α_2-Adrenoreceptors Profile Modulation and High Antinociceptive Activity of (*S*)-(−)-2-[1-(Biphenyl-2-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole

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A number of derivatives structurally related to cirazoline (**1**) were synthesized and studied with the purpose of modulating α_2-adrenoreceptors selectivity versus both α_1-adrenoreceptors and I_2 imidazoline binding sites. The most potent α_2-agonist was 2-[1-(biphenyl-2-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole (**7**), whose key pharmacophoric features closely matched those found in the α_2-agonist 2-(3-*exo*-(3-phenylprop-1-yl)-2-*exo*-norbornyl)amino-2-oxazoline (**15**).[30] (*S*)-(−)-**7** was the most potent of the two enantiomers, confirming the stereospecificity of the interaction with α_2-adrenoreceptors. This eutomer was tested on two algesiometric paradigms and, because of the interaction with α_2-adrenoreceptors, showed a potent and long-lasting antinociceptive activity, since it was abolished by the selective α_2-antagonist RX821002.

Introduction

Adrenoreceptors are membrane proteins belonging to the superfamily of G-protein-coupled receptors and are pharmacologically divided into α_1-, α_2-, and β-types.[1] For a long time, they have been attractive therapeutical targets because they serve a large variety of physiological actions such as regulation of the cardiovascular, bronchial, and gastrointestinal apparatuses.[2]

Very often, the use of new ligands as valuable pharmacological tools has been limited because of lack of selectivity toward different receptor systems and/or receptor subtypes. This is frequently observed in the search for α_2-adrenoreceptor ligands, whose structural features may be compatible with the recently discovered imidazoline binding sites.[3] There is indeed a large number of compounds able to interact with both α_2-adrenoreceptors and imidazoline binding sites, as, for example, clonidine, moxonidine, oximetazoline, idazoxan, and rilmenidine.[4] Therefore, in the search for α_2-adrenergic ligands, potentially useful in the treatment of various pathological states such as hypertension, shock, opiate withdrawal, attention deficit disorder, and glaucoma,[5] the reduction of the affinity at imidazoline binding sites can be useful in improving selectivity.

It has recently been shown that α_2-adrenergic agonists induce a potent and dose-dependent antinociceptive action, exerted through inhibition of the release of algogen neurotransmitters such as substance P and glutamate and not by the activation of μ-opioid receptors.[6] This proves to be very beneficial because the unwanted side effects associated with opioid ligands, such as respiratory depression, inhibition of gastric motility, and, more important, risk of abuse and dependence, are highly reduced.

α_2-Adrenoreceptors are a heterogeneous population, and at present three different subtypes, α_{2A}, α_{2B}, and α_{2C}, have been identified and each of them seems to mediate specific physiological functions.[7] For example, activation of the α_{2A} subtype mediates the hypotensive and sedative effects and inhibits neurotransmitter release, while activation of the α_{2B} subtype mediates vasoconstriction. Finally, the α_{2C} subtype participates in many central nervous system processes, and analogously to the α_{2A} subtype, it controls the presynaptic inhibition of norepinephrine release. It has recently been suggested that the α_{2A} subtype may primarily mediate also the antinociception effect, as revealed by the findings that in knock-out mice lacking the α_{2A} subtype, α_2-adrenoreceptor agonists do not induce any analgesic effect.[8a,b]

In a previous paper, we showed that it is possible to modulate α_1- and α_2-adrenoreceptor activity and selectivity by slight modifications of the aromatic substituent and the oxymethylene bridge of cirazoline (**1**).[9] To obtain selective α_2-adrenoreceptor agonists, if possible with antinociceptive activity, we designed **6**−**9**, **12** (Chart 1), and the two enantiomers of compound **7**. To make relevant structure−activity relationships, pharmacological tests have also been extended to the structurally related imidazolines **2**,[9−11] **3**,[12,13] **4**,[13,14] **5**,[13,15] **10**,[11,16] and **11**.[16] Besides, the pharmacological evaluation includes determination of the affinity at I_2 imidazoline binding sites because they also seem to be involved in nociception modulation.[17]

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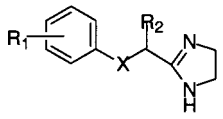
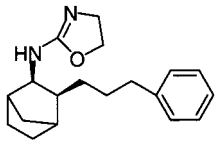
Chart 1



1, R_1=2-cyclopropyl, R_2=H, X=O
2, R_1=R_2=H, X=O
3, R_1=H, R_2=CH_3, X=O
4, R_1=2-phenyl, R_2=H, X=O
5, R_1=2-benzyl, R_2=H, X=O
6, R_1=2-benzyl, R_2=CH_3, X=O
7, R_1=2-phenyl, R_2=CH_3, X=O
8, R_1=3-phenyl, R_2=CH_3, X=O
9, R_1=4-phenyl, R_2=CH_3, X=O
10, R_1=R_2=H, X=NH
11, R_1=2-phenyl, R_2=H, X=NH
12, R_1=2-phenyl, R_2=CH_3, X=NH



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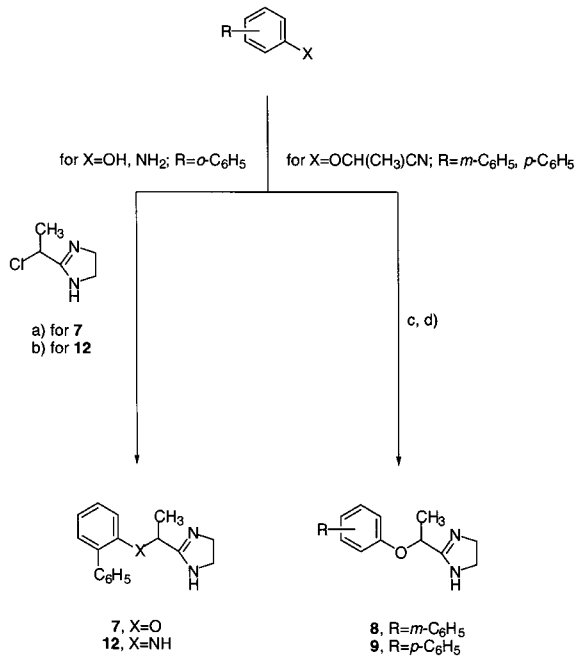
Scheme 1[a]



[a] Reagents: (a) Na/EtOH; (b) Δ/NaI; (c) HCl/MeOH; (d) $H_2NCH_2CH_2NH_2$/MeOH.

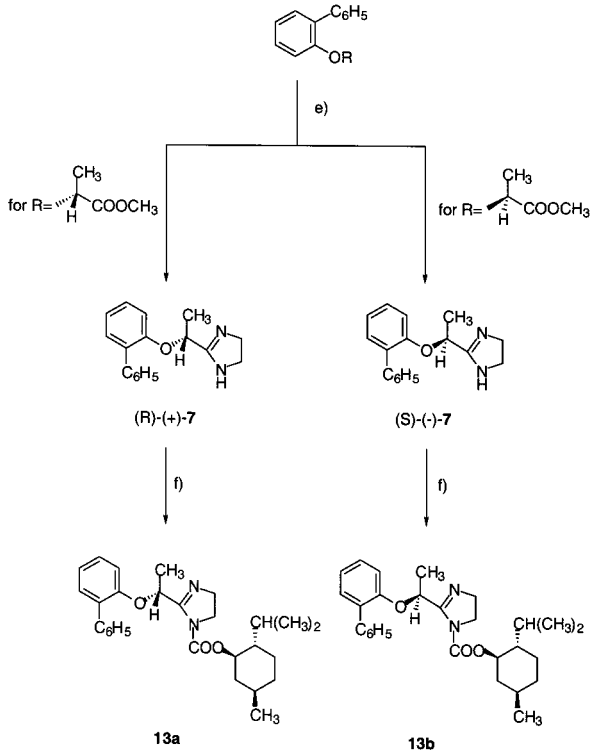
Chemistry

Compounds **7**−**9** and **12** were synthesized according to standard methods by alkylation of the appropriate substituted biphenyls with 2-(2-chloroethyl)imidazoline hydrochloride[18] or by reaction of the appropriate propionitriles[19a,b] with ethylenediamine (Scheme 1).

The two enantiomers (*R*)-(+)-**7** and (*S*)-(−)-**7** were prepared, respectively, by treatment of (*R*)-(−)- and (*S*)-(+)-2-(biphenyl-2-yloxy)propionic acid methyl esters[20] with ethylenediamine in the presence of $Al(CH_3)_3$ (Scheme 2).

Enantiomeric purity of the corresponding propionic acids, obtained by saponification of the above-mentioned

Scheme 2[a]



[a] Reagents: (e) $H_2NCH_2CH_2NH_2/Al(CH_3)_3$/toluene; (f) (−)-menthyl chloroformate.

esters, was determined by ^{1}H NMR spectroscopy, comparing their spectra with that of racemic acid in the presence of quinine. It was found to be >98% for both enantiomers. The spectrum of racemic acid in the presence of quinine showed a double quartet at δ 4.64 ppm for the CHCO proton, whereas only one quartet was observed for (*S*)-(+)- and (*R*)-(−)-2-(biphenyl-2-yloxy)propionic acid at δ 4.65 and 4.61 ppm, respectively.

Unfortunately, the subsequent reaction produced a partial racemization and the final imidazolines (*R*)-(+)-**7** and (*S*)-(−)-**7** possessed an enantiomeric purity of <98%, which was determined by HPLC and ^{1}H NMR spectroscopy of their corresponding diastereomeric carbamates **13a** and **13b**, obtained by reacting (*R*)-(+)-**7** and (*S*)-(−)-**7** with (−)-menthyl chloroformate. Therefore, the two final imidazolines [(*R*)-(+)-**7** and (*S*)-(−)-**7**] were subsequently resolved by fractional crystallization of the (+)-di-*O*,*O*′-*p*-toluyl-D-tartrate and (−)-di-*O*,*O*′-*p*-toluyl-L-tartrate salts, respectively; their enantiomeric purity, determined by HPLC and further confirmed by ^{1}H NMR spectroscopy of their corresponding diastereomeric carbamates **13a** and **13b**, was found to be >98% for both enantiomers. Concerning ^{1}H NMR spectroscopy, the difference of chemical shifts of the OCHCH_3 protons was quite evident and of diagnostic value; the (−)-menthyl derivative of (*R*)-(+)-**7**, **13a**, showed a doublet at δ 1.54, whereas for the (−)-menthyl derivative of (*S*)-(−)-**7**, **13b**, the corresponding signal was at δ 1.52.

Finally, imidazoline **6** was synthesized according to the procedure described for enantiomers (*R*)-(+)-**7** and (*S*)-(−)-**7**, starting from 2-(2-benzylphenoxy)propionic acid methyl ester **14**, which was obtained by alkylation of 2-hydroxydiphenylmethane with 2-bromopropionic acid methyl ester.

Table 1. Binding Affinities (pK_i [a]) and Functional Activities (pD_2,[b] pK_b [c]) of Compounds **2**−**12** and Enantiomers (*R*)-(+)-**7** and (*S*)-(−)-**7**

compd	pK_i I_2	pK_i α_2	pD_2 α_2 (ia)[d]	pD_2 α_1 (ia)[d]	α_2/α_1[e]	α_2/I_2[f]
2	9.05 ± 0.15	7.28 ± 0.16	pK_b 6.71 ± 0.10	5.26 ± 0.17 (α = 0.95)		0.017
3	5.57 ± 0.11	7.01 ± 0.08	pK_b 7.29 ± 0.12	5.20 ± 0.07 (α = 0.75)		27.5
4	6.52 ± 0.13	7.10 ± 0.15	8.46 ± 0.06 (α = 1)	6.68 ± 0.08 (α = 0.76)	60.3	3.8
5	6.70 ± 0.09	6.52 ± 0.17	pK_b 7.14 ± 0.10	5.27 ± 0.20 (α = 0.39)		0.66
6	5.12 ± 0.10	6.80 ± 0.03	pK_b 6.87 ± 0.15	5.99 ± 0.20 (α = 0.61)		47.9
7	5.79 ± 0.19	7.91 ± 0.05	8.52 ± 0.10 (α = 1)	7.20 ± 0.09 (α = 0.66)	20.9	131.8
(*R*)-(+)-**7**	4.31 ± 0.06	7.00 ± 0.08	7.40 ± 0.15 (α = 0.6)	6.23 ± 0.12 (α = 0.61)	14.8	489.8
(*S*)-(−)-**7**	5.14 ± 0.08	7.50 ± 0.13	8.55 ± 0.09 (α = 1)	7.51 ± 0.04 (α = 0.7)	11.0	229.1
8	6.57 ± 0.18	6.92 ± 0.15	6.66 ± 0.11 (α = 0.32)	4.53 ± 0.20 (α = 0.45)	134.9	2.2
9	6.74 ± 0.07	7.50 ± 0.12	pK_b 5.81 ± 0.08	pK_b 5.92±0.06	0.78	5.8
10	7.48 ± 0.16	7.14 ± 0.09	pK_b 6.38 ± 0.05	4.89 ± 0.23 (α = 1)		0.46
11	6.09 ± 0.15	6.45 ± 0.04	7.84 ± 0.06 (α = 1)	6.37 ± 0.18 (α = 0.74)	29.5	2.3
12	4.9 ± 0.06	7.00 ± 0.07	8.36 ± 0.15 (α = 1)	6.73 ± 0.08 (α = 0.68)	42.7	125.9

[a] pK_i affinity values for I_2 imidazoline binding sites and α_2-adrenoreceptors were assessed by measuring the ability of the test compounds to displace [^{3}H]idazoxan (rabbit kidney membranes) and [^{3}H]clonidine (rat cortex membranes), respectively. [b] pD_2 is −log ED_{50}, where ED_{50} is the concentration required to produce 50% inhibition of the twitch response. [c] pK_b values, calculated according to van Rossum,[23] using (−)-norepinephrine (α_1) or clonidine (α_2) as agonist. [d] Intrinsic activity (ia) is the maximum effect obtained with the agonist under study, expressed as a percentage of those of cirazoline (α_1) and clonidine (α_2), both taken as equal to 1. Values are means ± SEM of, in each case, a minimum of six experiments. [e] Antilog of the difference between pD_2 α_2 and pD_2 α_1 values. [f] Antilog of the difference between pK_i α_2 and pK_i I_2 values.

Pharmacology

The α_1- and α_2-adrenergic properties of the compounds reported in Table 1 were determined using epididymal and prostatic portions, respectively, of isolated rat vas deferens.[21,22] Agonist and antagonist potencies were respectively expressed as pD_2 and pK_b values, calculated according to van Rossum.[23]

Affinity values at α_2-adrenoreceptors and I_2 imidazoline binding sites were evaluated using membranes of rat cortex and rabbit kidney, respectively.[11] The radioligands used were [^{3}H]clonidine (2 nM, α_2) and [^{3}H]-idazoxan (5 nM, I_2), and nonspecific binding was defined by inclusion of 10 μM phentolamine (α_2, 25%) and 10 μM cirazoline (I_2, 10%).

Furthermore, compound **7** was tested at the three α_2-adrenoreceptor subtypes using HT29 cells expressing α_{2A}[24] and membrane preparations of COS-7 cells, stably transfected with cloned human α_{2b} and α_{2c} adrenoreceptor subtypes, respectively. Binding studies were performed as previously described[24,25] with [^{3}H]rauwolscine (5 nM), as radioligand, and phentolamine (10^{-3} M) to define nonspecific binding. Competition binding curves were performed with eight concentrations (10^{-3}–10^{-10} M) of drugs. IC_{50} values were determined by nonlinear regression analysis of binding data with the aid of the Graphpad Prism computer program. K_i values were calculated by the equation of Cheng and Prusoff[26] and reported as pK_i ± SEM.

On account of its interesting binding and intrinsic activity profile, compound (*S*)-(−)-**7** was also screened in vivo in mouse tail-flick and hot-plate methods for antinociceptive activity.[27]

Modeling. QXP (Quick EXPlore) Studies

QXP (Quick EXPlore) is a module of Flo96, a software for structure-based drug design including fast and efficient algorithms for flexible docking and fitting.[28] For the molecular fitting, QXP uses a procedure (TFIT) based on a full conformational search and similarity match (fitting) of two or more molecules simultaneously. The TFIT procedure is based on a mixed AMBER/MM2 force field, a superposition force field, a Monte Carlo conformational search, and a rigid body alignment algorithm. QXP automatically assigns short-range attractive forces between similar atoms in different molecules. Atoms are defined to be "similar" on the basis of their chemical properties, mainly polarity, charge, and hydrogen bond ability. In the template fitting procedure (molecular superposition), the typical intramolecular nonbonded energies are replaced by the superposition energies, while internal energies (E_{int}) are calculated by the normal force field ignoring nonbonded energies. The combined minimization of these two energies (E_{sup} and E_{int}) yielded structures with optimal superposition and relatively low internal energy. Within a defined energy range, the program affords different solutions ranked according to their total energy ($E_{tot} = E_{sup} - E_{int}$).

Results and Discussion

The pharmacological data of compounds **3**−**12** are reported in Table 1, together with those of **2**,[9,11] taken as reference compound.

The most important finding of this investigation is the effect on the biological profile of the insertion of a

second phenyl ring in the structure of cirazoline (**1**) related compounds. In functional studies, compound **2** behaved as an α_1-adrenoreceptor agonist while being an antagonist toward α_2-adrenoreceptors. The C-methylation of the oxymethylene bridge of **2**, yielding **3**, did not significantly affect α_1- and α_2-adrenergic properties, while it strongly modified the affinity for I_2 imidazoline binding sites, as **3** was more than 3000-fold less potent than **2**. Surprisingly, the introduction of a phenyl ring in the ortho position of **2**, yielding **4**, caused, together with an increase of the α_1-agonist activity, a reversal of the biological profile at α_2-adrenoreceptors because **4** was a potent ($pD_2 = 8.46$) and full agonist. Conversely, the phenyl ring was detrimental for affinity at I_2 imidazoline binding sites, as revealed by the pK_i value of **4**, which was more than 300-fold lower than that of the congener **2**. A further decrease in the affinity for I_2 imidazoline binding sites was observed for **7**, which bears a methyl in the oxymethylene bridge. This structural modification, which was strongly detrimental for I_2 imidazoline binding sites affinity, was consistent with α_2-adrenoreceptor affinity and potency, perhaps by the interaction of the C-bridge methyl group with a small lipophilic cavity (methyl pocket) defined by Leu_{110} in TM3, Leu_{169} in TM4, and Phe_{391} and Thr_{395} in TM6.[29] A similar trend was also observed for the isosteric aniline derivatives **10**−**12**, confirming the peculiar role played by both the phenyl ring substituent and the C-bridge methylation on the biological profile at I_2 imidazoline binding sites and α_2-adrenoreceptors.

The maximum effect produced by the phenyl ring was obtained when it was in the ortho position and directly linked to the aromatic ring. In fact, the *o*-benzyl derivative **5**, its methyl derivative **6**, and the phenyl regioisomers of **7**, namely, **8** and **9**, showed lower affinity and/or potency values relative to reference compounds **4** and **7**, respectively. Of interest was the finding that compounds **4** and **7** were potent α_2-adrenoreceptor agonists, whereas **5** and **6** were antagonists. Therefore, it appears that when the phenyl ring is moved farther away by a methylene unit, the agonist activity disappears. This could also be ascribed to a higher conformational flexibility of the benzylic compound compared to the more rigid biphenyl ring.

The striking effect observed, following the insertion of a phenyl substituent, on the potency of cirazoline-related compounds at α_2-adrenoreceptors was consistent with the recent observation that the presence of a phenyl group in 2-(3-*exo*-(3-phenylprop-1-yl)-2-*exo*-norbornyl)amino-2-oxazoline (**15**) may be responsible for the selective activation of $\alpha_{2A/C}$-adrenoreceptors.[30] This was explained by the ability of the phenyl ring to reach and interact with the Phe residue conserved in transmembrane helix IV of both receptor subtypes. In an attempt to find common molecular determinants for the α_2-adrenoreceptor activity, we superposed compounds **7** and **15**. The fitting module of QXP was applied to **7** and **15** in their protonated state, and the best fitting model has an E_{tot} and E_{sup} energy of −119 and −121 kcal/mol, respectively, since the energy of the selected conformers is only about 0.5 kcal/mol greater than their absolute energy minima. A simple visual inspection of the molecular overlay reported in Figure 1 reveals that putative pharmacophore moieties, like the charged

Figure 1. Molecular overlay of molecules **15** (in red) and **7** (in green) derived by QXP.

azolidine rings (imidazoline in **7** and oxazolidine in **15**) and the extended biphenyl (compound **7**) and phenylpropyl (compound **15**) hydrophobic moieties, occupied similar spatial regions. In particular, a relatively good superposition is observed between the two phenyl rings. Similar results were obtained by taking into account neutral molecules. Therefore, it is conceivable that **7** and **15** activate the receptor by the same interactions.

Since compound **7** has a chiral center, the two enantiomers (*R*)-(+)-**7** and (*S*)-(−)-**7** were prepared and separately tested in order to evaluate whether the interaction is stereospecific as has been observed for other α_2-adrenergic agonists such as medetomidine,[31] α-methylnoradrenaline,[32] and lofexidine.[33] As expected, the interaction at both α_1- and α_2-adrenoreceptors is stereospecific, since compound (*S*)-(−)-**7** is the eutomer.

Presynaptic α_2-adrenoreceptors of the rat vas deferens, the preparation used in the present study, have been reported to be the α_{2A}-subtype.[34] To assess the selectivity profile, compound **7** was tested at α_{2A}-, α_{2b}-, and α_{2c}-subtypes. The results obtained showed that **7** binds preferentially to the α_{2A}-subtype, as revealed by its affinity values: $pK_i\alpha_{2A} = 7.15$, $pK_i\alpha_{2b} = 6.20$, and $pK_i\alpha_{2c} = 6.50$.

The eutomer (*S*)-(−)-**7** was tested for antinociceptive activity on two algesiometric paradigms, mouse hot-plate and mouse tail-flick tests. As can be seen from Figures 2−5, (*S*)-(−)-**7**, with ED_{50} values of 0.063 (0.04−0.09) and 0.12 (0.07−02) mg/kg, respectively, with 95% confidence limits in parentheses, is a potent and long-lasting, at least up to 2 h, antinociceptive agent. Its action is abolished by pretreatment with RX821002 (Figure 6), a selective α_2-adrenergic antagonist,[35] demonstrating that the analgesic effect is exerted through the interaction with α_2-adrenoreceptors. Besides spinal I_2 imidazoline binding sites,[17] α_1-adrenoreceptors[36] may also act antinociceptively. However, the low affinity ($pK_i = 5.14$) for I_2 imidazoline binding sites shown by (*S*)-(−)-**7** and the fact that Prazosin, an α_1-adrenergic antagonist, does not affect the analgesic action of (*S*)-

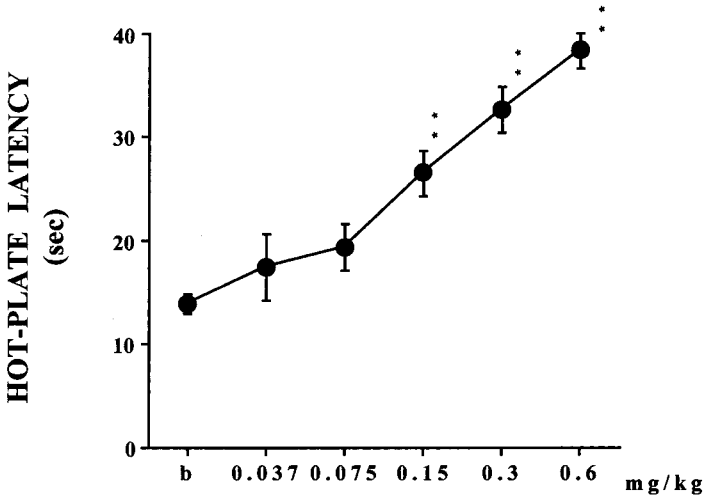


Figure 2. Baseline nociception (b) and antinociceptive effect in the hot-plate test 30 min after subcutaneous injection of different doses of the agonist (*S*)-(−)-**7**. The data are expressed in absolute values and are means ± SEM. The ∗∗represents the difference from controls with $p < 0.01$. Where not indicated, the difference was not statistically significant.

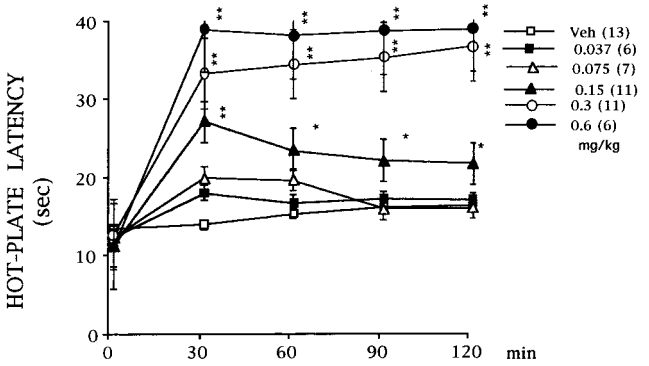


Figure 3. Antinociceptive effect in the hot-plate test at different times following subcutaneous injection of different doses of the agonist (*S*)-(−)-**7**. The data are expressed in absolute values and are means ± SEM. The ∗∗represents the difference from controls with $p < 0.01$. Where not indicated, the difference was not statistically significant. In parentheses is the number of animals.

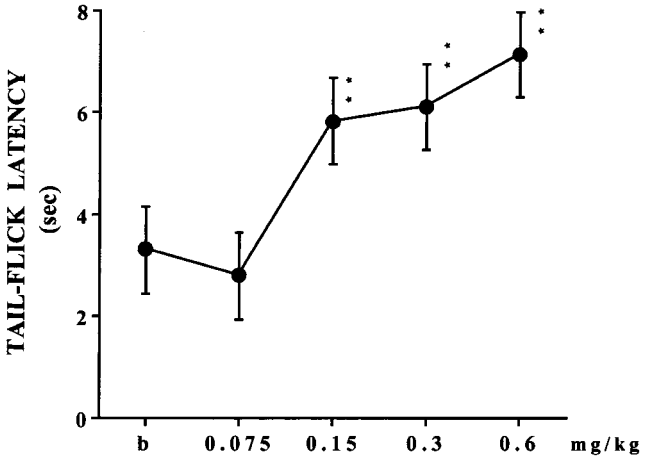


Figure 4. Baseline nociception (b) and antinociceptive effect in the tail-flick test 30 min after subcutaneous injection of different doses of the agonist (*S*)-(−)-**7**. The data are expressed in absolute values and are means ± SEM. The ∗∗represents the difference from controls with $p < 0.01$. Where not indicated, the difference was not statistically significant.

(−)-**7** (result not shown) speak against the implication of these biological systems in the observed antinociceptive activity.

In conclusion, we have shown that introduction of a phenyl ring in the ortho position of the 2-phenoxymethyl (or isosteric anilinomethyl) imidazoline basic structure and the simultaneous methylation of the carbon bridge address selectivity toward the α_2-adrenoreceptors with respect to α_1-adrenoreceptors and particularly to I_2 imidazoline binding sites. Compound (*S*)-(−)-**7**, the most interesting of the series, shows potent antinociceptive activity. These results may offer leads to the development of new analgesic agents.

Experimental Protocols

Chemistry. Melting points were taken in glass capillary tubes on a Buchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian EM-390 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Although the IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and are consistent with the assigned structures. Optical activity was measured at 20 °C with a Perkin-Elmer 241 polarimeter. HPLC analyses were recorded on an HP 1090 I series chromatograph on a LiChrosphere Si 60 4 mm × 250 mm stainless steel column (packed with 5 μm spherical silica particles, Merck). The mobile phase was hexane/AcOEt (55/45). The flow rate was set at 1.0 mL/min. The microanalyses were performed by the Microanalytical Laboratory of our department, and the elemental compositions of the compounds agreed to within ±0.4% of the calculated value. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040−0.063 mm, Merck) by flash chromatography. The term "dried" refers to the use of anhydrous sodium sulfate.

2-[1-(Biphenyl-2-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole Hydrochloride (7). 2-Phenylphenol (2.58 g, 15.16 mmol) was added to a solution of Na (0.6 g, 26.10 mmol) in absolute EtOH (40 mL). After 1 h of stirring, 2-(2-chloroethyl)imidazoline hydrochloride[18] (1.28 g, 7.57 mmol) was added to the reaction mixture, which was heated to reflux for 8 h under vigorous stirring. The solution was evaporated to dryness to give a residue, which was taken up in $CHCl_3$ and washed with water, 3 N NaOH, and water. Removal of dried solvent gave an oil, which was purified by flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (8/4/1/0.1) as eluent. The free base (0.6 g; yield 30%) was transformed into the

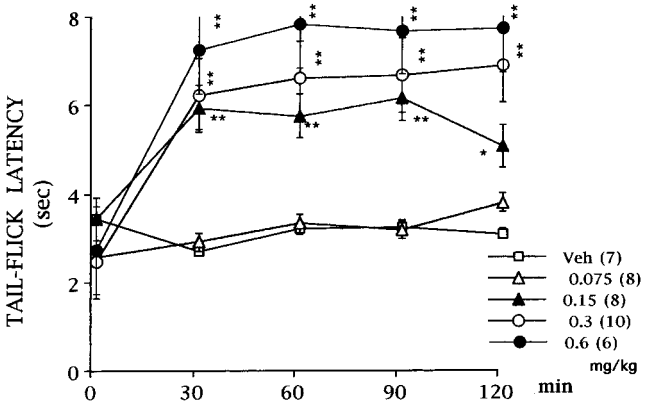


Figure 5. Antinociceptive effect in the tail-flick test at different times following subcutaneous injection of different doses of the agonist (*S*)-(−)-**7**. The data are expressed in absolute values and are means ± SEM. The ∗∗represents the difference from controls with $p < 0.01$. Where not indicated, the difference was not statistically significant. In parentheses is the number of animals.

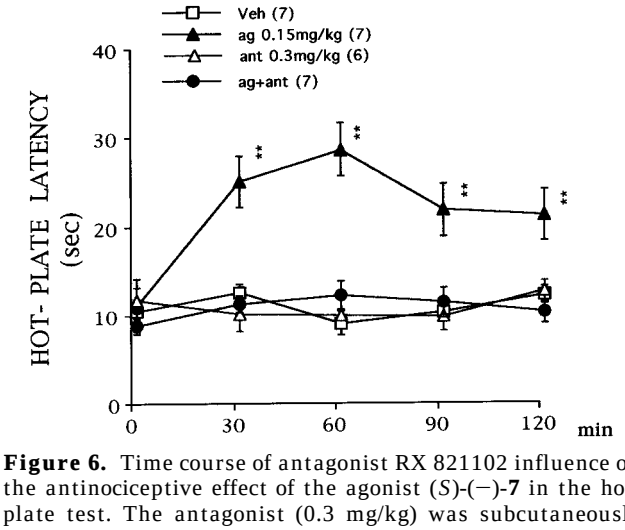


Figure 6. Time course of antagonist RX 821102 influence on the antinociceptive effect of the agonist (*S*)-(−)-**7** in the hot-plate test. The antagonist (0.3 mg/kg) was subcutaneously administered 30 min prior to the agonist (0.15 mg/kg). The data are expressed in absolute values and are means ± SEM. The ∗∗represents the difference from controls with $p < 0.01$. Where not indicated, the difference was not statistically significant. In parentheses is the number of animals.

hydrochloride salt, which was recrystallized from 2-PrOH/Et_2O (mp 179−180 °C): ^{1}H NMR (DMSO) δ 1.5 (d, 3, CH_3), 3.88 (s, 4, NCH_2CH_2N), 5.22 (q, 1, CH), 7.08−7.62 (m, 9, ArH), 10.48 (br s, 1, NH, exchangeable with D_2O). Anal. ($C_{17}H_{18}N_2O$·HCl) C, H, N.

2-(2-Benzylphenoxy)propionic Acid Methyl Ester (14). A mixture of 2-hydroxydiphenylmethane (4.33 g, 23.50 mmol), methyl 2-bromopropionate (3.92 g, 23.50 mmol), and K_2CO_3 (3.25 g, 23.50 mmol) was refluxed for 8 h. The mixture was cooled and filtered. The solvent was removed under reduced pressure to give a residue, which was taken up in ether and washed with cold 2 N NaOH. Removal of dried solvent afforded an oil (2.73 g; yield 43%), which was used in the next step without further purification: ^{1}H NMR ($CDCl_3$) δ 1.58 (d, 3, CHC*H*$_3$), 3.75 (s, 3, OCH_3), 4.05 (dd, 2, CH_2), 4.75 (q, 1, CHCO), 6.68−7.31 (m, 9, ArH).

2-[1-(2-Benzylphenoxy)ethyl]-4,5-dihydro-1*H*-imidazole Oxalate (6). A solution of ethylenediamine (1.12 mL, 16.70 mmol) in dry toluene (5 mL) was added dropwise to a mechanically stirred solution of 2 M trimethylaluminum (8.35 mL, 16.70 mmol) in dry toluene (13.80 mL) at 0 °C in a nitrogen atmosphere. After being stirred at room temperature for 1 h, the solution was cooled to 0 °C and a solution of **14** (2.27 g, 8.40 mmol) in dry toluene (5 mL) was added dropwise. The reaction mixture was heated to 70 °C for 3 h, cooled to 0 °C, and quenched cautiously with MeOH (2.48 mL) followed by water (0.45 mL). After addition of $CHCl_3$ (19.80 mL), the mixture was refluxed for 1 h to ensure the precipitation of the aluminum salts. After cooling, the mixture was filtered and the solvent was evaporated in vacuo. The residue was dissolved in Et_2O and extracted with 2 N HCl. The aqueous layer was made basic with 2 N NaOH and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo to give the free base as an oil, which was purified by flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (6/4/1/0.1) as eluent. The free base (0.3 g; yield 13%) was transformed into the oxalate salt, which was recrystallized from $CHCl_3$ (mp 150−151 °C): ^{1}H NMR (DMSO) δ 1.50 (d, 3, CH_3), 3.88 (s, 4, NCH_2CH_2N), 4.01 (dd, 2, CH_2), 5.40 (q, 1, CH), 6.92−7.35 (m, 9, ArH), 9.3 (br s, 1, NH exchangeable with D_2O). Anal. ($C_{18}H_{20}N_2O$·$H_2C_2O_4$) C, H, N.

(*S*)-(−)-2-[1-(Biphenyl-2-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole Oxalate [(*S*)-(−)-7]. Similarly, compound (*S*)-(−)-**7** was obtained from (*S*)-(+)-2-[(1,1′-biphenyl)]-2-yloxypropionic acid methyl ester.[20] Unlike compound **6**, the reaction mixture was heated to a lower temperature (30−40 °C).

The free base of (*S*)-(−)-**7** was purified through flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (6/4/1/0.1) (0.27 g; yield 10%) as eluent. The enantiomeric purity, determined by HPLC of its corresponding diastereomeric carbamate, obtained by reacting (*S*)-(−)-**7** with (−)-menthyl chloroformate, was 89%. To increase this enantiomeric purity, the free base was further purified by fractional crystallization of its (−)-di-*O*,*O*′-*p*-toluyl-L-tartrate salt.

A solution of (−)-di-*O*,*O*′-*p*-toluyl-L-tartaric acid (0.37 g, 0.98 mmol) in a minimum amount of *i*-PrOH was added to a stirred solution of (*S*)-(−)-**7** (0.26 g, 0.98 mmol) in a minimum amount of dry EtOH. After 24 h at room temperature, the white solid was filtered. The salt was dissolved in water, and the ice-cooled solution was made basic with 2.5% NH_4OH. The resulting mixture was extracted with $CHCl_3$. Removal of dried solvent gave (*S*)-(−)-**7** (0.21 g), which was transformed into the oxalate salt: $[\alpha]^{20}_D$ −3.43° (*c* 1, MeOH); mp 191−192 °C; ^{1}H NMR (DMSO) δ 1.46 (d, 3, CH_3), 3.85 (s, 4, NCH_2CH_2N), 5.18 (q, 1, CH), 7.02−7.62 (m, 9, ArH), 9.41 (br s, 1, NH, exchangeable with D_2O). Anal. ($C_{17}H_{18}N_2O$·$H_2C_2O_4$·0.5H_2O) C, H, N.

The enantiomeric purity of the free base was determined by HPLC and ^{1}H NMR spectroscopy of its corresponding diastereomeric carbamate **13b**, which was prepared as follows.

(−)-Menthyl chloroformate (0.17 mL, 0.81 mmol) was added to a cooled (0 °C) solution of (*S*)-(−)-**7** (0.18 g, 0.68 mmol) and Et_3N (0.09 mL, 0.68 mmol) in dry $CHCl_3$ (1.5 mL). After 10 min at room temperature, the solvent was evaporated and the residue was purified by flash chromatography using cyclohexane/AcOEt (8/2) as eluent: ^{1}H NMR ($CDCl_3$) δ 0.75−2.15 (m, 18, menthyl), 1.52 (d, 3, CHC*H*$_3$), 3.82 (s, 4, NCH_2CH_2N), 4.66 (m, 1, COOCH), 5.68 (q, 1, OC*H*CH_3), 6.89−7.69 (m, 9, ArH). The enantiomeric purity, determined by HPLC, was 98.7%.

(*R*)-(+)-2-[1-(Biphenyl-2-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole Oxalate [(*R*)−(+)-7]. Compound (*R*)-(+)-**7** was prepared following the same procedure described for **6** starting from (*R*)-(−)-2-[(1,1′-biphenyl)]-2-yloxypropionic acid methyl ester.[20] The time of heating was 45 min, which was different from the 3 h of compound **6**.

The free base of (*R*)-(+)-**7** was purified through flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (6/4/1/0.1) (0.26 g; yield 8.8%) as eluent. The enantiomeric purity, determined by HPLC of its corresponding diastereomeric carbamate, obtained by reacting (*R*)-(+)-**7** with (−)-menthyl chloroformate, was 96%. To increase this enantiomeric purity, the free base was then further purified by fractional crystallization of its (+)-di-*O*,*O*′-*p*-toluyl-D-tartrate salt.

A solution of (+)-di-*O*,*O*′-*p*-toluyl-D-tartaric acid (1.11 g, 2.93 mmol) in CH_3CN was added to a stirred solution of (*R*)-(+)-**7** (0.78 g, 2.93 mmol) in CH_3CN. After 24 h at room temperature, the white solid was filtered and crystallized four times from CH_3CN. The salt was dissolved in water, and the ice-cooled

solution was made basic with 2.5% NH_4OH. The resulting mixture was extracted with $CHCl_3$. Removal of dried solvent gave (*R*)-(+)-**7** (0.22 g), which was transformed into the oxalate salt: $[\alpha]^{20}_D$ +3.60° (*c* 1, MeOH); mp 192−193 °C; ^{1}H NMR (DMSO) δ 1.46 (d, 3, CH_3), 3.85 (s, 4, NCH_2CH_2N), 5.18 (q, 1, CH), 7.02−7.62 (m, 9, ArH), 10.19 (br s, 1, NH, exchangeble with D_2O). Anal. ($C_{17}H_{18}N_2O\cdot H_2C_2O_4\cdot 0.25H_2O$) C, H, N.

The enantiomeric purity of the free base was determined by HPLC and ^{1}H NMR spectroscopy of its corresponding diastereomeric carbamate **13a**, obtained following the procedure described for **13b**.

13a: ^{1}H NMR ($CDCl_3$) δ 0.75−2.06 (m, 18, menthyl), 1.54 (d, 3, CHC*H*$_3$), 3.85 (s, 4, NCH_2CH_2N), 4.63 (m, 1, COOCH), 5.62 (q, 1, OC*H*CH_3), 6.96−7.65 (m, 9, ArH). The enantiomeric purity determined by HPLC was 99%.

2-[1-(Biphenyl-4-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole Oxalate (9). HCl gas was bubbled through a stirred and cooled (0 °C) solution of 2-(4-biphenylyloxy)propionitrile[19b] (2.40 g, 10.75 mmol) and MeOH (0.43 mL, 10.75 mmol) in dry $CHCl_3$ (8.6 mL) for 45 min. After 24 h at 0 °C, dry ether was added to the reaction mixture to give an intermediate imidate, which was filtered (3.13 g). This solid (3.13 g) was added to a cooled (0 °C) and stirred solution of ethylenediamine (0.71 mL, 10.62 mmol) in dry MeOH. After 1 h at 0 °C, concentrated HCl (0.83 mL) in dry MeOH (8.70 mL) was added to the reaction mixture, which was stored overnight in the refrigerator and then was heated at 70 °C for 5 h. After cooling, the mixture was evaporated to dryness to give a residue, which was taken up in 2 N NaOH and extracted with $CHCl_3$. Removal of dried solvents gave an oil as the free base, which was purified by flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (8/4/1/0.1) as eluent. After evaporation in vacuo of the solvents, a solid (1.0 g; yield 35%; mp 140−141 °C) was obtained. The free base was transformed into the oxalate salt, which was recrystallized from dry EtOH (mp 179−180 °C); ^{1}H NMR (DMSO) δ 1.63 (d, 3, CH_3), 3.9 (s, 4, NCH_2CH_2N), 4.29 (br s, 1, NH, exchangeable with D_2O), 5.5 (q, 1, CH), 7.09−7.71 (m, 9, ArH). Anal. ($C_{17}H_{18}N_2O\cdot H_2C_2O_4$) C, H, N.

2-[1-(Biphenyl-3-yloxy)ethyl]-4,5-dihydro-1*H*-imidazole Hydrochloride (8). Similarly, compound **8** was obtained starting from 2-(3-biphenylyloxy)propionitrile.[19a] The free base was purified by flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (8/1/1/0.1) as eluent. After evaporation in vacuo of the solvents, an oil (1.25 g; yield 44%) was obtained. The free base was transformed into the hydrochloride salt, which was recrystallized from acetone/Et_2O (mp 172−174 °C): ^{1}H NMR (DMSO) δ 1.65 (d, 3, CH_3), 3.9 (s, 4, NCH_2CH_2N), 5.61 (q, 1, CH), 7.0−7.72 (m, 9, ArH), 10.45 (br s, 1, NH, exchangeable with D_2O). Anal. ($C_{17}H_{18}N_2O\cdot HCl\cdot 0.25H_2O$) C, H, N.

Biphenyl-2-yl-[1-(4,5-dihydro-1*H*-imidazol-2-yl)ethyl]-amine Oxalate (12). A mixture of 2-(2-chloroethyl)imidazoline hydrochloride[18] (0.73 g, 4.32 mmol), 2-aminobiphenyl (1.81 g, 10.69 mmol), and NaI (0.022 g, 0.14 mmol) was heated at 135−145 °C for 5 h under a stream of dry nitrogen. Then it was cooled to 80 °C and poured into H_2O (6.35 mL). After addition of 2 N NaOH (1.83 mL) at 0 °C, the mixture was extracted with Et_2O. Removal of dried solvents gave an oil, which was purified by flash chromatography using cyclohexane/AcOEt/MeOH/33% NH_4OH (4/1/0.5/0.1) as eluent. The free base (0.2 g; yield 18%) was transformed into the oxalate salt, which was recrystallized from dry EtOH (mp 210−211 °C): ^{1}H NMR (DMSO) δ 1.44 (d, 3, CH_3), 3.86 (s, 4, NCH_2CH_2N), 4.51 (m, 1, CH), 4.85 (d, 1, N*H*C_6H_4, exchangeable with D_2O), 6.6−7.6 (m, 9, ArH), 10.2 (br s, 1, NH, exchangeable with D_2O). Anal. ($C_{17}H_{19}N_3\cdot H_2C_2O_4\cdot 0.25H_2O$) C, H, N.

Pharmacology. 1. Functional Assays. In all cases, contractions were recorded isometrically by means of a force transducer connected to a two-channel Gemini polygraph (Basile, Comerio, Italy).

2. Rat Vas Deferens. Male albino rats (125−150 g) were killed by a sharp blow on the head, and both vasa deferentia were carefully removed, freed from adhering connective tissue, and transversally bisected. The 14-mm-long epididymal portion was used to assess the agonist activity on α_1-adrenoreceptors. It was mounted in 20-mL organ baths containing a physiological salt solution (PSS) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1. Propanolol hydrochloride (1 μM) and cocaine hydrochloride (10 μM) were present in the Krebs solution throughout the experiments outlined below in order to block β-adrenoreceptors and the neuronal uptake mechanism, respectively. The initial loading tension was set at 1 g. The medium was maintained at 37 °C and was aerated with 95%/5% O_2/CO_2. The tissues were allowed to equilibrate for at least 1 h before the addition of any drug. Two dose−response curves were constructed by cumulative addition of the reference agonist [(−)-norepinephrine]. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, a new dose−response curve to the agonist under study was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. It was verified in parallel experiments that the second (−)-norepinephrine concentration−response curve was identical to the first because the change in ratio of equieffective concentrations was less than 2, which usually entails a minimal correction. The results are expressed in terms of pD_2, which is $-\log ED_{50}$, the concentration of agonist required to produce 50% of the maximum contraction.

Compounds devoid of agonist activity were tested as antagonist against (−)-norepinephrine. Dose ratios at ED_{50} values of (−)-norepinephrine were calculated at one concentration that was tested six times. Dissociation constants were calculated according to the method of van Rossum.[23]

The 12-mm-long prostatic portion was used to assess the agonist activity on α_2-adrenoreceptors. PSS composition was as given above for the epididymal portions except that the $MgCl_2$ concentration was reduced to 0.54 mM and that it contained prazosin (0.1 mM) in order to block postsynaptic α_1-adrenoreceptors. The tissues were stimulated electrically at 0.1 Hz with square pulses of 3 ms duration at a voltage of 10−15 V. When the twitch response became constant, cumulative concentration−response curves were run in each tissue, which was treated only once with the agonist under study. The results are expressed in terms of pD_2, $-\log ED_{50}$, where ED_{50} is the concentration required to produce 50% inhibition of the twitch response.

Compounds devoid of agonist activity were tested as antagonist against clonidine. Dose ratios at ED_{50} values of clonidine were calculated at one concentration that was tested six times. Dissociation constants were calculated according to the method of van Rossum.[23]

Binding Assays. 1. α_2-Adrenoreceptors Binding Assays. Affinity for α_2-adrenergic receptors in the rat brain was assessed by measuring the ability of the test compounds to displace [^{3}H]clonidine from these receptors. Although [^{3}H]-clonidine may bind to the I_1 site, there is no indication of any I_1 binding in rat cortex. It was previously shown that [^{3}H]-clonidine bound to rat cortical membranes is completely displaced by noradrenaline in a monophasic manner.[37] In this assay, the cerebral cortex of rat brain was homogenized in 20 volumes of Tris buffer (50 mM, pH 7.4) with 5 mM EDTA, with a 30 s burst from a PT10 Polytron homogenizer set at 6. The homogenate was centrifuged at 500*g* for 10 min. The supernatant obtained was then centrifuged at 65 000*g* for 25 min, and the resulting pellet washed twice with 50 mM Tris-HCl without EDTA. The final pellet was resuspended in the same buffer and stored at −80 °C until required. Competition binding assays were performed by incubating washed rat cerebral membranes (200 μg of protein) with 5 nM [^{3}H]-clonidine (NEN, 60−63 Ci/mmol) in the absence or presence of a range of 10−12 concentrations of the competing ligand in a total volume of 400 μL of Tris assay buffer (50 mM Tris-HCl, pH 7.4). Nonspecific binding was defined as the concentration of bound ligand in the presence of 10 μM phentolamine.

Specific binding represented about 75% of the total binding at 5 nM [^{3}H]clonidine. Following equilibrium (45 min at 25 °C), bound radioactivity was separated from free radioactivity by filtration through a GF/B filter with a Brandel cell harvester. Bound radioactivity on the glass fiber filter was determined by liquid scintillation counting. Each point was performed in triplicate.

2. I_2 Imidazoline Binding Sites Binding Assays. Rabbit kidney was homogenized in 10 volumes of Tris-HCl buffer (50 mM, pH 7.4) and 250 mM sucrose and was centrifuged at 500*g* for 10 min. The supernatant was centrifuged at 28 000*g* for 30 min, and the resulting pellet was washed twice with the same buffer without sucrose. The final pellet was resuspended in Tris-HCl buffer (50 mM, pH 7.4) and stored at −80 °C until use. Rabbit kidney membranes (200 μg of protein) were incubated with 5 nM [^{3}H]idazoxan (Amersham, 43 Ci/mmol) in the absence or presence of a range of 10−12 concentrations of competing ligand drug in a total volume of 400 μL of assay buffer. To mask adrenoreceptors, 10 μM (−)-norepinephrine (in the presence of 0.005% ascorbic acid) was added to all tubes. Nonspecific binding was determined with 10 μM of cirazoline. Specific binding represented about 90% of the total binding at 5 nM [^{3}H]idazoxan. Following equilibrium (45 min at 25 °C), bound radioactivity was separated from free radioactivity by filtration as described above. Each point was performed in triplicate.

3. α_{2A}-Adrenoreceptors Binding Assays. 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 5% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were harvested at confluence after 48 h of incubation in fresh DMEM without FBS, and membranes were prepared immediately.

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.

HT29 membrane binding assays were performed with 5 nM [^{3}H]rauwolscine. Incubation was initiated by the addition of membranes (100 μg of protein/assay) and was carried out at 25 °C for 45 min in a total volume of 400 μL. Assays were then processed as described above, and radioactivity retained on the filters was determined in a β TriCarb counter (Packard). Nonspecific binding was defined with 10 μM phentolamine for [^{3}H]rauwolscine binding.

4. α_{2b}- and α_{2c}-Adrenoreceptors Binding Assays. Membranes of transfected COS-7 cells were obtained from Dr. H. Paris (Toulouse). Briefly, COS-7 cells were cultured in Dulbecco's modified eagle medium supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 50 μg/mL streptomycin. Each of the expression vectors was transfected by the DEAE−dextran method.[38] Culture dishes were collected 48 h after transfection. The cells transfected with pBCα_2C2 and pDPα_2C4 will be referred to as COS-α_2C2 and COS-α_2C4, respectively.

Cells were harvested in phosphate-buffered saline, pelleted by gentle centrifugation (800*g*, 10 min), and stored at −80 °C until analysis. Crude membrane fractions were prepared as follows. Cell pellets were resuspended in Tris-Mg^{2+} buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5), disrupted with a glass pestle homogenizer, and centrifuged for 10 min at 39 000*g* (4 °C). The crude membrane pellet was resuspended in the required volume of Tris-Mg^{2+} buffer and was immediately used for binding studies.

Binding experiments were conducted as previously described.[39] Briefly, 100 μL of membrane suspension were incubated for 45 min at 25 °C in the presence of [^{3}H]-rauwolscine in a 400 μL final volume of Tris-Mg^{2+} buffer. Membrane-bound radioligand was separated from the free radioligand by rapid filtration through GF/C Whatman filters using a Brandel cell harvester. Filters were washed with cold Tris-Mg^{2+} buffer, air-dried, transferred into vials, and counted for radioactivity by liquid scintillation spectrometry. Specific binding was defined as the difference between total and nonspecific binding determined in the presence of 10^{-4} M phentolamine.

5. Computer Analysis of Binding Data. IC_{50} were determined by nonlinear regression analysis of binding data with the aid of the Graphpad program. K_i values were calculated by the equation of Cheng and Prusoff.[26] Each curve was repeated at least three times, and the results are given as means ± SEM.

In Vivo Assays. Male Swiss mice (Charles River, Italy) weighing 25−30 g were used. The animals were housed in colony cages (four mice in each) with free access to food and water. They were maintained in a climate- and light-controlled room (22 ± 1 °C, 12/12 dark/light cycle) at least 7 days before testing. Testing took place during the light phase. The animals were brought to the test room at least 2 h before testing. In all the experiments with animals, special attention was paid to the ethical guidelines for pain investigations in conscious animals[40] and to international European ethical standards (86/609-EEC). Each mouse was used in only one experimental session.

1. Hot-Plate Method. The hot-plate latency was assessed by placing the mouse on a metal plate at a constant temperature on which a Plexiglas cylinder was placed (U. Basile, Italy). The hot-plate was set to 55 ± 0.5 °C. The time of hind paw licking was taken as the end point. At this moment, the latency to respond was recorded and the mouse was immediately removed. Maximum latency accepted was 40 s. Each mouse was tested 0.5 and 1 h before vehicle or agonist treatment in baseline latency determination and 0.5, 1, 1.5, and 2 h after subcutaneous administration.

For antagonist experiments, vehicle or selective antagonist RX821002 was subcutaneously injected 30 min before the agonist.

2. Tail-Flick Method. The tail-flick latency was assessed by a tail-flick analgesymeter (U. Basile, Italy), consisting of an infrared source whose radiant light of adjustable intensity was focused by an aluminized parabolic mirror on a photocell. Radiant heat was focused on a blackened spot 1−2 cm from the tip of the tail, and latency was recorded until the mouse flicked its tail. Beam intensity was adjusted to give a tail-flick latency of 2−3 s in controls. Each mouse was tested 0.5 and 1 h before vehicle or agonist treatment in the baseline latency determination and 0.5, 1, 1.5, and 2 h after subcutaneous administration. To avoid tissue damage, a maximum tail-flick latency of 8 s was used.

3. Statistical Analysis. The data are means ± SEM of absolute values and were analyzed by split-plot ANOVA within group comparisons for drug treatment and within group comparisons for time. Parity comparisons were made by means of the Dunnett test. Statistical significance was set at $p < 0.05$.

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