37 °C (onset of the experiment) and the cells were then incubated until the end of the experiment with prewarmed (37 °C) [^{3}H]noradrenaline-free HEPES-buffered salt solution, and veratridine was administered 2 h after the end of exposure to clonidine. Basal efflux of tritium was determined in the 5 min period before and in that 20 min after onset of stimulation. Veratridine-evoked tritium overflow was determined in the four 5 min periods after the onset of veratridine application. At the end of the experiment, the cells were solubilized by 0.1% v/v Triton X-100 (in 5 mM Tris–HCl; pH 7.4). The radioactivity of the solubilized cells and of the wash-out samples was determined by liquid scintillation counting.

2.6. Materials

Rauwolscine hydrochloride, agmatine sulfate, noradrenaline base, 1-oleoyl-lysophosphatidic acid, sphingosine-1-phosphate (Sigma, Munich, Germany); cirazoline hydrochloride (Synthélabo, Paris, France); aganodine, moxonidine, 4-chloro-2-(2-imidazolin-2-ylamino)-isoindoline hydrochloride (BDF 6143; Beiersdorf, Hamburg, Germany); desipramine hydrochloride (Ciba-Geigy, Wehr, Germany); clonidine hydrochloride (Boehringer, Ingelheim, Germany); *N*-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1*H*-pyrazole-3-carboxamide (SR141716A; Sanofi, Montpellier, France); [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)-cyclohexane (CP55,940; Tocris-Cookson-Biotrend, Cologne, Germany); [6-methoxy-2-(4-methoxy phenyl)benzo[b]thien-3-yl][4-cyanophenyl]methanone (LY320135; Lilly, Indianapolis, USA); 1,3-di(2-tolyl)guanidine (DTG), anandamide (RBI, Natick, USA); *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl](1-naphthalenyl)methanone mesylate (WIN55,212-2), WIN55,212-3 (*S*(−)enantiomer of WIN55,212-2) (RBI-Biotrend, Cologne, Germany); 1-palmitoyl-LPA (Avanti Polaris Lipids, Alabaster, USA); (−)-[2,5,6-^{3}H]noradrenaline (specific activity, 46 Ci/mmol; NEN, Dreieich, Germany); [*O*-methyl-^{3}H]rauwolscine (specific activity, 76 Ci/mmol), [^{3}H]SR141716A (specific activity, 44.0 Ci/mmol; Amersham, Braunschweig, Germany). Drugs were dissolved

in saline with the following exceptions: DTG was dissolved in methanol; and SR141716A, LY320135, anandamide, WIN55,212-2, WIN55,212-3, CP55,940, 1-oleoyl-LPA, 1-palmitoyl-LPA and S1P were dissolved in dimethylsulfoxide (final maximum concentration in the buffer solution, 1%). The stock solutions were further diluted in the buffer. Corresponding control experiments were run with the solvent only.

3. Results

3.1. [^{3}H]Noradrenaline release experiments

At a KCl concentration of 4.8 mM in the incubation buffer (i.e. the KCl concentration in most classical physiological salt solutions), 1 mM veratridine induced only an inconsistent and small tritium overflow above basal outflow from PC12 cells preincubated with [^{3}H]noradrenaline ($n = 18$; data not shown). When the KCl concentration was increased to 15 mM for 5 min after 60 min exposure to 4.8 mM KCl in the incubation buffer, also no consistent tritium overflow occurred ($n = 5$; data not shown). However, in the presence of 15 mM KCl in the incubation buffer, 1 mM veratridine induced a tritium overflow above basal efflux that in a representative series of control experiments amounted to $1.39 \pm 0.29\%$ of the tritium present in the cells before the respective collection period ($n = 24$; corresponding to 1760 ± 285 dpm/well). This veratridine (1 mM)-evoked tritium overflow was abolished in the absence of Ca^{2+} ions in the incubation buffer ($n = 6$); it was inhibited by 56% by 50 μM colchicine ($n = 6$). In all further experiments, the veratridine stimulus in the presence of 15 mM KCl was applied as the standard stimulation procedure with this depolarizing alkaloid. The veratridine-evoked tritium overflow reflects exocytotic release of tritiated and unlabelled noradrenaline (for details, see Section 4) and is denoted as [^{3}H]noradrenaline release in the following text, table and figures.

The basal tritium efflux from the cells incubated with the buffer containing 15 mM KCl during the 5 min period before stimulation with veratridine amounted to $1.31 \pm 0.10\%$ of the tritium present in the cells before this collection period, corresponding to 593 ± 71 dpm/well ($n = 24$ in a representative series of control experiments). It was not altered by the antagonists or agonists investigated in this study.

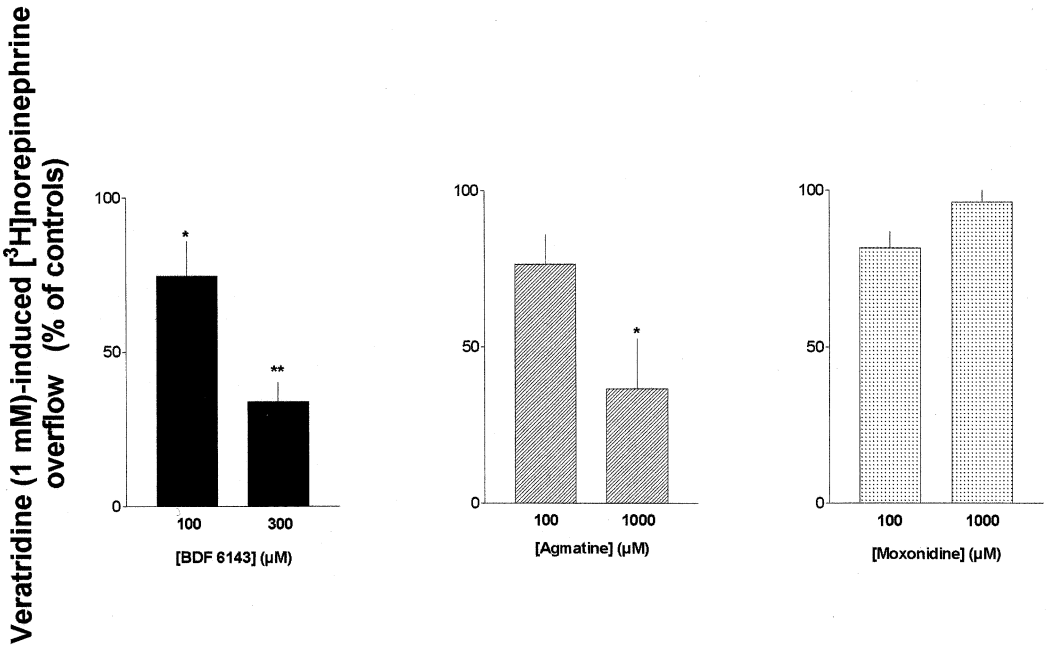

Fig. 1. Concentration dependence of the inhibitory effects of BDF6143 and agmatine, and lack of effect of moxonidine on veratridine-induced [^{3}H]noradrenaline release from PC12 cells. The cells were preincubated with culture medium containing 10 nM [^{3}H]noradrenaline and, after 60 min exposure to [^{3}H]noradrenaline-free culture medium, incubated with [^{3}H]noradrenaline-free HEPES-buffered salt solution containing 15 mM KCl, 1 μM desipramine and 3 μM rauwolscine throughout. BDF6143, agmatine or moxonidine was present from the onset of incubation with the HEPES buffer until the end of the experiment; 5 min stimulation with 1 mM veratridine was carried out 60 min after onset of incubation with the buffer. Evoked [^{3}H]noradrenaline release was expressed as the percentage of veratridine-evoked release in control experiments carried out in parallel without the drug under study. Means ± S.E.M. of six experiments in each group. * $P < 0.05$, ** $P < 0.01$ (compared with the corresponding controls).

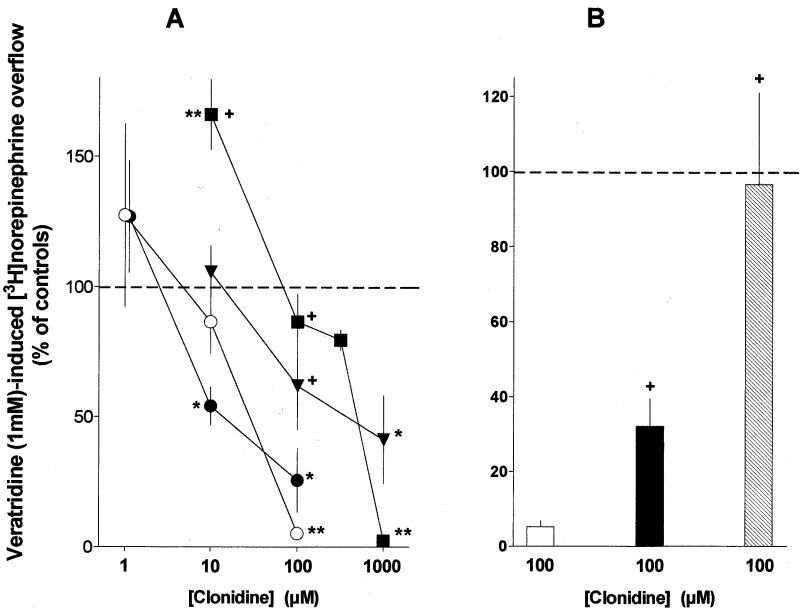

Fig. 2. Inhibitory effect of clonidine on [^{3}H]noradrenaline release from PC12 cells (A) and interaction with rauwolscine, SR141716A and LY320135 (B). The cells were preincubated with culture medium containing 10 nM [^{3}H]noradrenaline and, after 60 min exposure to [^{3}H]noradrenaline-free culture medium, incubated with [^{3}H]noradrenaline-free HEPES-buffered salt solution containing 15 mM KCl and 1 μM desipramine throughout. Clonidine and the interacting drug under study (rauwolscine, 3 μM (●), 30 μM (▼); SR141716A, 1 μM (solid column), 10 μM (■); LY320135, 10 μM (dashed column)) or its solvent (○, open column) were present from the onset of incubation with the HEPES buffer until the end of the experiment; 5 min stimulation with 1 mM veratridine was carried out 60 min after onset of incubation with the buffer. Evoked [^{3}H]noradrenaline release was expressed as the percentage of evoked release in control experiments carried out in parallel without clonidine. Means ± S.E.M. of six experiments in each group; * $P < 0.05$, ** $P < 0.01$ (compared with the corresponding controls); $^+$ at least $P < 0.05$ (compared with the effect of clonidine in the absence of interacting drugs; Dunnett's test for multiple comparisons).

3.2. Effect of imidazoline and guanidine derivatives and interaction with antagonists

In the experiments with imidazoline and guanidine derivatives, 3 μM rauwolscine was present in the incubation buffer unless stated otherwise. This experimental condition was the same as in previous analogous experiments in the rat vena cava (Molderings and Göthert, 1998) and excluded the possibility that the compounds might mediate an effect via activation of α_2-adrenoceptors. Rauwolscine (3 μM) given alone did not significantly modify the veratridine-induced [^{3}H]noradrenaline release ($n = 6$; results not shown). In the presence of 3 μM rauwolscine (standard condition in the experiments with imidazolines, guanidines and cannabinoid ligands), BDF 6143 and agmatine (Fig. 1) as well as clonidine (Fig. 2A) concentration-dependently inhibited the veratridine-induced [^{3}H]noradrenaline release. In addition, several further imidazolines and guanidines were studied at a standard concentration of 100 μM; not only BDF6143, agmatine (Fig. 1 and Table 1) and clonidine (Fig. 2 and Table 1), but also cirazoline, aganodine and DTG inhibited the veratridine-induced [^{3}H]noradrenaline release (Table 1). Under the assumption that all active compounds produce the same maximum effect and exhibit approximately parallel concentration–response curves (which is supported by the similar shape of the concentration–response curves shown in Figs. 1 and 2), the inhibition obtained by 100 μM of the respective compound may be considered to be a rough qualitative estimate of their potency. Under this assumption, the results are compatible with the following rank order of inhibitory potency: cirazoline ≥ clonidine ≥ aganodine > DTG ≥ BDF6143 ≥ agmatine. In contrast, the catecholamine noradrenaline and the imidazoline moxonidine (the latter up to a concentration of 1000 μM) failed to modify the veratridine-induced [^{3}H]noradrenaline release (Fig. 1 and Table 1).

In the absence of rauwolscine, clonidine also concentration-dependently inhibited veratridine-evoked [^{3}H]noradrenaline release (Fig. 2A, open circles). The concentration–response curve of clonidine in the presence of 3 μM rauwolscine did not substantially differ from that in the absence of rauwolscine (Fig. 2A, closed circles). However, this curve was shifted to the right by 30 μM rauwolscine (Fig. 2A, closed triangles) and SR141716A (10 μM; Fig. 2A, closed squares); in the presence of this SR141716A concentration, 10 μM clonidine even acted facilitatory on [^{3}H]noradrenaline release (Fig. 2A). SR141716A and LY320135 at concentrations of 1 and 10 μM, respectively, counteracted the inhibition produced by 100 μM clonidine (Fig. 2B). SR141716A and LY 320135 given alone did not affect the veratridine-evoked [^{3}H]noradrenaline release (result

not shown). When the PC12 cells were pretreated with 100 μM clonidine for 72 h, 100 μM clonidine present from the onset of incubation with HEPES buffer until the end of the experiment did not inhibit, but did increase evoked [^{3}H]noradrenaline release ($171 \pm 24\%$ of veratridine-evoked [^{3}H]noradrenaline release in the respective control experiments; $n = 6$, $P < 0.05$).

3.3. Effects of cannabinoid receptor agonists and interaction with antagonists

In the presence of 3 μM rauwolscine, WIN55,212-2 concentration-dependently inhibited evoked [^{3}H]noradrenaline release, whereas 100 μM WIN55,212-3 did not (Fig. 3). CP55,940 at a concentration of 0.3 μM also significantly inhibited evoked tritium overflow, whereas at 1 μM no inhibition was found (Fig. 3) and at 10 μM even a facilitation was induced (Fig. 3, open columns). The inhibitory effect of 0.3 μM CP55,940 was antagonized by 1 μM SR141716A (Fig. 3, solid column). Anandamide at a concentration of 1 μM did not affect, whereas 100 μM anandamide enhanced veratridine-evoked [^{3}H]noradrenaline release (Fig. 3).

3.4. Effects of lysophospholipid receptor agonists and interaction with antagonists

In the absence of interacting drugs, 1-oleoyl-LPA concentration-dependently inhibited the evoked [^{3}H]noradrenaline release (Fig. 4A). In contrast, 10 μM S1P was ineffective ($101.7 \pm 31.3\%$ of the veratridine-evoked [^{3}H]noradrenaline release in the respective control experiments; $n = 6$). The concentration–response curve for 1-palmitoyl-LPA was bell shaped (Fig. 4A); evoked [^{3}H]noradrenaline release was concentration-dependently increased by up to 1 μM 1-palmitoyl-LPA, whereas 10 μM did not alter evoked [^{3}H]noradrenaline release. The inhibitory effect of 10 μM 1-oleoyl-LPA was counteracted by 30 μM rauwolscine and 1 μM SR141716A (Fig. 4B); 10 μM LY320135 even reversed the inhibitory effect of 10 μM 1-oleoyl-LPA to a facilitation ($198.8 \pm 39.9\%$ of the evoked [^{3}H]noradrenaline release in the respective control experiments, $n = 6$; $P < 0.05$ compared with the respective control experiments). When the PC12 cells were pretreated with 100 μM clonidine for 72 h, 10 μM 1-oleoyl-LPA did not inhibit but tended to increase evoked [^{3}H]noradrenaline release (Fig. 4B).

Table 1
Influence of imidazoline derivatives (cirazoline, clonidine, BDF 6143, moxonidine), guanidine derivatives (aganodine, DTG, agmatine) and noradrenaline at a concentration of 100 μM on [^{3}H]noradrenaline release from PC12 cells[a]

Drug (100 μM)	Veratridine(1 mM)-induced [^{3}H]noradrenaline release (% of controls without drug)
Cirazoline	$17.4 \pm 7.5\%$***
Clonidine	$25.6 \pm 12.2\%$*
Aganodine	$32.3 \pm 5.7\%$**
DTG	$63.3 \pm 7.0\%$*
BDF 6143	$74.7 \pm 11.1\%$*
Agmatine	$76.4 \pm 9.5\%$
Moxonidine	$81.6 \pm 5.2\%$
Noradrenaline	$93.0 \pm 11.5\%$

[a] The cells were preincubated with culture medium containing 10 nM [^{3}H]noradrenaline and, after 60 min exposure to [^{3}H]noradrenaline-free culture medium, incubated with [^{3}H]noradrenaline-free HEPES-buffered salt solution containing 15 mM KCl, 1 μM desipramine and 3 μM rauwolscine throughout. Unlabelled noradrenaline or the imidazoline or guanidine derivatives under study was present from the onset of incubation with the HEPES buffer until the end of the experiment; 5 min stimulation with 1 mM veratridine was carried out 60 min after onset of incubation with the buffer. Evoked [^{3}H]noradrenaline release was expressed as the percentage of veratridine-evoked release in control experiments carried out in parallel without the drug under study. Means ± S.E.M. of six to 11 experiments in each group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (compared with the corresponding controls).

3.5. Radioligand binding experiments

In the radioligand binding experiments, 16.8 nM [^{3}H]rauwolscine failed to label specific α_2-adrenoceptor binding sites as defined by addition of unlabelled rauwolscine (10 μM). [^{3}H]Rauwolscine binding amounted to 134.0 ± 7.1 ($n = 4$) and 132.8 ± 7.0 fmol/mg protein ($n = 4$) in the absence and presence of 10 μM rauwolscine, respectively (corresponding to 0.19 ± 0.01 and $0.19 \pm 0.01\%$ of the radioactivity added to each assay, respectively). Analogously, 10 nM [^{3}H]SR141716A did not label specific cannabinoid CB_1 binding sites as defined by addition of CP55,940 (3 μM). In the absence and presence of 3 μM CP55,940, binding of [^{3}H]SR141716A amounted to 84.1 ± 4.8 ($n = 4$) and 85.2 ± 4.1 fmol/mg protein ($n = 4$), corresponding to 4.64 ± 0.13 and $4.71 \pm 0.12\%$ of the radioactivity added to each assay, respectively.

3.6. Evidence for receptor expression by PCR experiments

Primer pairs suitable to identify the cDNAs for the rat α_{2A} and CB_1 cannabinoid receptors were applied in PCR experiments. In control experiments, fragments were amplified by PCR from the genomic DNA from rat blood with the same primer pairs. Sequencing of these fragments revealed that they code for the rα_{2A}-adrenoceptor and the rCB_1 receptor. In contrast, no message for the rα_{2A} and the rCB_1 receptors were found with the respective primer pairs in the cDNA from the undifferentiated PC12 cells (results not shown).

Genomic DNA prepared from rat blood was also used to prove whether the chosen primer pairs for lysophospholipid receptors were suitable to identify the corresponding edg messages. After PCR amplification

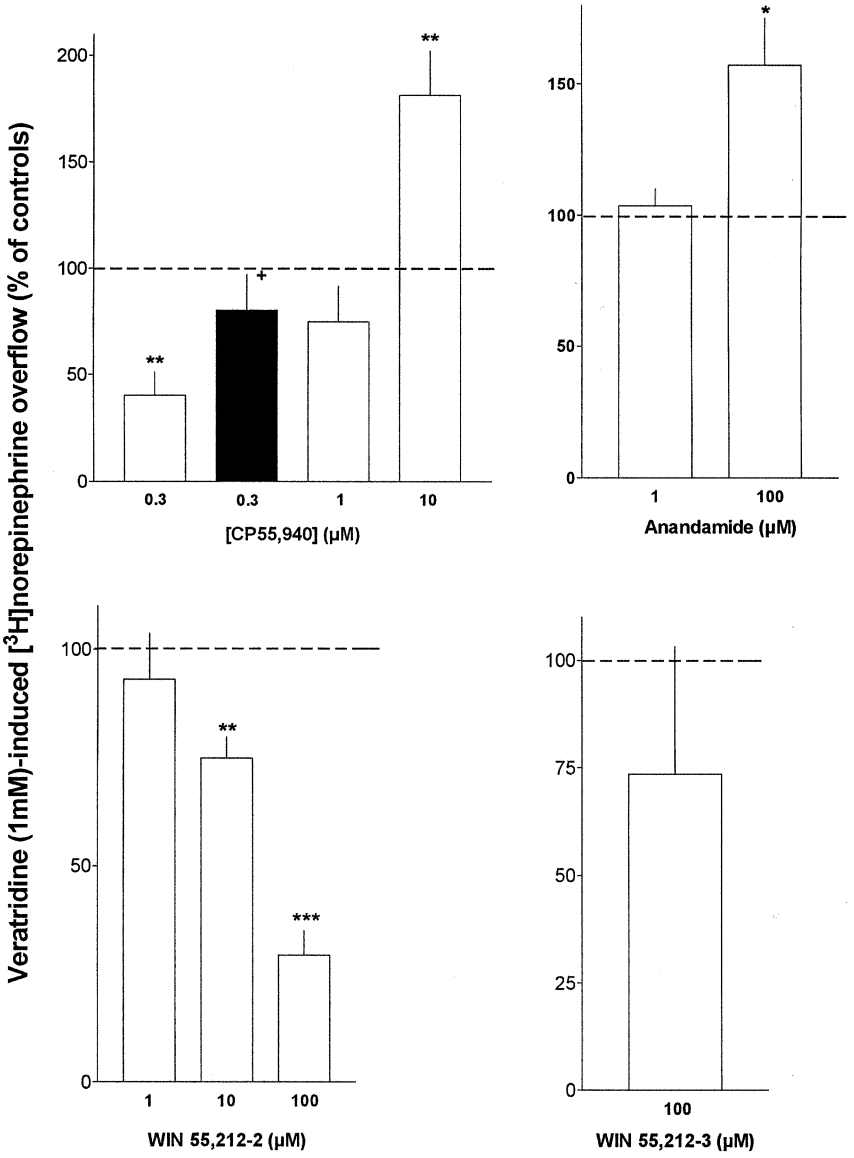

Fig. 3. Effects of CB_1 receptor agonists (CP55,950, anandamide, WIN55,212-2 and WIN55,212-3) on veratridine-induced [^{3}H]noradrenaline release from PC12 cells. The cells were preincubated with culture medium containing 10 nM [^{3}H]noradrenaline and, after 60 min exposure to [^{3}H]noradrenaline-free culture medium, incubated with [^{3}H]noradrenaline-free HEPES-buffered salt solution containing 15 mM KCl, and 1 μM desipramine throughout. The agonist and antagonist under study (3 μM rauwolscine, open columns; 1 μM SR141716A + 3 μM rauwolscine, solid column) were present from the onset of incubation with the HEPES buffer until the end of the experiment; 5 min stimulation with 1 mM veratridine was carried out 60 min after onset of incubation with the buffer. Evoked [^{3}H]noradrenaline release was expressed as the percentage of veratridine-evoked release in control experiments carried out in parallel without CP55,950, anandamide, WIN55,212-2 or WIN55,212-3. Means ± S.E.M. of six experiments in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (compared with the corresponding controls); $^+$ $P < 0.05$ (compared with the effect of 0.3 μM CP55,940 in the presence of 3 μM rauwolscine).

of this genomic DNA, PCR products were found that were identified by sequencing as partial sequences of edg1, edg2, edg3, edg4, edg5, edg6, edg7 and edg8 (results not shown). PCR amplification of PC12 cell cDNA using the same primer pairs resulted in PCR products coding for partial sequences of edg2, edg3, edg5 and edg7 but not of edg1, edg4, edg6 and edg8 (results not shown).

4. Discussion

In the present study, we investigated whether PC12 cells, which represent a neuronal-like rat pheochromocytoma cell line, are endowed with release-inhibiting receptors that resemble previously characterized presynaptic imidazoline receptors on sympathetic nerves (Molderings and Göthert, 1999), but lack α_2-adreno-

ceptors and cannabinoid CB_1 receptors. The ultimate aim was to investigate whether the release-inhibiting receptors can be classified as belonging to a receptor family that has already been defined by its pharmacological and molecular properties.

Our PCR experiments with primer pairs flanking the coding region of the rα_{2A}-adrenoceptor gene on cDNA prepared from undifferentiated PC12 cells revealed that PC12 cells do not express α_{2A}-adrenoceptors that have been proved to act as presynaptic autoreceptors (Trendelenburg et al., 1997). This finding is in accordance with the lack of specific high affinity binding of [^{3}H]rauwolscine (Duzic and Lanier, 1992; present study), but appears to be in contrast to the results of functional studies on undifferentiated PC12 cells (Gatti et al., 1988; Gollasch et al., 1992; Kim et al., 1993), in which clonidine was used as a purported selective α_2-adrenoceptor agonist (Gatti et al., 1988) or yohimbine acted as an antagonist at the very high concentration of 100 μM (Gollasch et al., 1992; Kim et al., 1993). However, these drugs (in particular at the concentrations used) are by no means selective α_2-adrenoceptors ligands; thus, the results described by those authors are in line with the data of the present study and are compatible with our final conclusions (see later). The failure to detect specific high affinity [^{3}H]rauwolscine binding that labels α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors, and mRNA for α_{2A}-adrenoceptors in undifferentiated PC12 cells provides clear evidence that these cells are not endowed with any subtype of α_2-adrenoceptors.

In view (1) of the ability of PC12 cells to produce the endogenous cannabinoid receptor agonist anandamide (Bisogno et al., 1998) and to exhibit responses to cannabinoid receptor ligands (Tahir et al., 1992; Kiss, 1999), and (2) of the putative relationship of the presynaptic imidazoline receptor to (but not identity with) the CB_1 cannabinoid receptor (Molderings et al., 1999), it was of importance to examine whether PC12 cells express CB_1 receptors. Our results from the PCR experiments with primers for the rat CB_1 receptor gene suggest that undifferentiated PC12 cells do not express detectable amounts of CB_1 receptors. This conclusion is supported by the failure to demonstrate specific high-affinity binding of [^{3}H]SR141716A, a highly selective CB_1 receptor radioligand to membranes from undifferentiated PC12 cells.

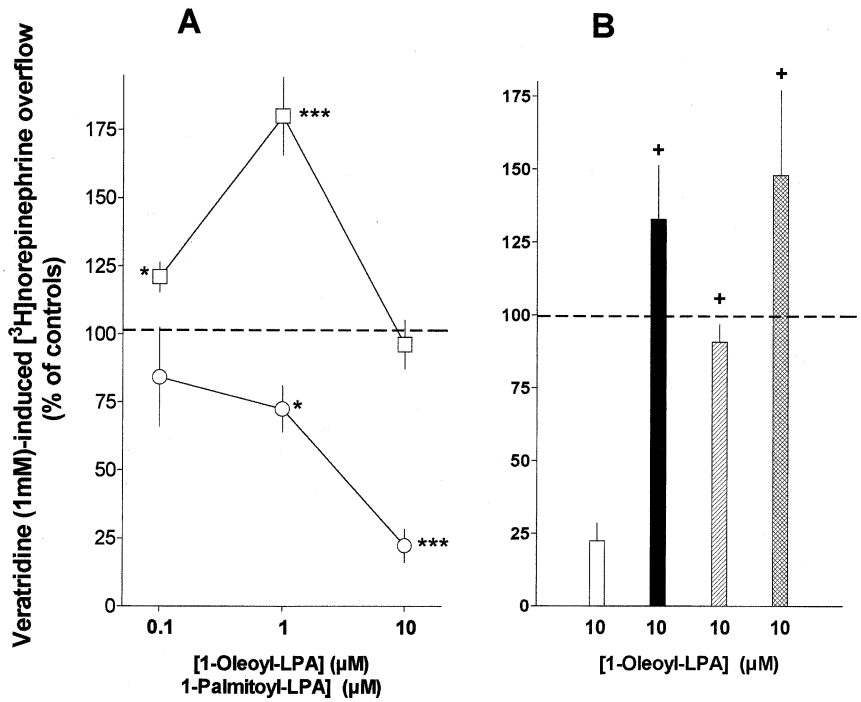

Fig. 4. Effects of 1-oleoyl-LPA and 1-palmitoyl-LPA on [^{3}H]noradrenaline release from PC12 cells (A) and interaction with rauwolscine, SR141716A and clonidine (B). The cells were preincubated with culture medium containing 10 nM [^{3}H]noradrenaline and, after 60 min exposure to [^{3}H]noradrenaline-free culture medium, incubated with [^{3}H]noradrenaline-free HEPES-buffered salt solution containing 15 mM KCl and 1 μM desipramine throughout. 1-Oleoyl-LPA and the interacting drug under study (rauwolscine, 30 μM (second solid column); SR141716A, 1 μM (third dashed column); pretreatment with 100 μM clonidine (fourth cross-hatched column)), or its solvent (○, first column) or 1-palmitoyl-LPA (□) were present from the onset of incubation with the HEPES buffer until the end of the experiment; 5 min stimulation with 1 mM veratridine was carried out 60 min after onset of incubation with the buffer. Evoked [^{3}H]noradrenaline release was expressed as the percentage of evoked release in control experiments carried out in parallel without the lysophosphatidic acid derivatives. Means ± S.E.M. of six experiments in each group; * $P > 0.05$, *** $P < 0.001$ (compared with the corresponding controls); $^+$ at least $P < 0.05$ (compared with the effect of 1-oleoyl-LPA, in the absence of interacting drugs; Dunnett's test for multiple comparisons).

In order to identify receptors on PC12 cells with the characteristic features of presynaptic imidazoline receptors, veratridine-evoked tritium overflow from PC12 cells preincubated with [^{3}H]noradrenaline was determined, which under the present conditions (blockade of the neuronal noradrenaline transporter) reflects release of tritiated and endogenous noradrenaline from the PC12 cells (for details, see Bönisch et al., 1990). Veratridine stimulates Na^+ influx mainly through voltage-dependent Na^+ channels (Ulbricht, 1998). Since [^{3}H]noradrenaline release was completely suppressed by omission of Ca^{2+} ions and markedly reduced by colchicine (which impairs the microtubular system involved in exocytosis), veratridine-induced [^{3}H]noradrenaline release from PC12 cells resembles action-potential-induced exocytotic [^{3}H]noradrenaline release from sympathetic neurones. Undifferentiated PC12 cells, i.e. cells not treated with nerve growth factor, express only a low number of veratridine-sensitive Na^+ channels (Reed and England, 1986; Rudy et al., 1987). To elicit a detectable and reproducible noradrenaline release by veratridine, the signal had to be increased by partial depolarization with elevated K^+ (15 mM).

The present results prove that PC12 cells express receptors with the characteristic features of presynaptic imidazoline receptors mediating inhibition of noradrenaline release in cardiovascular tissue (Molderings and Göthert, 1999; Molderings et al., 1999). (1) Moxonidine and the catecholamine noradrenaline, which stimulate presynaptic α_2-adrenoceptors but not presynaptic imidazoline receptors, were inactive in the present experiments on PC12 cells; in contrast, aganodine and clonidine, which are preferential agonists at presynaptic imidazoline receptors, inhibited [^{3}H]noradrenaline release in the present study. (2) Further imidazoline and guanidine derivatives such as cirazoline, BDF6143 and DTG also inhibited release irrespective of whether or not they possess affinity as agonists or antagonists for α_2-adrenoceptors. Their rank order of potency in inhibiting [^{3}H]noradrenaline release from PC12 cells was similar to that in cardiovascular tissue. (3) Rauwolscine acted as an antagonist versus clonidine at the imidazoline-recognizing receptors but with markedly lower potency than at α_2-adrenoceptors. (4) The highly selective CB_1 cannabinoid receptor antagonists SR141716A (Rinaldi-Carmona et al., 1995) and LY320135 (Felder et al., 1998) counteracted the inhibitory effect of clonidine but with potency lower than at CB_1 receptors.

The cannabinoid receptor agonist WIN55,212-2 also produced a low potency inhibition of [^{3}H]noradrenaline release, whereas WIN55,212-3, an enantiomer that is inactive at CB_1 cannabinoid receptors but shares the lipophilicity of WIN55,212-2, did not. The cannabinoid receptor agonist CP55,940 at a concentration of 0.3 μM also inhibited [^{3}H]noradrenaline release, an effect which was antagonized by 1 μM SR141716A. The more the concentration of CP55,940 was increased above 0.3 μM, the more the inhibitory effect disappeared and was finally reversed to a facilitation. Anandamide, an endogenous agonist at cannabinoid receptors, also facilitated [^{3}H]noradrenaline release from PC12 cells. Similarly, a facilitation of [^{3}H]noradrenaline release has previously been observed in the human heart with CP55,940 and anandamide (Molderings et al., 1999), when LY320135 was present in the superfusion fluid. In conclusion, the results with the cannabinoid receptor agonists suggest that these drugs may activate not only a release-inhibiting receptor, but also a release-facilitating receptor. The latter is unmasked on PC12 cells and sympathetic nerves by increasing the agonist concentration and addition of a CB_1 cannabinoid receptor antagonist, respectively; no attempt has been made in this study to classify this receptor.

Taken together, the receptors mediating inhibition of [^{3}H]noradrenaline release in cardiovascular tissue and PC12 cells resemble each other with respect to their pharmacological properties and share properties with the α_2-adrenoceptor and the CB_1 cannabinoid receptor. However, they are not identical with those receptors because the expression of α_2 and CB_1 cannabinoid receptors on PC12 cells was ruled out by our radioligand binding and PCR experiments. A comparison of the nucleotide sequences of the α_2-adrenoceptors and the CB_1 cannabinoid receptor with those of G-protein-coupled receptors listed in the gene databases revealed that only lysophospholipid receptors possess a significant homology with both receptor classes (about 40%). Accordingly, 1-oleoyl-LPA (but not S1P) inhibited evoked [^{3}H]noradrenaline release, and this effect was antagonized at low potency by rauwolscine, SR141716A and LY320135. Moreover, pretreatment of the cells for 72 h with 100 μM clonidine, which has been assumed to desensitize the presynaptic imidazoline receptors (Göthert and Molderings, 1991; Fuder and Schwarz, 1993), abolished the inhibitory effect of 1-oleoyl-LPA. This finding indicates that 1-oleoyl-LPA and at least clonidine may exert their inhibitory effect via the same receptor. On the other hand, similar to the cannabinoids CP55,940 and anandamide (see earlier) 1-palmitoyl-LPA, which has been shown to be able to discriminate between the LPA-sensitive lysophospolipid receptors (Bandoh et al., 1999; Im et al., 2000), up to 1 μM increased evoked [^{3}H]noradrenaline release; at 10 μM, the facilitation appeared to be counterbalanced by a simultaneously elicited inhibitory effect. Higher concentrations to study the latter putative inhibitory effect could not be investigated because vehicle-induced effects made an exact evaluation impossible.

The ability of 1-oleoyl-LPA to mimic the pharmacological properties of the imidazoline and guanidine derivatives at the release-inhibiting receptor points to the possibility that this receptor may represent a lysophos-

pholipid receptor. At present, eight lysophospholipid receptor types have been identified and cloned. They are provisionally termed edg1–edg8 according to the endothelial differentiation genes 1–8 that code for these receptors. 1-Oleoyl-LPA is an agonist at edg2, edg4 and edg7, whereas it is almost not active at edg1, edg3, edg5, edg6 and edg8. In contrast, S1P is an agonist at the latter receptors, whereas it is inactive at the former ones. Using the PCR technique, no cDNA coding for edg1, edg4, edg6 and edg8 receptors was detectable in PC12 cells. However, we demonstrated the presence of cDNAs encoding edg2, edg3, edg5 and edg7 receptors in PC12 cells. Hence, these results of the PCR experiments together with those of our release experiments and with the pattern of edg receptors that are activated by 1-oleoyl-LPA (see earlier) are at least compatible with the possibility that the release-inhibiting receptor may be identical with the edg2 or the edg7 receptor or a so far unknown lysophospholipid receptor at which 1-oleoyl-LPA acts as an agonist.

In conclusion, the three questions addressed in Section 1 can be answered as follows. (1) In contrast to the argumentation by Gatti et al. (1988), Gollasch et al. (1992), Kim et al. (1993), our data from [^{3}H]rauwolscine binding and PCR experiments unequivocally provide evidence that PC12 cells are not endowed with α_2-adrenoceptors. Also, as proved by binding and PCR techniques, these cells are devoid of CB_1 receptors. (2) The present [^{3}H]noradrenaline release experiments revealed that the release-inhibiting receptor on undifferentiated PC12 cells exhibits the same pharmacological properties (such as low potency activation by imidazolines, guanidines and CB_1 receptor agonists, and low potency blockade by rauwolscine and CB_1 receptor antagonists) as the release-inhibiting receptor on sympathetic nerve terminals previously denoted as presynaptic non-I_1/non-I_2-imidazoline receptor (Molderings and Göthert, 1999). In view of the absence not only of α_2-adrenoceptors, but also of CB_1 receptors in PC12 cells, the identity of the pharmacological properties suggests that the release-inhibiting receptor may be identical to the cardiovascular presynaptic imidazoline receptor. (3) Since 1-oleoyl-LPA also inhibits noradrenaline release from PC12 cells in a manner sensitive to receptor desensitization by clonidine and to low potency antagonism by CB_1 receptor antagonists and rauwolscine, 1-oleoyl-LPA may also act via such a so-called imidazoline receptor. Furthermore, PC12 cells were found to express mRNA for 1-oleoyl-LPA-activated edg2 and edg7 receptors. Taken together, these results are compatible with the possibility that the release-inhibiting receptors so far known as presynaptic non-I_1/non-I_2-imidazoline receptors are edg-encoded lysophospholipid receptors. More direct support for this hypothesis will be sought in future experiments on cells transfected with cDNAs coding for edg receptors.

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