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Separation of α-Adrenergic and Imidazoline/Guanidinium Receptive Sites (IGRS) Activity in a Series of Imidazoline Analogues of Cirazoline†

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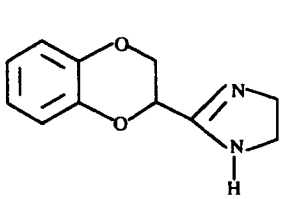
Abstract—To characterize the structure–activity relationship between α_1-adrenergic receptors and the family of imidazoline/guanidinium receptive sites (IGRS), we synthesized and characterized a series of analogues of cirazoline, an imidazoline with high affinity for α_1-adrenergic receptors and IGRS. Analysis of potency, affinity and efficacy of the synthesized molecules indicate different structure–activity relationships for IGRS and α-adrenergic receptors. Cirazoline exhibits a 25-fold higher affinity for IGRS (pK_i 7.9) than for α_1-adrenergic receptors. Replacement of the cyclopropyl ring with an isopropoxy group resulted in a molecule that was 20-fold more selective for α_1-adrenergic receptors than for IGRS, i.e. a 500-fold increase in selectivity relative to cirazoline. The unsubstituted derivative **3** and the methyl and allyl substituted analogues **4** and **12** are of particular interest: compounds **3** and **4** recognize IGRS with high affinity (pK_i 7.83 and 8.17) and high selectivity (398 and 123) with respect to the α_1-adrenergic receptor; compound **12** also recognizes IGRS with high affinity (pK_i 8.08) and high selectivity (228 and 138) with respect to the α_{2B} and α_{2C}-adrenergic receptor subtypes. Thanks to their IGRS selectivity, these compounds represent novel and valuable pharmacological tools for the characterization and elucidation of the physiological role of these novel sites.

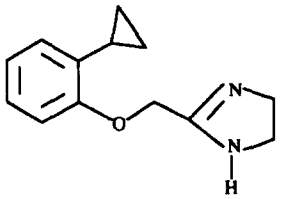
Introduction

Imidazolines are one of the most studied classes of α-adrenergic drugs. Discrete structural modifications of these ligands reveal agonist or antagonist properties with varying degrees of selectivity for α-adrenergic receptor subtypes. In addition, several of these ligands also exhibit high affinity for a family of membrane proteins termed imidazoline receptors or imidazoline/guanidinium receptive sites (IGRS). Idazoxan (**1**), for example, a selective α_2-adrenergic receptor antagonist[1] belonging to the imidazoline class, has been shown to recognizes these sites in a wide range of tissues, with an affinity comparable with that determined at α_2-adrenergic receptors.[2] The functionality of these proteins is poorly understood, but such entities may participate in metabolic regulation[3–5] and in various centrally-mediated effects on wakefulness[6–8] or blood pressure.[9–11] However, a full characterization of these novel sites, and of their physiological relevance has yet to be completed. This has been hampered by the lack of selective ligands, which is a mandatory issue when studying receptor structure and function. There is, therefore, an urgent need to develop selective ligands which would be of paramount importance for a more definitive structural and functional characterization.

Cirazoline (**2**), a potent α_1-adrenergic receptor agonist and α_2-adrenergic receptor antagonist,[12] exhibits high affinity for IGRS in a variety of tissues.[2] Thus, this molecule can serve as a useful starting point to characterize the structure–activity restrictions of this diverse group of ligand-binding pockets. As an initial approch to this issue, we synthesized a series of cirazoline derivatives and evaluated their interaction with the three groups of membrane proteins.



1 Idazoxan



2 Cirazoline

Results

In the present investigation, α_1-adrenergic and IGRS activities were determined and compared with those of cirazoline. For a few selected compounds, affinities for

†Part of this work was presented at the Ist International Symposium on Imidazoline Preferring Receptors, held in Paris, 29–30 June, 1992.

IGRS and for the four cloned α_2-adrenergic receptor subtypes, α_{2A-D}, were also compared. The results are shown in Tables 1 and 2.

All the compounds were tested for α_1-adrenergic activity on isolated rabbit aorta and they were found to be full agonist or nearly so, with varying degrees of potency. In studying agonists it should be borne in mind that the two key parameters that govern agonist–receptors interaction, namely, affinity and efficacy, could have independent structure–activity relationships. Thus potency, affinity, and efficacy were determined in rabbit aorta by the technique of Furchgott and Bursztyn.[13] As can be seen, replacement of the cyclopropyl group with other substituents generally decreases potency, the exception being compound **14**, which is equipotent to cirazoline (**2**). When the two parameters, affinity and efficacy, are considered separately, opposite effects of replacement of the cyclopropyl by the isopropoxy group are observed. Affinity is slightly increased while the efficacy is significantly decreased. The same results are obtained with compounds **6**, **8** and **12**, where the cyclopropyl is replaced by ethyl, isopropyl, or the allyl group, respectively. The insertion of a methyl group in the bridge separating the phenyl and imidazoline rings, also differentially affects affinity and efficacy (**4** versus **5** and **8** versus **9**). In this case, however, affinity is decreased and efficacy is increased.

Removal of the cyclopropyl group to give the unsubstituted compound **3** results in a 10-fold decrease in potency which parallels the decrease in affinity, while efficacy is virtually unaffected. It is worth noting that, if compound **3** is considered as the reference, substitution at *ortho* position either with an alkyl or alkoxy group increases both potency, with exception of compound **10**, and affinity. On the other hand, efficacy is negatively affected, with the exception of cirazoline and compound **5**, in which the presence of the cyclopropyl group or of the two methyl groups seems to favor retention of efficacy. This latter finding would tend to support the hypothesis put forward by Ruffolo *et al.*[12] concerning the role played by the cyclopropyl ring in increasing efficacy.

The affinity of synthesized compounds for IGRS was determined by radioligand binding experiments in rabbit kidney membranes, using [^{3}H]idazoxan as radioligand and increasing concentration of competing ligand. In order to prevent [^{3}H]idazoxan binding to the α_2-adrenergic receptor, membranes were preincubated with 10 μM rauwolscine. Non-specific binding was determined with 10 μM cirazoline.

Table 1. Potencies (pD_2) affinities (pK_A) and efficacies (e_r) at α_1-adrenergic receptor and inhibition constant (pK_i) at IGRS

Compd	pD_2[a]	i.a.[a]	pK_A[a]	e_r	pK_i[b]	SI[c]
2	7.47 ± 0.05	1.00	6.50 ± 0.02	1	7.90 ± 0.10	25
3	6.26 ± 0.10	0.86 ± 0.05	5.23 ± 0.12	0.89	7.83 ± 0.21	398
4	6.70 ± 0.04	0.91 ± 0.04	6.08 ± 0.15	0.47	8.17 ± 0.10	123
5	6.65 ± 0.04	0.80 ± 0.07	5.64 ± 0.16	1.04	< 5	—
6	7.19 ± 0.08	0.93 ± 0.02	6.70 ± 0.03	0.38	7.84 ± 0.16	14
7	7.14 ± 0.05	0.81 ± 0.03	6.50 ± 0.08	0.54	7.84 ± 0.24	22
8	7.18 ± 0.08	0.99 ± 0.07	6.92 ± 0.07	0.26	6.73 ± 0.13	0.7
9	7.00 ± 0.02	0.99 ± 0.01	6.27 ± 0.08	0.60	< 5	—
10	6.21 ± 0.09	0.96 ± 0.04	5.94 ± 0.05	0.27	6.33 ± 0.03	2.5
11	6.45 ± 0.03	0.91 ± 0.05	6.11 ± 0.09	0.30	5.61 ± 0.12	0.3
12	7.19 ± 0.09	0.81 ± 0.06	6.97 ± 0.04	0.25	8.08 ± 0.22	13
13	6.64 ± 0.05	0.91 ± 0.04	6.33 ± 0.12	0.29	6.92 ± 0.13	3.9
14	7.46 ± 0.03	1.00 ± 0.05	6.85 ± 0.03	0.47	5.58 ± 0.14	0.05

[a] pD_2, i.a.(intinsic activity) and pK_A values are the mean ± SE of at least five experiments.
[b] pK_i values are the mean ± SE of two to four experiments performed in duplicate.
[c] SI is the antilog of the difference between pK_i and pK_A values.

Table 2. Inhibition constants (pK_i)[a] at IGRS and at [^{3}H]rauwolscine binding sites (α_{2A-D})

Compd	IGRS	α_{2A}		α_{2B}		α_{2C}		α_{2D}	
	pK_i	pK_i	SI[b]	pK_i	SI[b]	pK_i	SI[b]	pK_i	SI[b]
2	7.90 ± 0.10	7.05 ± 0.09	7	6.11 ± 0.09	62	6.74 ± 0.01	14	6.46 ± 0.11	28
3	7.83 ± 0.21	6.71 ± 0.19	13	5.93 ± 0.09	85	6.80 ± 0.12	11	6.43 ± 0.11	25
6	7.84 ± 0.16	7.00 ± 0.21	7	5.89 ± 0.08	89	6.14 ± 0.07	50	6.72 ± 0.08	13
11	5.61 ± 0.12	6.73 ± 0.02	0.08	5.30 ± 0.40	2	5.73 ± 0.13	0.8	6.22 ± 0.12	0.3
12	8.08 ± 0.22	6.60 ± 0.08	30	5.73 ± 0.03	223	5.94 ± 0.02	138	6.63 ± 0.13	28

[a] pK_i values are the mean ± SE of two to four experiments performed in duplicate.
[b] SI is the antilog of the difference between pK_i at IGRS and pK_i at α_{2A-D} -adrenergic receptors.

The synthesized compounds exhibited a distinct profile in their ability to interact with α_1-adrenergic receptor versus IGRS. Cirazoline exhibits high affinity, with a pK_i of 7.90 and a SI of 25. Either removal (**3**) or replacement of the cyclopropyl ring by methyl (**4**), ethyl (**6**), *n*-propyl (**7**), or allyl (**12**) group conserves the high degree of affinity. Particularly interesting are the results obtained with compounds **3** and **4**. They have the same affinity as cirazoline for IGRS, with pK_i values of 7.83 and 8.17, and, as mentioned above, they also have lower pK_A values (5.23 and 6.08, respectively) at the α_1-adrenergic receptor. The two compounds are therefore most selective for IGRS with SIs of 398 and 123, respectively. If cyclopropyl replacement is carried out by branched alkyl (**8, 10, 11**) or alkoxy (**13, 14**) groups a large decrease in affinity, spanning two orders of magnitude, is observed. Compound **14**, with a pK_i of 5.58 at IGRS and a pK_A of 6.85 at the α_1-adrenergic receptor, exhibits a reversed selectivity with a SI of 0.05. A more dramatic drop in affinity is observed with the introduction of a methyl group in the bridge; compounds **5** and **9** are thus virtually inactive at IGRS.

In the third series of experiments, the affinities of compounds **2, 3, 6, 11,** and **12** for the α_2-adrenergic receptor subtypes were determined in competition binding studies ([^{3}H]rauwolscine) using membranes prepared from Cos cells transfected with receptor subtype cDNA/gene. In this case, non specific binding was determined in the presence of 10 μM rauwolscine. As can be seen in Table 2, cirazoline displays moderate affinity for α_2-subtypes, with a 10-fold selectivity for α_{2A} with respect to α_{2B}. If a comparison is made between IGRS and α_2-subtypes, cirazoline displays preferential affinity for IGRS with a selectivity ratio ranging from seven-fold, in the case of α_{2A}, to 62-fold in the case of α_{2B}. The most pronounced effect in terms of affinity and selectivity is seen with compounds **11** and **12**. In the first case, where a *t*-butyl group replaces the cyclopropyl ring, a significant drop in affinity is observed at α_{2B} and α_{2C}, while at α_{2A} and α_{2D} sites it remains unchanged. This effect, together with the large decrease in affinity at IGRS, abolishes IGRS selectivity with respect to α_{2B-D} and even reverses it in the case of the α_{2A} site. In the second case, where the allyl group replaces the cyclopropyl ring, the decrease in affinity for the α_{2B} and α_{2C} sites, together with the slight improvement at IGRS, makes compound **12** the most selective for IGRS with respect to α_{2B} and α_{2C}, with SIs of 223 and 138, respectively.

Discussion

Bosquet's insight,[9] namely, that the central antihypertensive actions of imidazoline clonidine could be related to the imidazoline structure rather than to the interaction with the α_2-adrenergic receptor, has led to the concept of imidazoline receptor or imidazoline/guanidinium receptive sites (IGRS) being developed. Recent studies have shown that IGRS are widely distributed in both the CNS and peripheral nerve endings and may subserve several physiological actions.[3-11] Furthermore, ligand binding data[2,14,15] show that IGRS, like other receptors, exist as multiple subtypes: one is a clonidine-preferring subtype (which also binds idazoxan), while the other is a idazoxan-preferring subtype (which poorly binds clonidine). In addition, it seems that the latter subtype may be further subdivided on the basis of whether it displays high or low affinity for the guanidinium amiloride,[14,15] an agent that effects a number of ion transport systems.

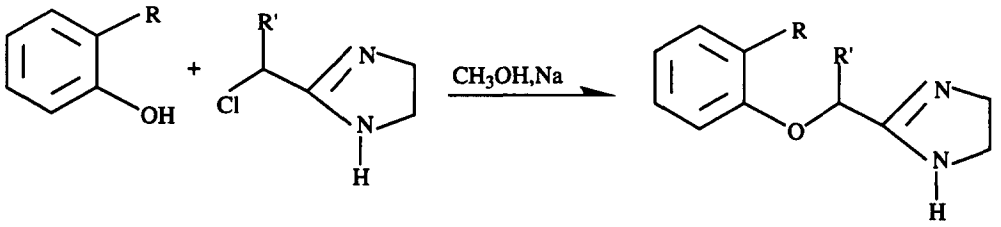
However, a definitive subclassification, with a statement of physiological function(s), has yet to be decided upon, mainly because all the ligands used for IGRS characterization suffer from lack of selectivity with respect to α-adrenergic receptors.

With this in mind, and with the aim of developing IGRS selective ligands, we prepared a series of analogues of cirazoline, a very potent α_1-adrenergic receptor agonist which also recognizes with high affinity IGRS and with moderate affinities α_2-adrenergic receptor subtypes. The results presented here show that the cyclopropyl moiety in the cirazoline structure is not an essential feature for either α-adrenergic or IGRS recog-nition. As far as the latter is concerned, it is clear that removal of the cyclopropyl ring, or its replacement with a linear alkyl group, is permissible, while its replace-ment with branched alkyl or alkoxy groups is not. Moreover, the strong differential effect of methylation of the carbon bridge further supports the view that IGRS and α-adrenergic receptors are two distinct entities and that the binding pocket possesses different steric requirements.

Thus compound **14** is selective for α_1-adrenergic receptor with a selectivity ratio of 0.05, which represents a more than 500-fold improvement over cirazoline. Compound **11**, on the other hand, is more selective for the α_{2A} subtype than the other receptors studied. However, the most interesting results are obtained with compounds **3** and **4**, which display a IGRS/α_1 selectivity of 398 and 123, respectively, and compound **12**, which has IGRS/α_{2B} and IGRS/α_{2C} selectivities of 223 and 138, respectively.

In conclusion, with a simple structural modification of cirazoline, α-adrenergic and IGRS activity may be separated, thus generating selective ligands for one or other receptor system. Some of them (**3**, **4**, and **12**) represent novel valuable pharmacological tools useful in IGRS characterization in preparations where the presence of α-adrenergic receptor subtypes may complicate the understanding of the physiological role played by these novel sites.

Furthermore, from the results presented in this report it emerges quite clearly that (a) the methylation of the carbon bridge is detrimental for IGRS activity, and (b) the removal of substituents on the phenyl ring favors IGRS selectivity. These two main findings have to be kept in mind when designing selective compounds for



15 R'=H
16 R'=CH3

5-14

Scheme 1.

one or the other receptor system. Compound **3**, with the highest IGRS/α_1 selectivity, constitutes a new starting point in the attempt to produce more selective ligands. Work along this direction is in progress and will be the object of forthcoming papers.

Experimental

Chemistry

All the compounds were characterized by ^{1}H NMR, IR and elemental analyses and were synthesized by standard procedures as shown in Scheme 1; thus, alkylation of the appropriate phenol with **15** or **16**[16] gave compounds **3–14**. 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. The microanalyses were performed by the Microanalytical Laboratory of our department (Università di Camerino) and the elemental compositions of the compounds agreed to within ± 0.4% of the calculated value.

2-(2-Allylphenoxymethyl)-4,5-dihydro-1H-imidazolhydrochloride (12). 2-Allylphenol (0.52 g, 3.8 mmol) was added to a solution of Na (0.15 g, 6.5 mmol) in abs EtOH (10 mL). After 1 h, imidazoline **15**[16] as hydrochloride (0.3 g, 1.9 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 $CHCl_3$ (40 mL) and washed with H_2O (15 mL), 3 N NaOH (3 × 15 mL) and H_2O (15 mL). Removal of the dried solvent gave 0.053 g (12% yield) of **12** as the free base, which was transformed into the hydrochloride salt with HCl gas in abs EtOH and purified by recrystallization. ($C_{13}H_{16}N_2O_2$·HCl) C,H,N.

Similarly compounds **3–8**,[17-22] **9–11,13**,[23] and **14**[21] were obtained from the corresponding phenol and imidazolines (**15** or **16**)[16] (Scheme 1).

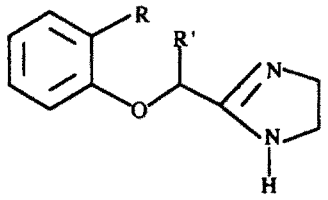
The physical characteristics of all the compounds are reported in Table 3.

Pharmacology

Rabbit aorta. Freshly isolated aorta (male New Zealand rabbits, 2.5–3.0 kg) was freed of fat and connective tissue. Spirally cut strips were placed in 20 mL organ baths containing a solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.3, and glucose 11. The solution was aerated with 95% O_2–5% CO_2. The bath temperature was 37 °C and the loading tension of the strips was 2.0 g. Contraction were recorded isometrically by means of a force transducer connected to a two-channel Gemini polygraph (Basile, Comerio, Italy). After 2 h of equilibration, agonist dose–response curves were obtained in a cumulative manner, the first one being discarded and the second one taken as a control. Dissociation constants and relative efficacies were determined as previously described according to the method of Furchgott and Bursztyn.[13] After the control dose–response curve, the preparation was treated with an adequate amount of dibenamine (0.3 mM, 15 min) to occlude a fraction of the receptors. The tissue was then washed for 20 min and a new dose–response curve constructed for the dibenamine-treated tissue. Several equipotent doses of the agonist before (*A*) and after (*A'*) dibenamine treatment were determined graphically. 1/*A* was plotted versus 1/*A'* and the points were fitted to a straight line by linear regression analyses. The dissociation constant (K_A) was calculated from the slope of the regression line and the intercept on the ordinate scale. In some experiments K_A values were shown to be independent of the percentage of receptor inactivation since further incubation with the irreversible antagonist gave the same results. The efficacy (e_r) of the agonist under study relative to that of cirazoline was evaluated by the ratio RACz:RAx, where RACz and RAx are the percentages of the receptor to be occupied by cirazoline and the compound under study respectively, to elicit 50% of the maximal response. pD_2(–log ED_{50}), i.a. (intrinsic activity, defined as the height of maximum contraction referred to that of cirazoline which is taken equal to 1) and pK_A values are the mean ± SE of at least five experiments.

Transient expression. Cos-7 cell tranfection was performed as previously described.[24] Cos-7 cells were grown to 60% confluency on 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10%) and glutamine (2 mM). Each plate was then incubated with 7 mL of a DNA mixture containing DEAE-dextrane (0.25 mg mL^{-1}), Tris–HCl (100 mM, pH 7.4), and DNA (1.6 μg mL^{-1}),

Table 3. Physicochemical characteristics of compounds **3–14**



Compd	R	R'	Formula	Recrytn solvent	mp °C[a]	y[b] %	^{1}H NMR(DMSO)	Analyses[c]
3	H	H	$C_{10}H_{12}N_2O\cdot HCl$	EtOH/Et_2O	135–137	30	δ 3.85 (4H, *s*, NCH_2CH_2); 5.13 (2H, *s*, OCH_2); 7.00–7.50 (5H, *m*, Ar-H)	C,H,N
4	CH_3	H	$C_{11}H_{14}N_2O\cdot HCl$	*i*-PrOH	187–190	22	δ 2.25 (3H, *s*, CH_3); 3.85 (4H, *s*, NCH_2CH_2); 5.13 (2H, *s*, OCH_2); 6.85–7.30 (4H, *m*, Ar-H)	C,H,N
5	CH_3	CH_3	$C_{12}H_{16}N_2O\cdot C_2H_2O_4$	EtOH	139–145	35	δ 1.61 (3H, *t*, $CHCH_3$); 2.25 (3H, *s*, $ArCH_3$); 3.88 (4H, *s*, NCH_2CH_2); 5.43 (1H, *q*, $CHCH_3$); 6.85–7.27 (4H, *m*, Ar-H)	C,H,N
6	C_2H_5	H	$C_{12}H_{16}N_2O\cdot HCl$	EtOH/Et_2O	95–98	9	δ 1.13 (3H, *t*, CH_3); 2.68 (2H, *q*, CH_2CH_3); 3.85 (4H, *s*, NCH_2CH_2); 5.13 (2H, *s*, OCH_2); 6.89–7.35 (4H, *m*, Ar-H)	C,H,N
7	*n*-C_3H_7	H	$C_{13}H_{18}N_2O\cdot HCl$	*i*-PrOH	169–171	17	δ 0.90 (3H, *t*, CH_3); 1.59 (2H, *m*, CH_2CH_3); 2.58 (2H, *t*, CH_2CH_2); 3.90 (4H, *s*, NCH_2CH_2); 5.12 (2H, *s* , OCH_2); 6.90–7.32 (4H, *m*, Ar-H)	C,H,N
8	*i*-C_3H_7	H	$C_{13}H_{18}N_2O\cdot HCl$	*i*-PrOH	86–89	19	δ 1.18 [6H, *d*, $(CH_3)_2$]; 3.45 [1H, *m*, $CH(CH_3)_2$]; 3.91 (4H, *s*, NCH_2CH_2); 5.12 (2H, *s*, OCH_2); 6.90–7.40 (4H, *m*, Ar-H)	C,H,N
9	*i*-C_3H_7	CH_3	$C_{14}H_{20}N_2O\cdot HCl$	EtOH	181–183	26	δ 1.18 [6H, *d*, $(CH_3)_2$]; 1.62(3H, *d*, $CHCH_3$); 3.38 [1H, *m*, $CH(CH_3)_2$]; 3.90 (4H, *s*, NCH_2CH_2); 5.48 (1H, *q*, $OCHCH_3$); 6.90–7.38 (4H, *m*, Ar-H)	C,H,N
10	*sec*-C_4H_9	H	$C_{14}H_{20}N_2O\cdot C_2H_2O_4$	EtOH/Et_2O	189–192	15	δ 0.80 (3H, *t*, CH_2CH_3); 1.15 (3H, *d*, $CHCH_3$); 1.55 (2H, *m*, CH_2CH_3); 3.28 (1H, *m*, $CHCH_2$); 3.90 (4H, *s*, NCH_2CH_2); 5.08 (2H, *s*, OCH_2); 6.90–7.25 (4H, *m*, Ar-H)	C,H,N
11	*ter*-C_4H_9	H	$C_{14}H_{20}N_2O\cdot HCl$	EtOH/Et_2O	207–211	18	δ 1.32 [9H, *s*, $(CH_3)_3$]; 3.92 (4H, *s*, NCH_2CH_2); 5.11 (2H, *s*, OCH_2); 6.95–7.30 (4, *m*, Ar-H)	C,H,N
12	$CH_2CH{=}CH_2$	H	$C_{13}H_{16}N_2O_2\cdot HCl$	EtOH/Et_2O	127–129	12	δ 3.48 (2H, *d*, CH_2CH); 3.92 (4H, *s*, NCH_2CH_2); 5.05 (2H, *m*, $CH{=}CH_2$); 5.12 (2H, *s*, OCH_2); 5.98 (1H, *m*, CH_2CH); 6.97–7.30 (4H, *m*, Ar-H)	C,H,N
13	OCH_3	H	$C_{11}H_{14}N_2O_2\cdot HCl$	EtOH/Et_2O	151–154	35	δ 3.81 (3H, *s*, OCH_3); 3.89 (4H, *s*, NCH_2CH_2); 5.06 (2H, *s*, OCH_2); 6.88–7.25 (4H, *m*, Ar-H)	C,H,N
14	O*i*-C_3H_7	H	$C_{13}H_{18}N_2O_2\cdot C_2H_2O_4$	EtOH/Et_2O	156–158	49	δ 1.28 [6H, *d*, $(CH_3)_2$]; 3.38 (4H, *s*, NCH_2CH_2); 4.60 [1H, *m*, $CH(CH_3)_2$]; 5.03 (2H, *s*, OCH_2); 6.85–7.11 (4H, *m*, Ar-H)	C,H,N

[a] The heating rate was 1°C min^{-1}.
[b] Yield.
[c] Analyses for C, H, and N were within ± 0.4% of the theoretical value required.

encoding α_{2A}- α_{2B}- α_{2C}- α_{2D}-adrenergic receptor gene, in DMEM, supplemented as above. Following 16-h incubation at 37 °C, the DNA mixture was removed and cells exposed to 10% dimethyl sulfoxide (v/v) in phosphate buffered saline (pH 7.4) for 3 min at room temperature. The 10% dimethyl sulfoxide solution was then replaced with the supplemented DMEM containing 0.1 mM chloroquine, and the cells were incubated for 2 h at 37 °C. Fresh DMEM was then added and the cells were harvested after 48–72 h for membrane preparation.

Membrane preparations. Frozen rabbit kidney cortices were purchased from Pel-Freez (Rogers, Arkansas). Crude rabbit kidney cortex membranes were prepared as previously described.[25] Kidney cortices were immediately placed in buffer A (5 mM EDTA, 5 mM EGTA, 0.01 mM phenylmethylsulfonyl fluoride, 5 mM Tris[hydroxymethyl]aminomethane–HCl, pH 7.4, 4°C). The tissue was disrupted, minced and homogenized by eight passes in a Dounce homogenizer. The homogenate was filtered through two layers of cheese cloth mesh and centrifuged at 1000 *g* for 10 min at 4 °C (Sorvall RC 5B, type SS34 rotor) to yield a low-speed pellet. The supernatant was decanted and centrifuged at 47,800 *g* for 10 min at 4 °C to yield a high-speed pellet generally used for analysis of plasma membrane receptors. The high speed pellet was washed twice (40 mL) and resuspended by homogenization in ice-cold buffer B (0.6 mM EDTA, 5 mM $MgCl_2$, 0.01 mM PMSF, and 50 mM Tris–HCl, pH 7.4).

Cos-7 cell membranes were prepared as described previously.[26] Briefly, cells were washed twice with washing solution (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and harvested with a rubber policeman. Cells were pelleted at 4 °C at 200 *g* in Sorvall RT6000 centrifuge. The pellet was resuspended in 5 mL/dish of buffer A and homogenized in a Dounce homogenizer. The cell lysate was then centrifuged at 17,000 *g* (Sorvall RC5B, type SS34 rotor) for 15 min and the pellet resuspended in membrane buffer (50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 0.6 mM EDTA).

Binding studies. Radioligand binding studies were performed as previously described.[27] In competition binding studies, membranes (200–250 μg of protein) were incubated with 8–9 nM [^{3}H]idazoxan (IGRS) or [^{3}H]rauwolscine (α_2-adrenergic receptor) and increasing concentrations of competing ligands at 24 °C, in a total volume of 100 μL. The concentration of [^{3}H]rauwolscine in each experiment corresponded to the radioligand K_d determined for the particular receptor subtype/omologue. After 45 min incubation, the reaction was terminated by adding 4 mL of 100 mM Tris–HCl, pH 7.4, at 4 °C followed by rapid filtration over glass fiber filters. The filters were washed with 3 × 4 mL aliquots of buffer at 4 °C and placed in 7 mL of Ecoscint scintillation fluid. Radioactivity retained by the filter was determined in a liquid scintillation spectrometer with 50% efficiency. Non-specific binding was determined in the presence of 10 μM cirazoline (IGRS) and 10 μM rauwolscine (α_2-adrenergic receptor). Membranes used in studies with [^{3}H]idazoxan were preincubated with 10 μM rauwolscine to prevent [^{3}H]idazoxan binding to the α_2-adrenergic receptor. Binding data were analyzed utilizing a non-linear least square curve fitting procedure, LIGAND.[28] All binding studies were performed in fresh membrane preparation following determination of the protein concentration by the method of Lowry *et al.*[29] Inhibition constants K_i were derived from the Cheng–Prusoff equation,[30] $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and dissociation constant of radiolabeled ligand used, respectively. pK_i values are the mean ± SE of two to four separate experiments performed in duplicate.

In order to quantitate selectivity, selectivity indexes (SI) were calculated. SI is the antilog of the difference between pK_i at IGRS and pK_A at α_1- or pK_i at α_{2A-D}-adrenergic receptors.

Statistical evaluation. The results are expressed as the means ± S.E.M. Student's *t*-test was used to assess the statistical significance of the difference between two means.

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