0026-895X/00/061142-10$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:1142–1151, 2000 /12895/824598

Coupling of I_1 Imidazoline Receptors to the cAMP Pathway: Studies with a Highly Selective Ligand, Benazoline

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Received July 29, 1999; accepted February 9, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Clonidine and benazoline are two structurally related imidazolines. Whereas clonidine binds both to α_2-adrenoceptors (α_2R) and to I_1 imidazoline receptors (I_1R), benazoline showed a high selectivity for imidazoline receptors. Although the α_2R are negatively coupled to adenylate cyclase, no effect on cAMP level by activation of I_1R has been reported so far. We therefore aimed to compare the effects of clonidine and benazoline on forskolin-stimulated cAMP levels in cell lines expressing either I_1R only (PC12 cells), α_2R only (HT29 cells), or I_1R and α_2R together (NG10815 cells). Clonidine proved able to decrease the forskolin-stimulated cAMP level in the cells expressing α_2R and this effect could be blocked by rauwolscine. In contrast, in cells lacking these adrenoceptors, clonidine had no effect. On the other hand, benazoline and other I_1 receptor-selective imidazolines decreased forskolin-stimulated cAMP level in the cells expressing I_1R, in a rauwolscine- and pertussis toxin-insensitive manner. These effects were antagonized by clonidine. According to these results, we demonstrated that 1) α_2R and I_1R are definitely different entities because they are expressed independently in different cell lines; 2) α_2R and I_1R are both implicated in the cAMP pathway in cells (one is sensitive to pertussis toxin and the other is not); and 3) I_1R might be coupled to more then one transduction pathway. These new data will be essential to further understand the physiological implications of the I_1R and the functional interactions between I_1 receptors and α_2-adrenoceptors.

Most imidazolines and related compounds bind both to α-adrenoceptors and to imidazoline-specific receptors (for review, see Regunathan and Reis, 1996). During the last decade, imidazoline-binding proteins different from adrenoceptors have been characterized by extensive binding studies, photoaffinity labeling, purification procedures in different tissues, and immunological analysis with specific antibodies. All these results, taken together, support the existence of a heterogeneous family of imidazoline-specific binding sites/proteins resolved in at least three different subtypes, defined as I_1, I_2, and non-I_1/non-I_2 imidazoline receptors (for review, see Regunathan and Reis, 1996). I_1 receptors corresponding to clonidine high-affinity binding sites have been implicated in blood pressure regulation (Ernsberger and Haxhiu, 1997) as well as in ocular pressure decrease and catecholamine release from chromaffin cells (Regunathan and Reis, 1996). Such I_1 high-affinity binding sites have been detected with tritiated clonidine or iodinated paraiodoclonidine in several models, including bovine brainstem membranes (Heemskerk et al., 1998), human brainstem membranes (Dontenwill et al., 1999), bovine chromaffin cells (Molderings et al. 1993), PC12 cells (Separovic et al., 1996), canine prostate (Felsen et al., 1994), and human platelets (Piletz and Sletten, 1993). Moreover, the subcellular localization of I_1 receptors to the plasma membrane has been assessed in the bovine brainstem (Ernsberger and Shen, 1997; Heemskerk et al., 1998), in the human platelets (Piletz and Sletten, 1993) and in the PC12 cells (Ernsberger et al., 1995; Separovic et al., 1996).

Identification of the transduction pathway associated to the stimulation of I_1 receptors has been approached in different ways. The coupling of I_1 receptors to G proteins has been suggested by the sensitivity of the imidazoline-specific binding to GTP or nonhydrolysable analogs in the canine prostate (Felsen et al., 1994), in the chromaffin cells (Molderings et al., 1993; Ernsberger et al., 1995), and in the bovine brainstem (Ernsberger and Shen, 1997).

Effects of imidazolines on classical second messenger systems of G protein-coupled receptors, either cAMP or inositol-phosphates and diacylglycerol (DAG), have been studied in various models, including rat adrenal glands, bovine chro-

ABBREVIATIONS: DAG, diacylglycerol; PC, phosphatidyl choline; PL, phospholipase; PTX, pertussis toxin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PIC, paraiodoclonidine; PDE, phosphodiesterase; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; $[Ca^{2+}]_i$, intracellular free calcium concentration.

maffin cells, and rat brain. No effect on P_i turnover could be detected with moxonidine and clonidine in adrenal glands or chromaffin cells (Regunathan et al., 1990, 1991). Recently, however, an increase of DAG through activation of a specific phosphatidylcholine-phospholipase C (PC-PLC) was shown for moxonidine in PC12 cells. This effect of moxonidine was blocked by efaroxan, a putative I_1 receptor antagonist (Separovic et al., 1996). Whether this PC-PLC activation is associated with G proteins remains to be determined.

The decrease of cAMP that was observed with clonidine, rilmenidine, or moxonidine in rat brain cortex (Regunathan and Reis, 1994; Regunathan et al., 1995), was clearly caused by the stimulation of α_2-adrenoceptors; however, these imidazolines had no effect on cAMP in tissues that only express I_1 receptors, such as adrenal glands or chromaffin cells (Regunathan et al., 1990, 1991). In the rat brainstem, where α_2-adrenoceptors (Guyenet et al., 1994) and I_1 receptors coexist (Kamisaki et al., 1990), an inhibitory interaction between the two types of receptor was suggested, because no effect on cAMP could be seen with moxonidine or rilmenidine in this tissue (Regunathan and Reis, 1994; Regunathan et al., 1995).

All these studies were performed with hybrid imidazoline ligands (clonidine, rilmenidine, and moxonidine) able to bind to imidazoline receptors and α_2-adrenoceptors. Therefore, we reassessed the capability of imidazolines to affect the cellular cAMP turnover using a highly selective imidazoline receptor ligand, benazoline (Pigini et al., 1997). Three different cell lines expressing either I_1 receptors alone (PC12 cells), I_1 receptors and α_2-adrenoceptors together (NG10815 cells), or α_2-adrenoceptors alone (HT29 cells) were used to compare the effects of benazoline and clonidine on forskolin-stimulated cAMP values. In cells expressing α_2-adrenoceptors (NG10815 cells and HT29 cells), clonidine elicited a decrease of forskolin-stimulated cAMP level by a rauwolscine and pertussis toxin (PTX)-sensitive mechanism as expected for an α_2-adrenoceptor agonist. In contrast, benazoline proved able to dose dependently decrease forskolin-stimulated cAMP content only in cells expressing I_1 receptors (PC12 cells and NG10815 cells). This effect was rauwolscine- and PTX-insensitive. Other I_1 receptor ligands exhibited properties similar to those of benazoline, whereas clonidine acted as an antagonist. We propose, therefore, that the I_1 receptors are negatively coupled to the cAMP pathway.

Experimental Procedures

Cell Cultures. PC12 cells were obtained from Dr. G. Rebel (IRCAD, Strasbourg, France). They were cultured in 75-cm^2 flasks at 37°C with 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM; 1000 mg/ml glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. When the cells reached confluence (3 to 4 days after plating), they were harvested by 1-min exposure to 0.25% trypsin at 37°C. For binding assays, after removing the medium, cells at confluence were frozen in the flasks at −20°C until use to prepare membranes.

NG10815 cells were obtained from Dr. B. Kieffer (ESBS, Illkirch, France). They were cultured in 75-cm^2 culture flasks at 37°C with 10% CO_2 in DMEM (4500 mg/ml glucose) with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and HAT medium (0.1 μM hypoxanthine, 4 μM aminopterin, and 16 μM thymidine). After reaching confluence, cells were harvested for passaging by gentle shaking. For binding assays, cells were harvested at confluence after 24-h incubation in DMEM without FBS, and membranes were prepared immediately.

HT29 cells were obtained from Dr H. Paris (INSERM U338, Toulouse, France) and cultured in 75-cm^2 culture flasks at 37°C with 10% CO_2 in DMEM (4500 mg/ml glucose) with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were harvested at confluence after 48-h incubation in fresh DMEM without FBS, and membranes were prepared immediately.

Membrane Preparations. Frozen PC12 cells were scraped into cold Tris-HEPES buffer (5 mM Tris-HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM $MgCl_2$) and homogenized with a Potter homogenizer. After centrifugation at 75,000*g* for 20 min, the pellet was washed in cold Tris-HEPES buffer and centrifuged again. Pellets were resuspended in Tris-HEPES buffer at 2 to 4 mg protein/ml and used immediately for binding assays.

NG10815 and HT29 cell membrane preparations were obtained after homogenization of the cells in 50 mM cold Tris·HCl buffer containing 5 mM EDTA with a Polytron homogenizer. The homogenate was then centrifuged at 65,000*g* for 25 min and the pellet was washed thrice with Tris·HCl buffer without EDTA. Membrane preparations were stored at −80°C until use.

Binding Assays. Binding assays on PC12 cell membranes were performed with [^{125}I]paraiodoclonidine (PIC). Incubation was initiated by the addition of membranes (200 μg of protein/400-μl final volume) and were carried out at 25°C during 30 min. For saturation experiments, concentrations of [^{125}I]PIC ranging from 0.05 to 5 nM were used. For competition experiments, increasing concentrations of drugs (10^{-10} to 10^{-4} M) were added with 0.5 nM [^{125}I]PIC (corresponding to the K_D value of the radioligand). To stop the incubation, samples were filtered very quickly through GF/B glass fiber filters, incubated for 3 h in 0.03% polyethylenimine with a Brandel harvester, and filters washed twice with 3 ml of 50 mM cold Tris·HCl buffer, pH 7.7. Radioactivity retained on the dried filters was determined in a Minaxi gamma counter (Packard, Meriden, CT). NG10815 membrane binding assays were performed as described in Greney et al. (1999). HT29 membrane binding assays were performed with 0.5 nM [^{125}I]PIC or with 5 nM [^{3}H]clonidine. Incubation was initiated by the addition of membranes (100 μg protein/assay) and were carried out at 25°C during 45 min in a total volume of 400 μl. Assays were then processed as described above and radioactivity retained on the filters determined in a beta TriCarb counter (Packard) or in a Minaxi gamma counter. Nonspecific binding was defined with 10 μM phentolamine for [^{3}H]clonidine and [^{3}H]PIC binding in HT29 cells, using 1 mM phentolamine for [^{3}H]clonidine binding in NG10815 cells and 10 μM BDF6143 for [^{125}I]PIC binding in PC12 cells. Phentolamine is able to bind to both I_1 imidazoline binding sites and α_2-adrenoceptors and was therefore chosen to define nonspecific binding in NG10815 cells and in HT29 cells. BDF6143 was chosen to define nonspecific binding in PC12 cells according to Separovic et al. (1996). Because of the low level of imidazoline binding sites in the PC12 cell membranes (B_{max} = 20 fmol/mg of protein) compared with the NG10815 cell membranes (B_{max} = 320 fmol/mg of protein, Greney et al., 1999), we used [^{125}I]PIC as the radioligand to detect the imidazoline receptors in the former cells.

cAMP Experiments. PC12 cells and HT29 cells at confluence were harvested by mild trypsinization and NG10815 cells were harvested by gentle shaking and centrifuged at 200*g* for 5 min. In a series of experiments, cells were treated with PTX (200 ng/ml culture medium) for 24 h before harvesting. They were washed thrice with DMEM containing 50 mM HEPES without FBS. Cells ($3–5 \times 10^5$ cells/assay) were incubated for 10 min at 37°C in 200 μl of DMEM-HEPES containing 250 μM 3-isobutyl-1-methylxanthine (a nonselective phosphodiesterase inhibitor), 10 μM forskolin, and increasing concentrations of drugs (10^{-9} to 10^{-3} M). The reaction was stopped by 800 μl of ice-cold methanol/formic acid (95:5, v/v) and cells were then sonicated for 5 min. Pellets obtained after centrifugation at 2000*g* for 15 min were discarded and supernatants were used to determine cAMP levels.

Dosage of cAMP was determined by a radioimmunoassay using specific rabbit anti-succinylated cAMP antibodies. Briefly, cAMP contained in the samples was submitted to succinylation by addition of succinic anhydride and incubated with the antibodies (diluted 1:8000) and ^{125}I-cAMP for 18 to 24 h at 4°C. Free radioactivity was separated from bound by absorption on ice-cold activated charcoal (2 mg/ml in 0.1 M phosphate buffer, pH 6.3, in the presence of 2.5 mg/ml BSA) and centrifugation for 20 min at 2000*g*. Radioactivity in the supernatant was counted in a Minaxi gamma counter. Results were extrapolated from a standard curve of cAMP constructed with increasing concentrations of cold cAMP (0.48 to 624 fmol). Alternatively, a radioreceptor assay kit for dosage of cAMP (Amersham, Orsay, France) was used according to the instructions of the manufacturer with similar results.

For experiments with PC12 cell membranes, membranes were prepared according to Hide et al. (1991). Adenylate cyclase assays were conducted in a total volume of 350 μl containing 50 mM Tris·HCl, pH 7.4, 10 μM GTP, 1 U of adenosine deaminase, 5 mM creatinine phosphate, 0.4 mg of creatinine kinase, 0.01 μM cAMP, 250 μM 3-isobutyl-1-methylxanthine, 30 μg BSA, and 5 mM $MgCl_2$. Benazoline was added from stock solution in 50 mM Tris·HCl, pH 7.4. Incubations were conducted for 10 min at 37°C and were initiated by the addition of PC12 cell membranes (about 10 μg) to reaction mixture that had been preincubated for 10 min at 37°C. Reactions were stopped by addition of 1 ml of ice-cold 65% methanol (v/v) solution. After drying the samples in a SpeedVac, dosage of cAMP was effected with a radioreceptor assay kit (Amersham).

Dosage of Phosphodiesterase Activity. Cytosolic cyclic nucleotide phosphodiesterase (PDE) isoforms (PDE1, PDE3, PDE4, and PDE5) were isolated from media layer of bovine aorta by a modification of the methods of Lugnier et al. (1986). Cytosolic PDE2 was isolated from cultured bovine aortic endothelial cells as described previously (Lugnier and Schini, 1990). PDE activities were measured by radioenzymatic assay at a substrate concentration of 1 μM cAMP or cGMP in the presence of 15,000 cpm [^{3}H]cAMP or [^{3}H]cGMP, respectively, as a tracer. The buffer solution was of the following composition: 50 mM Tris·HCl, pH 7.5, 2 mM magnesium acetate, and 1 mM EGTA. PDE1 was assayed in basal state (in presence of 1 mM EGTA) and calmodulin-activated states (with 10 μM $CaCl_2$ and 18 nM calmodulin) using [^{3}H]cGMP as substrate. PDE2 was assayed in basal (without cGMP) and cGMP-activated states (with 5 μM cGMP) using [^{3}H]cAMP. To prevent the influence of cross-contamination between isolated PDE3 and PDE4, the studies performed with [^{3}H]cAMP as substrate were always carried out in the presence of 10 μM rolipram or 100 μM cGMP, respectively. PDE5 was assayed using [^{3}H]cGMP as substrate. Dose-effect curves of PDE activity were made using six concentrations and IC_{50} values were determined using a nonlinear regression analysis with the computer program Prism 2.01 (GraphPAD Software, San Diego, CA). The results are expressed as percentage of inhibition of substrate hydrolysis.

PC-PLC Experiments. PC12 cells were seeded at about 1×10^6 cells/well in a six-well plate in DMEM containing 10% FBS. After 12-h culture at 37°C with 8% CO_2, wells were rinsed thrice with serum-free DMEM and drugs (10^{-6} M) were added for 1 min. The time of stimulation and the drug concentrations were chosen according to the method of Separovic et al. (1996). After three washes with serum- and drug-free DMEM, cells were lysed by addition of 1.5 ml of ice-cold 3 mM 1,4-piperazinediethanesulfonic acid (PIPES), 0.6 mM EDTA, 0.03% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and frozen at −20°C. After thawing at room temperature, lysed cells were scraped out of the wells. Phosphocholine levels were measured according to the protocol of the Amplex Red phosphatidylcholine-specific PLC assay kit (Molecular Probes, Interchim, France). In this enzyme-coupled assay, PC-PLC activity is monitored indirectly by using 10-acetyl-3,7-dihydrophenoxazine (Amplex red reagent), a sensitive fluorogenic probe for H_2O_2. First PC-PLC converts the phosphatidylcholine substrate to form phosphocholine and DAG. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase, reacts with Amplex red reagent in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. Thus 100 μl of a solution containing 400 μM Amplex red reagent, 2 U/ml horseradish peroxidase, 8 U/ml alkaline phosphatase, and 0.2 U/ml choline oxidase was added to each assay tube and fluorescence red in a fluorometer (Perkin-Elmer Cetus, Norwalk, CT) using excitation at 560 nm and emission detection at 590 nm. Fluorescence was recorded from 20 to 40 min after 30-min incubation at room temperature of the samples with the enzyme cocktail. Linear regression was used to determine the fluorescence of each sample at exactly 30-min incubation to accurately compare the values. Basal levels of phosphocholine were determined in samples run in parallel without adding drugs for the 1-min stimulation. Use of the Amplex Red phosphatidylcholine-specific PLC assay kit with cellular extracts can, however, also measure the free choline presumably generated by a PC-PLD. However, the activation of a PC-PLD by imidazoline receptors in PC12 cells has been ruled out by Separovic et al. (1996; see discussion).

Fluorimetric Measurement of Relative Internal Free Ca^{2+} Concentration in PC12 Cells. For determination of changes in internal free Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells, cells were harvested and washed with Ringer's solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 11 mM glucose, pH 7.4. The cells were then loaded with 10 μM fura-red (a fluorescent calcium indicator; Molecular Probes, Eugene, OR) for 45 min at 37°C using the acetoxymethyl ester (AM) derivative of the dye, washed and resuspended in Ringer's solution. Measurements of changes in Ca^{2+} levels in stirred cell suspensions were made using a Perkin-Elmer model LS50B luminescence spectrometer and were expressed as fluorescence emitted at 640 nm in response to excitation at 488 nm (data sampling interval, 0.5 s).

Materials. DMEM medium, FBS, penicillin, and streptomycin were obtained from Life Technologies (Cergy-Pontoise, France). Benazoline was synthetized by Prof. Pigini (Camerino, Italy), BDF6143 was kindly provided by Beiersdorf-Lilly (Hamburg, Germany). Clonidine and rauwolscine were purchased from Research Biochemicals (Bioblock, Strasbourg, France). [^{125}I]PIC (2200 Ci/mmol) and [^{3}H]clonidine (66.5 Ci/mmol) were purchased from New England Nuclear (Paris, France). All other chemicals were from Sigma Chemical (L'Isle d'Abeau Chesnes, France).

Results

Binding Characteristics of Imidazolines for I_1 Receptors and α_2-Adrenoceptors. PC12 cells express imidazoline binding sites corresponding to the I_1 subtype (Separovic et al., 1996). Because this cell line was shown to be very labile according to cell culture conditions, we attempted to characterize more extensively the clone used in our laboratory. For this purpose, saturation experiments were performed with [^{125}I]PIC on cell membrane preparations. In fact, specific binding to membrane receptors was saturable and of high affinity. The specific binding defined by 10 μM BDF6143 exhibited a K_D value of 0.5 nM and a B_{max} value of about 20 fmol/mg of protein. On the other hand, no specific binding could be obtained with 10 μM rauwolscine (an α_2-adrenoceptor antagonist) to define nonspecific binding. Competition experiments with rauwolscine confirmed the absence of α_2-adrenoceptors in this cell line because a K_i value as high as 70 μM ($n = 2$) was obtained for this drug. Imidazolines completely displaced the specific binding of [^{125}I]PIC to PC12 cell membranes. Clonidine and BDF6143 exhibited K_i values of 125 ± 75 nM ($n = 4$) and 28 ± 6 nM ($n = 3$), respectively. The competition curves of benazoline were better resolved by

two compartments, one with a high affinity (K_i = 1.3 nM, 48% of the total sites) and the other with an affinity of 2800 nM (n = 6) (Fig. 1A). Moxonidine also proved able to displace [^{125}I]PIC binding sites with two affinities (34 ± 5 nM and 24 ± 10 μM, respectively; n = 5). The high-affinity binding sites accounted for 59% of the total sites. Efaroxan behaved like moxonidine and benazoline in binding assays; its competition curves appeared biphasic and led to determination of two binding affinities, 144 ± 170 nM (33% of total sites) and 100 μM (n = 5). We checked the effect of a nonhydrolyzable GTP analog, GTPγS, on the competition curve of benazoline

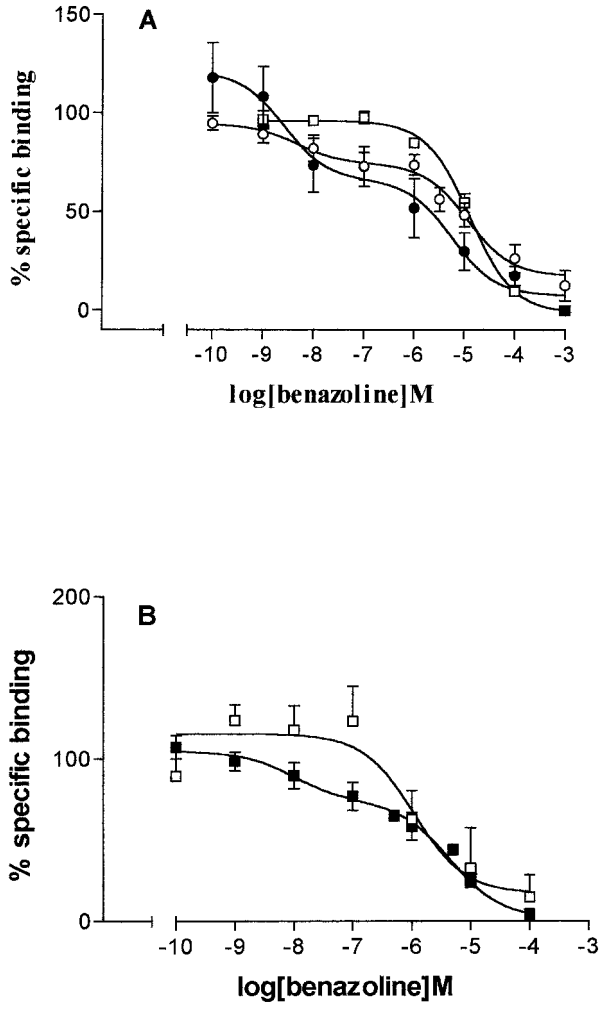


Fig. 1. A, concentration-dependent inhibition of specific I_1R and α_{2A}-R binding by benazoline. Increasing concentrations of benazoline (10^{-10} to 10^{-3} M) were incubated with membrane preparations of HT29 cells (□, α_{2A}-R), NG10815 cells (○, I_1R), and PC12 cells (●, I_1R) in the presence of 5 nM [^{3}H]clonidine, 20 nM [^{3}H]clonidine with 10 μM rauwolscine, and 0.5 nM [^{125}I]PIC, respectively. Nonspecific bindings were determined by 10 μM phentolamine, 1 mM phentolamine, and 10 μM BDF6143, respectively. Total and nonspecific binding were, respectively, 14634 ± 3147 and 6434 ± 2384 dpm on PC12 membranes, 4240 ± 770 and 2170 ± 680 dpm on NG10815 membranes, and 10230 ± 6 and 780 ± 25 dpm on HT29 membranes. Data points are means ± S.D. of three to nine experiments performed in triplicate. B, effect of 100 μM GTPγS on competition curve of benazoline for I_1 receptors in PC12 cells. Binding assays were performed as described under *Experimental Procedures* except that 100 μM GTPγS was included in each tube. Each point represents the mean of six experiments, each conducted in triplicate. In this set of experiments, high-affinity binding sites of benazoline accounted for 30% of total binding sites in the absence of GTPγS and were completely lost in the presence of 100 μM GTPγS. ■, control; □, 100 μM GTPγS.

(Fig. 1B). In the presence of 100 μM GTPγS, the competition curve of benazoline was better resolved by one compartment with an affinity of 1090 ± 1000 nM (n = 4).

HT29 cells express well characterized α_{2A}-adrenoceptors (Bylund et al., 1988). To check the presence of imidazoline binding sites in this cell line, competition experiments were performed with clonidine and noradrenaline on [^{3}H]clonidine and [^{125}I]PIC total binding. Clonidine and noradrenaline proved able to displace the total binding of either radioligand to a similar extent, with IC_{50} values of 1.8 ± 0.8 and 44 ± 10 nM, respectively, on [^{3}H]clonidine binding sites and 51 ± 9 and 153 ± 91 nM on [^{125}I]PIC binding sites. These results demonstrate that specific imidazoline binding sites of the I_1 receptor subtype, different from adrenoceptors, are not expressed in these cells. Competition curve of benazoline on [^{3}H]clonidine-specific binding to α_{2A}-adrenoceptors in this cell line led to the determination of a K_i of 3500 ± 2700 nM (n = 3) (Fig. 1A).

NG10815 cells expressed imidazoline receptors and α_{2B}-adrenoceptors. In these cells, [^{3}H]clonidine proved able to label high-affinity I_1 receptors when the experiments were performed in the presence of 10 μM rauwolscine to mask the α_{2B}-adrenoceptors (Greney et al., 1999). Benazoline completely displaced this imidazoline-specific binding. The competition curve was better resolved by two compartments with K_i values of 2.3 ± 1.8 nM (30% of the sites) and 5700 ± 500 nM, respectively (n = 9) (Fig. 1A).

Effect of Imidazolines on cAMP Level in the Cells. The PC12 cells were incubated with increasing concentrations of imidazolines after stimulation by 10 μM forskolin. Benazoline produced a dose-dependent decrease in forskolin-stimulated cAMP content of the cells. The dose-response curve of benazoline appeared biphasic (P = .02 for the comparison of fits) with EC_{50} values of 2.2 ± 2.1 nM (20% of maximal effect) and 27 ± 18 μM, respectively (n = 8), with a maximal inhibition of 51 ± 11%. Moxonidine and BDF6143 dose dependently decreased the forskolin-stimulated cAMP level in the cells with EC_{50} values of 35 ± 34 nM (n = 5) and 78 ± 8 nM (n = 3), respectively. Maximal inhibition values recorded with moxonidine and BDF6143 were 12 ± 2 and 27 ± 2%, respectively. Efaroxan dose-response curves were better resolved by two compartments (P < .001) with EC_{50} values of 0.4 ± 0.1 nM (14% of maximal effect) and 270 μM, respectively, and maximal inhibition of 56 ± 8%. Conversely, clonidine proved unable to modify significantly (n = 5) the forskolin-stimulated cAMP concentrations under the same conditions (Fig. 2). Clonidine (1 μM), which had no effect on its own, antagonized the decrease of cAMP induced by 1 μM benazoline (n = 4) (Fig. 3). Same antagonism on the effect of 10 μM benazoline was obtained with 10 μM clonidine. As expected from the results mentioned above, efaroxan did not antagonise the benazoline effect (n = 4) (Fig. 3).

When adenosine (20 μM) instead of forskolin was used to stimulate adenylate cyclase in the PC12 cells, no inhibition was obtained with increasing concentrations of benazoline (Fig. 4), suggesting the involvement of specific adenylate cyclase isoforms.

To confirm that the modulation of the forskolin-stimulated cAMP accumulation obtained by imidazolines in the PC12 cells involved membrane receptors, we next examined whether such a modulation could also occur in membrane preparations instead of whole cells. In this case, a monopha-

sic dose-response curve was obtained for benazoline (Fig. 5) with an EC_{50} value of 0.7 ± 0.3 nM (n = 3) and a maximal inhibition of 27 ± 4%.

To ascertain whether I_1 receptor-mediated cAMP decrease was dependent on an increase in intracellular calcium level, PC12 cells were loaded with the fluorescent calcium indicator fura-red, which displays a decrease in fluorescence upon binding of calcium. Application of 10 μM benazoline induced a small but significant increase in the relative level of $[Ca^{2+}]_i$ (Fig. 6). However, at lower concentrations, no significant effect could be observed.

To confirm the effects of imidazolines on cAMP levels we tested them in NG10815 cells known to express I_1 imidazoline receptors as well as α_{2B}-adrenoceptors. As shown in Fig. 7A, benazoline decreased the forskolin-stimulated cAMP content in a dose-dependent manner with an EC_{50} value of 25 ± 0.7 μM and a maximal inhibition of 60 ± 6% (n = 5). Clonidine also decreased forskolin-stimulated cAMP level in these cells with an EC_{50} value of 15 ± 18 nM and a maximal inhibition of 38 ± 3% (Fig. 7B). Because α_2-adrenoceptors were present in these cells, we checked whether the clonidine- and benazoline-induced effects were caused by any activation of these receptors. Rauwolscine (10 μM), an α_2-adrenergic blocking drug, did not significantly modify the effects of benazoline on cAMP levels, but it shifted the dose-response curve of clonidine to the right (EC_{50} = 350 μM; n = 2) (Fig. 7, A and B, respectively).

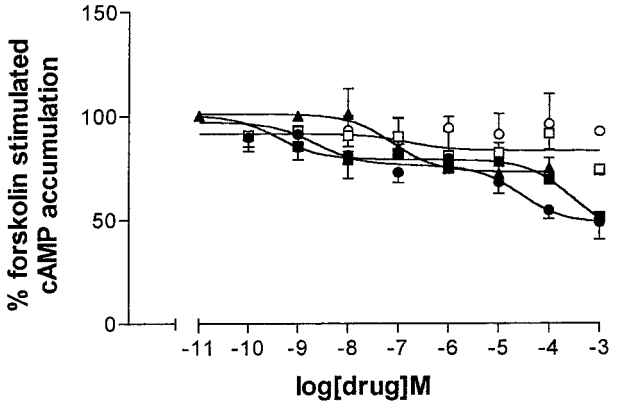


Fig. 2. Dose-dependent inhibition of forskolin-stimulated cAMP production in the PC12 cells by imidazolines. Inhibition curves of forskolin-stimulated cAMP production in PC12 cells by benazoline (●), efaroxan (■), BDF6143 (▲), moxonidine (□), and clonidine (○) are shown. Results from three to eight experiments performed in triplicate were averaged and expressed as percentage of control (in the presence of forskolin alone) ± S.E. —, nonlinear least-squares fit, determined as described under *Experimental Procedures*. Basal cAMP production was 118 ± 16 fmol/min/10^5 cells; forskolin-stimulated cAMP production was 2358 ± 508 fmol/min/10^5 cells.

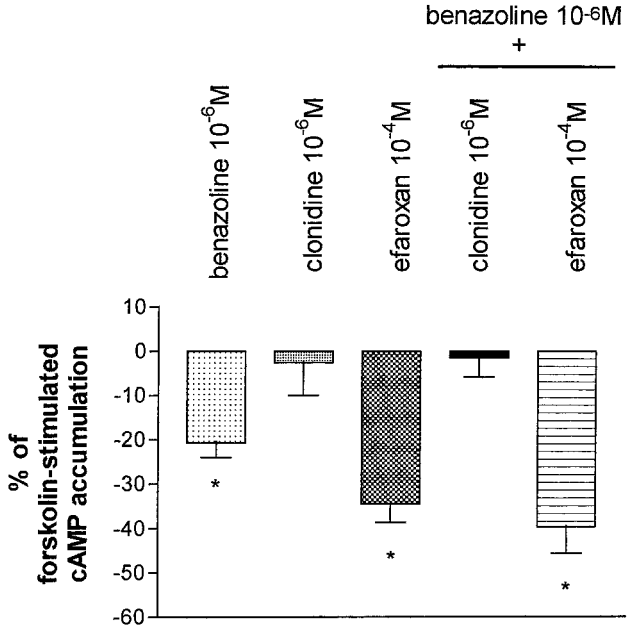


Fig. 3. Effect of clonidine on forskolin-stimulated cAMP level in response to benazoline in PC12 cells. To measure the effect of benazoline, cells (10^5) were incubated with benazoline and forskolin for 10 min at 37°C. For coincubation experiments, cells (10^5) were preincubated with 1 μM clonidine or 100 μM efaroxan in the presence of forskolin for 10 min at 37°C before addition of 1 μM benazoline and a subsequent incubation for 10 min at 37°C. In control experiments of the latter, clonidine and efaroxan were added for 20 min and cAMP content was measured. Results are expressed as percentage ± S.E.M. change from matched controls containing forskolin only. Bars, mean values from at least four separate experiments. * $P < .05$, paired t test; statistically different from control.

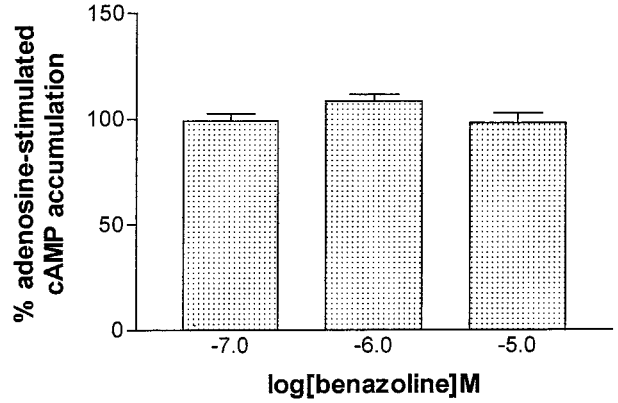


Fig. 4. Effect of benazoline on adenosine-stimulated cAMP level in PC12 cells. Cells (10^5) were incubated with benazoline (10^{-7} to 10^{-5} M) and adenosine (20 μM) for 10 min at 37°C and cAMP measured as described. Results are expressed as percentage ± S.E. of control containing adenosine only. Bars, mean values of three experiments performed in triplicate. Basal cAMP level was 45.3 ± 0.5 fmol/10^5 cells/min and adenosine-stimulated cAMP production was 252 ± 14 fmol/10^5 cells/min.

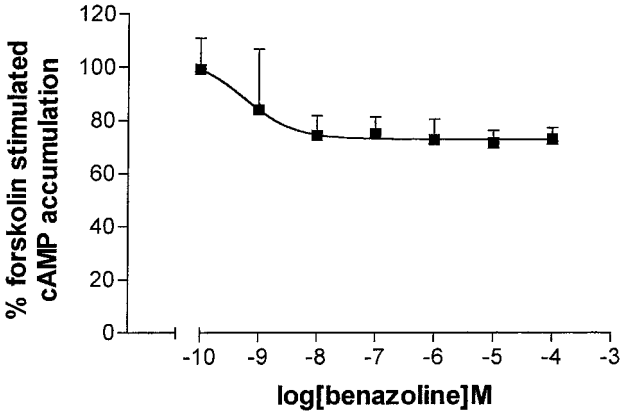


Fig. 5. Effects of benazoline on forskolin-stimulated cAMP accumulation in PC12 cell membranes. PC12 cell membranes (10 μg) were incubated with increasing concentrations of benazoline as described under *Experimental Procedures*. Data were analyzed using a nonlinear regression program (GraphPad). Values are mean ± S.E. of three experiments. Basal value of cAMP was 45 ± 16 pmol/mg protein/min and forskolin-stimulated value was 90 ± 14 pmol/mg protein/min.

These results confirmed that clonidine behaved as an α_2-adrenoceptor agonist in the NG10815 cells on the one hand and that benazoline elicited its effect on the cAMP content of the cells by an α_2-adrenoceptor-independent mechanism on the other hand.

Because α_{2A}-adrenoceptors are naturally expressed in HT29 cells without coexpression of imidazoline receptors, we used this cell line to further confirm the above results. In these cells, no significant effect on forskolin-stimulated cAMP accumulation could be recorded with increasing concentrations of benazoline (Fig. 8A). Moreover, the addition of 10 μM rauwolscine did not unmask any effect of benazoline (Fig. 8A). Clonidine decreased the forskolin-stimulated cAMP level with an EC_{50} value of 10 ± 3 nM and a maximal inhibition of 50 ± 5% (n = 3). Rauwolscine (10 μM) shifted the dose-response curve of clonidine to the right (EC_{50} = 40 ± 16 μM, maximal inhibition of 70 ± 8%; n = 2), demonstrating that the effect of clonidine was obviously mediated by the activation of α_{2A}-adrenoceptors (Fig. 8B).

The α_2-adrenoceptors are coupled to PTX-sensitive G proteins. When NG10815 cells were pretreated with PTX (200 ng/ml) for 24 h, the dose-response curve of benazoline was shifted to the left [EC_{50} = 25 μM without PTX (n = 5) and 2.8 μM with PTX (n = 3)] without change of the maximal cAMP decrease (maximal inhibition with PTX pretreatment, 57 ± 5%), suggesting that this effect did not depend on $G_{i/o}$ activation (Fig. 9A). On the other hand, similar PTX pretreatment markedly affected the effect of the α_2-adrenoceptor agonist clonidine in these cells (Fig. 9A). When PC12 cells were pretreated with PTX (200 ng/ml) during 24 h, the dose-response curve of benazoline was not significantly changed (Fig. 9B), confirming that this effect was independent of $G_{i/o}$ protein activation.

To preclude a direct effect of benazoline on PDE enzymes, experiments were conducted on purified PDE isoforms. Table 1 shows that in contrast with their respective specific inhibitors, the various PDE isoforms were not significantly activated or inhibited by 100 μM benazoline. Similar results were obtained with 10 μM benazoline (data not shown).

It has been shown (Separovic et al., 1996, 1997) that moxonidine activated a PC-PLC in the PC12 cells, leading to an increase of DAG and phosphocholine levels and that these effects involved activation of I_1 receptors, because they were blocked by efaroxan. We aimed to determine whether this transduction pathway was also associated to the I_1 receptors in the cell line used in the present study. In fact, similar results were obtained as moxonidine (1 μM) increased (after 1 min of stimulation) the phosphocholine content of the cells (37 ± 8% above control, n = 4), although efaroxan and BDF6143 proved inactive (Fig. 10). Moreover, benazoline (1 μM) significantly increased the phosphocholine level in these cells (57 ± 23% above control, n = 4), suggesting that, like moxonidine, it behaved as an agonist on this pathway (Fig. 10).

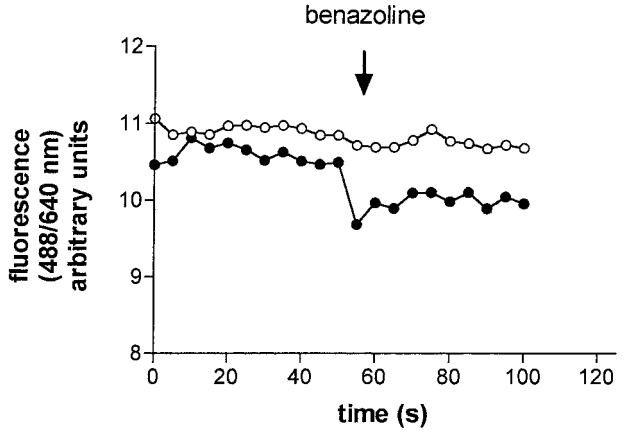


Fig. 6. Effect of benazoline on $[Ca^{2+}]_i$ in PC12 cells. PC12 cells were loaded with fura-red for 45 min at 37°C. Fluorescence emitted was recorded at 640 nm in response to excitation at 488 nm. One representative experiment of four is shown. Benazoline [10 μM (filled symbol) or 100 nM (open symbol)] was added to stirred cells at 60 s of incubation.

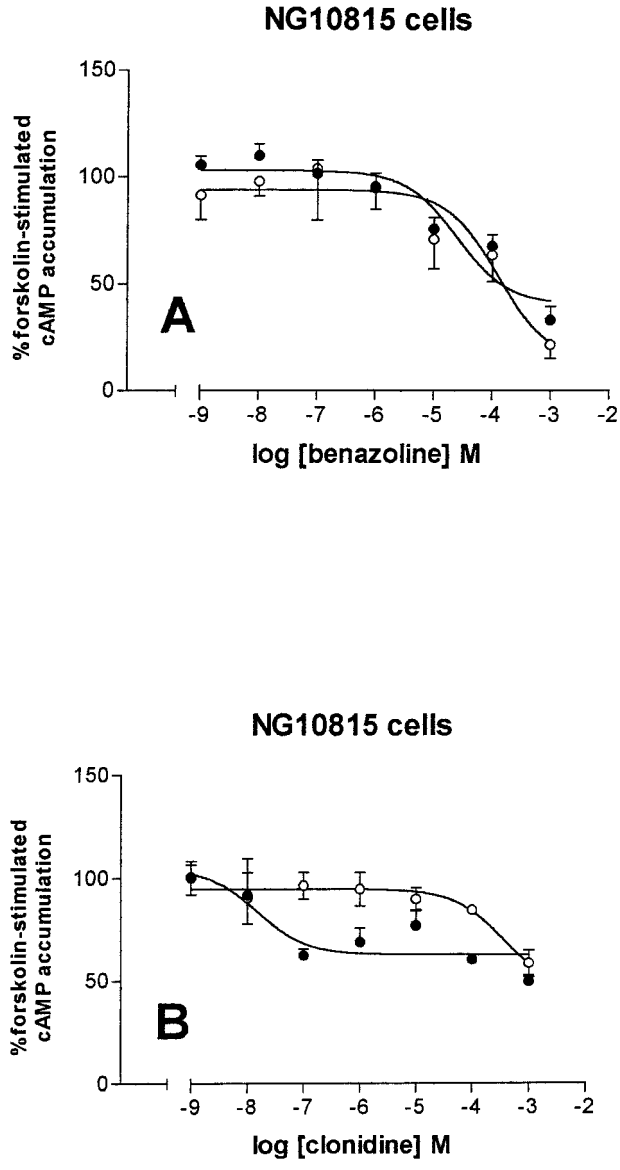
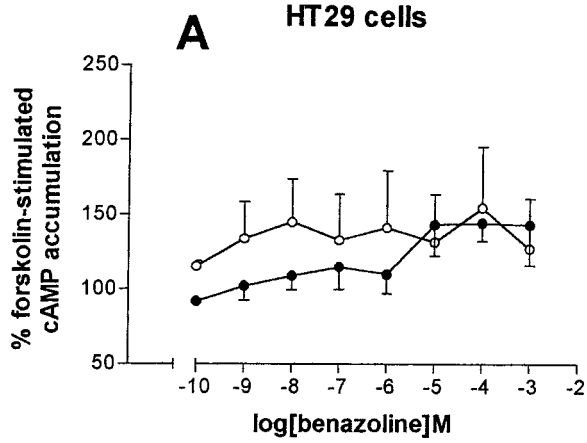


Fig. 7. Inhibition of forskolin-stimulated cAMP production in the NG10815 cells. Inhibition of forskolin-stimulated cAMP production by benazoline (A) and by clonidine (B) is shown. A, cells were incubated with increasing concentrations of benazoline only (●) or in the presence of 10 μM rauwolscine (○). B, cells were incubated with increasing concentrations of clonidine only (●) or in the presence of 10 μM rauwolscine (○). Results from two to five experiments performed in triplicate were averaged and expressed as percentage of control (with forskolin only) ± S.E. —, nonlinear least-squares fit, determined as described under *Experimental Procedures*. Basal cAMP production was 433 ± 186 fmol/min/10^5 cells; forskolin-stimulated cAMP production was of 8600 ± 2300 fmol/min/10^5 cells.

Discussion

The aim of this study was to get further insight into the transduction pathway(s) of I_1 receptors. We took advantage of the availability of a newly described selective imidazoline receptor ligand, benazoline, to reexamine the influence of imidazolines on the cAMP pathway in cells. Regunathan et al. (1991) clearly showed that clonidine, although able to bind to I_1 imidazoline receptors and to α_2-adrenoceptors, was unable to modulate cAMP levels in bovine adrenal chromaffin cells expressing I_1 receptors without coexpression of α_2-adrenoceptors. Nevertheless, we examined the effect of benazoline in PC12 cells (derived from a rat adrenal pheochromocytoma) and in two other cell lines, the NG10815 and HT29 cells on this pathway. In addition, we tested the capability of other imidazolines (including clonidine) known to interact with I_1 binding sites to modulate the cAMP pathway in these cells. All the results presented here confirm the hypothesis that benazoline can negatively modulate the forskolin-stimulated cAMP accumulation through the activation of I_1 imidazoline receptors and that clonidine behaves in this pathway as an antagonist.



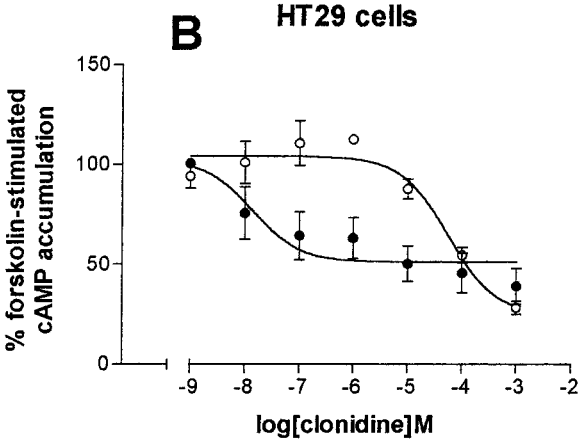
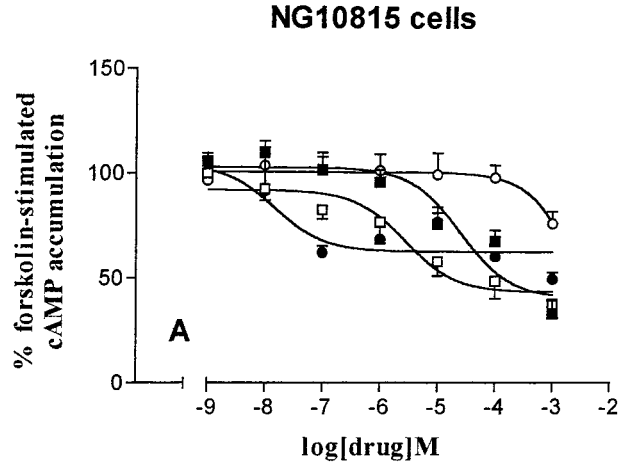


Fig. 8. Inhibition of forskolin-stimulated cAMP production in HT29 cells. Inhibition of forskolin-stimulated cAMP production in HT29 cells by benazoline (A) or clonidine (B). A, cells were incubated with increasing concentrations of benazoline only (●) or in the presence of 10 μM rauwolscine (○). B, cells were incubated with increasing concentrations of clonidine only (●) or in the presence of 10 μM rauwolscine (○). Results from two to five experiments performed in triplicate were averaged and are expressed as percentage of control (in the presence of forskolin only) ± S.E. —, nonlinear least-squares fit, determined as described under *Experimental Procedures*. Basal cAMP production was 49.6 ± 7.9 fmol/min/10^5 cells; forskolin-stimulated cAMP production was of 3526 ± 1200 fmol/min/10^5 cells.



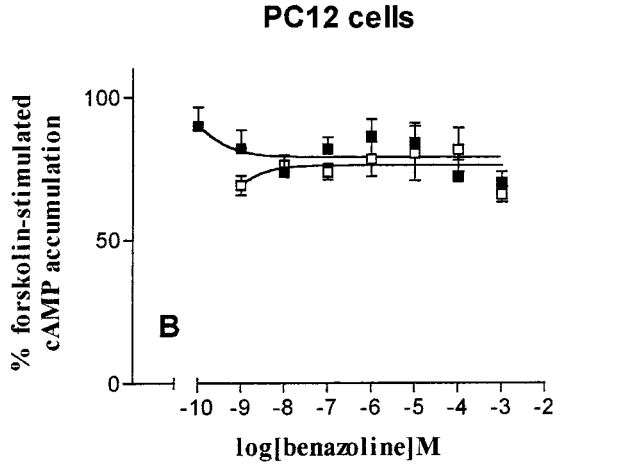


Fig. 9. Effect of imidazolines on forskolin-stimulated cAMP production after PTX pretreatment of the cells. Inhibition of forskolin-stimulated cAMP production by benazoline and clonidine without (■, ●) or with (□, ○) PTX (200 ng/ml during 24 h) pretreatment in NG10815 cells (A) or in PC12 cells (B). Results are from three experiments performed in triplicate and expressed as the percentage of control values (with forskolin only). In NG10815 cells, basal values of cAMP were 22 ± 7 fmol/min/10^5 cells and 74 ± 33 fmol/min/10^5 cells for control and PTX-treated cells, respectively, and forskolin-stimulated cAMP values were 1900 ± 640 fmol/min/10^5 cells and 1900 ± 780 fmol/min/10^5 cells for control and PTX-treated cells, respectively. In PC12 cells, basal values of cAMP were 126 ± 23 fmol/min/10^5 cells and 113 ± 27 fmoles/min/10^5 cells for control and PTX-treated cells, respectively, and forskolin-stimulated cAMP values 1130 ± 73 fmol/min/10^5 cells and 940 ± 126 fmol/min/10^5 cells for control and PTX-treated cells, respectively.

Imidazoline and Adrenergic Receptors Expressed by the Different Cell Lines Used in This Study. We have shown for the first time in this study that HT29 cells, expressing the α_{2A}-subtype of human adrenoceptors only (Bylund et al., 1988) did not express simultaneously the I_1 subtype of imidazoline binding sites labeled either by tritiated clonidine or iodinated PIC. It was previously reported that no I_2 imidazoline binding sites could be detected in these cells with [^{3}H]idazoxan (Cantiello and Lanier, 1989). Therefore, this cell line represents a uniquely suitable model to study the α_{2A}-adrenoceptors in the absence of the two subtypes of imidazoline receptors.

The second cell line used in this study, the NG10815 cells, expressed α_{2B}-adrenoceptors (Bylund et al.,1988), I_1 receptors, and I_2 binding sites as demonstrated by binding studies using [^{3}H]rauwolscine, [^{3}H]clonidine in the presence of 10 μM rauwolscine, and [^{3}H]idazoxan, respectively, as the radioligands (Greney et al., 1999).

PC12 cells were described previously as cells lacking α_2-adrenoceptors in binding assays using *p*-[^{125}I]iodoclonidine (Separovic et al., 1996) and in hybridization studies with adrenoceptor cDNA probes (Duzic and Lanier, 1992). We confirmed that it was also the case in the PC12 cell line used in this study, because rauwolscine, an α_2-adrenoceptor antagonist, did not displace the *p*-[^{125}I]iodoclonidine-specific binding with high affinity. On the other hand, imidazolines proved able to completely displace *p*-[^{125}I]iodoclonidine binding with high affinities. A high affinity for I_1 imidazoline receptors of PIC was described in the bovine brainstem (Heemskerk et al. 1998) and in the human platelets (Piletz and Sletten, 1993). The K_D and B_{max} values determined in our study for PIC were in close agreement with those described previously by Separovic et al. (1996) in the same cell line. The imidazoline binding sites detected in the PC12 cells were clearly different from I_2 binding sites, because clonidine, moxonidine, efaroxan, and BDF6143 displayed high affinities in this model, as was the case for I_1 receptors in human platelets (Piletz and Sletten, 1993) and in bovine chromaffin cells (Molderings et al., 1993), although they were only weak ligands for I_2 binding sites (Bricca et al., 1993; Piletz and Sletten, 1993). On the other hand, no specific high-affinity [^{3}H]idazoxan I_2 binding sites could be detected in the PC12 cells (Steffen et al., 1995). Thus PC12 cells represent an interesting model with which to study the I_1 receptors in the absence of α_2-adrenoceptors and of I_2 binding sites. The existence of cell lines expressing α_2-adrenoceptors in the absence of imidazoline receptors (HT29 cells) on the one hand and I_1 imidazoline receptors in the absence of α_2-adrenoceptors (PC12 cells) on the other hand definitely confirms that these receptors are different molecular entities.

TABLE 1

Effect of 100 μM benazoline on purified PDE isoforms

Experiments have been conducted as described under *Experimental Procedures*. The results are given in percentage inhibition compared with the inhibitory effects (EC_{50}) of the specific inhibitors of each PDE isoform. Results are the mean of three experiments. The standard error was <15%.

	100 μM Benazoline	Inhibitor	IC_{50}
	%		μM
Basal PDE1	11	Nimodipine	2.8
Activated PDE1	4		3.2
Basal PDE2	6	EHNA	N.S.
Activated PDE2	13		3.4
PDE3	16	CI930	0.8
PDE4	4	Rolipram	1.1
PDE5	13	Zaprinast	0.7

N.S., IC_{50} > 100 μM.

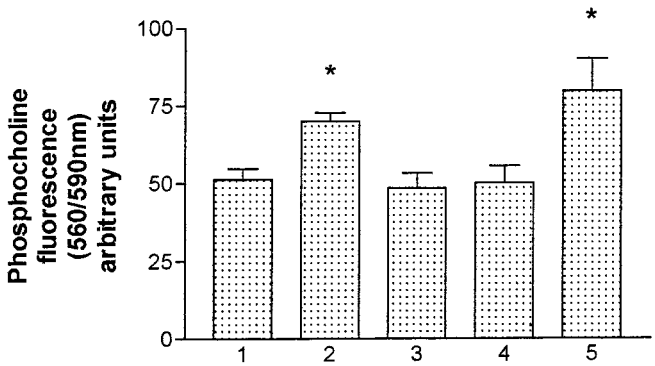


Fig. 10. Effect of imidazolines on the accumulation of phosphocholine in the PC12 cells. Cells were incubated with drugs (10^{-6} M) for 1 min. The results were determined from four experiments, each performed with separate cell cultures, and are shown as values of fluorescence (excitation at 560 nm and emission detection at 590 nm) red after 30-min development of the enzymatic reaction as described under *Experimental Procedures*. *$P < .05$ by nonparametric paired Mann-Whitney *U* test; significantly different from vehicle-treated control. 1, basal; 2, moxonidine; 3, efaroxan; 4, BDF6143; 5, benazoline.

We also confirmed that benazoline is a ligand that is highly selective for imidazoline receptors over α_2-adrenoceptors, as shown previously (Pigini et al., 1997). In addition, competition curves obtained on I_1 receptor bindings with benazoline, moxonidine, and efaroxan were best resolved in two compartments, one displaying a high affinity and the other a low affinity for these drugs. Resolution of I_1 binding sites in two compartments were also shown in human platelets (Piletz et al., 1996) and in bovine chromaffin cells (Molderings et al., 1993) defined either by *p*-[^{125}I]iodoclonidine binding or by [^{3}H]clonidine binding, respectively. However, our results differed from those of Separovic et al. (1996) in that they detected only the first high-affinity binding site for moxonidine and efaroxan in their PC12 cells. This discrepancy could be attributable to the fact that we used whole-cell membrane preparations in contrast with the purified plasma membranes in their binding studies.

I_1 Receptors Are Coupled to the cAMP Pathway. Clonidine decreased forskolin-stimulated cAMP in the cell lines expressing either α_{2A}- or α_{2B}-adrenoceptors (HT29 and NG10815 cells, respectively) as described previously (Bousquet et al., 1985; Convents et al., 1989). In PC12 cells, which express I_1 receptors able to bind clonidine (K_i = 125 ± 75 nM) but did not express α_2-adrenoceptors, this drug had no influence on the forskolin-stimulated cAMP accumulation. Similar results were reported previously in the rat adrenal gland (Regunathan et al., 1990) and bovine chromaffin cells (Regunathan et al., 1991).

In contrast, benazoline decreased forskolin-stimulated cAMP levels in the cell lines expressing I_1 receptors. These effects can hardly be attributed to the stimulation of α_2-adrenoceptors, which are negatively coupled to adenylate cyclase through the activation of $G_{i/o}$ proteins, because 1) rauwolscine, an α_2-adrenoceptor antagonist, was unable to antagonize the effects of benazoline, although it antagonized those of clonidine in NG10815 cells and in HT29 cells; and 2) pretreatment of cells by PTX, which inhibited $G_{i/o}$ proteins by

ADP-ribosylation, had no effect on cAMP decrease elicited by benazoline, although such a pretreatment abolished the activity of the α_2-adrenoceptor agonist clonidine; and, finally, 3) similar effects of benazoline could be recorded in NG10815 cells, which express α_2-adrenoceptors, and in PC12 cells, which do not.

In addition, benazoline is a ligand selective for imidazoline receptors, and its ability to dose dependently decrease the forskolin-stimulated cAMP level was observed only in cell lines expressing I_1 receptors. In fact, in cells lacking the I_1 receptors (HT29 cells), such a decrease was never observed. It was tempting, therefore, to propose that benazoline acted through the activation of I_1 receptors. This hypothesis was further confirmed by four lines of evidence: 1) other ligands (moxonidine, efaroxan, and BDF6143), shown to be I_1-selective ligands looked like benazoline; 2) clonidine (a high-affinity I_1 receptor ligand), which was devoid of any effect by itself, behaved as an antagonist in the PC12 cells; 3) benazoline activity took place in I_1 receptors containing membrane preparations; and 4) benazoline acted as an agonist on the PC-PLC pathway described as an I_1 receptor transduction mechanism (Separovic et al.,1996, 1997). In addition, the EC_{50} values recorded for the drugs on cAMP accumulation fit with the IC_{50} values obtained for the high-affinity binding sites (at least in the PC12 cells).

Another transduction pathway for the I_1 receptors in the PC12 cells has been proposed previously (Separovic et al., 1996, 1997). These authors clearly showed that moxonidine increased the DAG and phosphocholine levels in the cells by activation of a PC-PLC. We confirmed these results in the cell line used in our laboratory, because moxonidine increased the level of phosphocholine, which was formed by hydrolysis of PC through activation of PC-PLC. Although we cannot completely exclude an activation of a PC-PLD by benazoline (see *Experimental Procedures*), benazoline behaved like moxonidine in these experiments. These results are strongly in favor of the association of two transduction pathways with the I_1 receptors (again, at least in the PC12 cells). Interestingly, in the case of the I_1 receptor, although benazoline and moxonidine were agonists for both pathways, some agonists for the cAMP pathway (efaroxan) had antagonist effects on the PC-PLC pathway (Separovic et al., 1996). Recently, similar data were reported for the β3-adrenoceptor (Gerhardt et al., 1999). Our data open a new field of investigations aiming to determine the existence of a cross talk between the different transduction pathways and to identify their respective contributions to the physiological roles of the I_1 receptors.

We further characterized the cAMP transduction pathway associated with the I_1 receptors in the PC12 cells with benazoline. One of the questions addressed was the coupling of the I_1 receptors to G proteins. It has been shown that I_1 binding sites were sensitive to nonhydrolyzable analogs of GTP (Ernsberger and Shen, 1997). We confirmed these data and showed that benazoline behaved as a G protein-coupled receptor agonist in binding assays. In addition, benazoline also decreased cAMP in membrane preparations, suggesting that the cAMP signaling passed through the activation of an inhibitory G protein insensitive to PTX (Ho and Wong, 1998). Further work is needed to explore this hypothesis.

An intracellular target for imidazolines implicated in the cAMP pathway in whole cells might also exist. Benazoline and efaroxan decreased the cAMP level in whole PC12 cells with biphasic dose-response curves, although in membrane preparations, the second low-affinity compartment was lost. We showed that this putative intracellular target could not be the PDE enzymes. One explanation for the biphasic dose-response curves obtained with some ligands might be the existence of a cross talk between the cAMP and PC-PLC pathways using the intracellular machinery, leading to complex effects in whole cells. Alternatively, the increase of intracellular Ca^{2+} observed with high concentrations of benazoline might be involved in the second part of the dose-response curves. Additional work is required to understand these mechanisms.

In conclusion, we showed in this study that some ligands selective for I_1 receptors decreased forskolin-stimulated cAMP content in cells expressing these receptors and that clonidine proved able to antagonize these effects. The present results strongly support the hypothesis according to which I_1 receptors might be negatively linked with the cAMP pathway and that such a pathway coexists with the activation of a PC-PLC. Although a decrease of the cAMP level in cells can also be achieved by activation of α_2-adrenoceptors, we clearly demonstrated that the two receptors are different molecular entities acting through different mechanisms and in different cell lines. Our work may open new perspectives for the understanding of the transduction mechanism(s) and the physiological implications of the I_1 receptors.

Acknowledgments

We are grateful to Dr. B. Bucher (UMR Centre National de la Recherche Scientifique 7519, Strasbourg, France), who provided us with the specific anti-cAMP antibodies.

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