α_2 -Adrenoceptor subtypes and imidazoline-like binding sites in the rat brain

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1 The binding of [³H]-yohimbine and [³H]-idazoxan to rat cortex and hippocampus is rapid, reversible and of high affinity. Saturation data indicate that a single population of binding sites exist for [³H]-yohimbine in the cortex (B_{max} 121 ± 10 fmol mg⁻¹ protein; K_d 5.2 ± 0.9 nM) and hippocampus (B_{max} 72 ± 6 fmol mg⁻¹ protein; K_d 5.8 ± 0.7 nM). [³H]-idazoxan labels one site in the cortex (B_{max} 87 ± 8 fmol mg⁻¹ protein; K_d 4.1 ± 0.9 nM) and hippocampus (B_{max} 30 ± 6 fmol mg⁻¹ protein; K_d 3.5 ± 0.5 nM), when 3μ M phentolamine is used to define non-specific binding. A second distinct [³H]-idazoxan binding site (B_{max} 110 ± 21 fmol mg⁻¹ protein; K_d 3.6 ± 0.07 nM) is identified in rat cortex if 0.3μ M cirazoline is used to define non-specific binding and 3μ M yohimbine is included to prevent binding to α_2 -adrenoceptors.

2 Displacement studies indicate that the α_1 -adrenoceptor antagonist prazosin and the 5-HT₁ ligands 8-OH-DPAT, RU 24969 and methysergide differentiate [³H]-yohimbine binding into two components; a high and low affinity site. In contrast the displacement of [³H]-idazoxan by each ligand was monophasic.

3 The affinities of 8-OH-DPAT, RU 24969 and methysergide determined against [³H]-idazoxan binding to the cortex and hippocampus correlate significantly with the binding site displaying low affinity for prazosin and previously designated α_{2A} . In contrast, a poor correlation exists for the high affinity site for prazosin designated α_{2B} .

4 [³H]-idazoxan, in the presence of 3μ M yohimbine, labels a site that displays high affinity towards cirazoline, naphazoline and guanabenz, but low affinity towards clonidine, *p*-aminoclonidine, adrenaline, noradrenaline and the α_2 -adrenoceptor antagonists yohimbine, rauwolscine, WY 26703 and BDF 6143.

5 The results of this study indicate that [³H]-yohimbine labels two sites; the α_{2A} - and α_{2B} -adrenoceptors whereas [³H]-idazoxan labels an α_2 -adrenoceptor with a profile consistent with the α_{2A} -adrenoceptor subtype. In addition, [³H]-idazoxan labels an imidazoline binding site in the rat cortex that is pharmacologically distinct from α_2 -adrenoceptors. The low affinity of clonidine and *p*-aminoclonidine indicates that the imidazoline-like binding site in rat cortex is different from the site labelled by [³H]-clonidine and [³H]-*p*-aminoclonidine in human, rat and bovine brain stem, providing evidence of potential heterogeneity within this class of binding sites.

Introduction

Evidence indicates that α_2 -adrenoceptors are heterogeneous. Prazosin inhibits [³H]-yohimbine binding in the rat CNS in a biphasic manner (Bylund, 1985), with an approximately 50 fold separation between the high affinity site (4–20 nM) and the low affinity site (200–1000 nM). This moderately high affinity of prazosin has led to some hesitation in defining the binding site labelled by [³H]-yohimbine as the α_2 -adrenoceptor subtype (Yamada *et al.*, 1980). However, the low affinity exhibited by yohimbine for truly 'high' affinity [³H]-prazosin (0.01–0.05 nM) sites suggests that [³H]-yohimbine binding is not to an α_1 -adrenoceptor site. This also means that the affinity ratio between yohimbine and prazosin is not as useful for differentiating between α_1 - and α_2 -adrenoceptor subtypes as originally suggested (Bylund & U'Prichard, 1983). A moderately high potency of prazosin at rat α_2 -adrenoceptors has also been demonstrated in functional studies of noradrenaline release in the rat submandibular gland (Turner *et al.*, 1984). On the basis of the high and low affinities for prazosin against [³H]-yohimbine, two subtypes of α_2 -adrenoceptors have been suggested; α_{2A} (yohimbine = rauwolscine $\gg$ prazosin) and α_{2B} (rauwolscine > yohimbine $\geq$ prazosin) (Nahorski *et al.*, 1985). Although originally proposed as a non-rodent (α_{2A}), rodent (α_{2B}) classification, both subtypes have been shown to exist in the rat cerebrum (Bylund, 1985) and human caudate (Petrash & Bylund, 1986). 'Selective tissues', such as human platelet

(α_{2A}) (Nahorski *et al.*, 1985), neonatal rat lung (α_{2B}) (Latifpour *et al.*, 1982) and selective cell lines HT29 (α_{2A}) and NG108-15 (α_{2B}) have also been described (Bylund *et al.*, 1988) which may be useful in further studies of the proposed subtypes.

Recent autoradiographical and pharmacological evidence has been presented suggesting that [³H]-idazoxan labels two distinct α_2 -adrenoceptor populations in the rat brain with high affinity, only one of which is labelled by [³H]-rauwolscine (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987). A non- α_2 -adrenoceptor site labelled by [³H]-idazoxan has been reported to exist in a number of peripheral tissues (Yablonsky *et al.*, 1988; Langin & Lafontan, 1989; Michel *et al.*, 1989; MacKinnon *et al.*, 1989). A central imidazoline preferring binding site labelled by [³H]-clonidine and [³H]-*p*-aminoclonidine has been identified in human, rat and bovine brain-stem (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989).

In this study we have examined the binding of [³H]-yohimbine and [³H]-idazoxan to α_2 -adrenoceptors in rat cerebrocortical and hippocampal membranes and [³H]-idazoxan binding to imidazoline-like binding sites in the rat cerebral cortex. The results suggest that the α_2 -adrenoceptor labelled by [³H]-idazoxan in the rat cortex and hippocampus correlates with the α_{2A} -adrenoceptor subtype, and that the imidazoline-like binding site in rat cortex is distinct from the imidazoline preferring binding site identified in human, rat and bovine brain stem. In addition, the affinities of the 5-HT_{1A} ligands 8-hydroxy-2-(di *n*-propylamino) (8-OH-DPAT), RU 24969 and methysergide were examined as we have shown previously that these 5-HT_{1A} ligands show selectivity for an α_2 -adrenoceptor site labelled by [³H]-yohimbine (Brown *et al.*, 1990).

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Methods

Membrane preparation

Male Sprague-Dawley rats (150–200 g) were killed by cervical dislocation, the brains rapidly removed and dissected on ice. Cerebral cortices or hippocampi (5 hippocampi/membrane preparation) were homogenized in 25 volumes of Tris buffer (50 mM Tris HCl, 5 mM EDTA; pH 7.4 at 25°C) using a polytron PT 10 (setting 10; 2 × 10 s bursts). The homogenate was then centrifuged at 49,000 *g* for 15 min at 4°C. After a second wash, the pellet obtained was resuspended in Tris assay buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C) before undergoing two further washes. The final pellet was resuspended in assay buffer for direct use in binding assays or stored under liquid nitrogen.

α_2 -Adrenoceptor binding assays

[³H]-yohimbine binding to rat cerebral membranes α_2 -Adrenoceptor competition binding assays were performed by incubating washed rat cerebral membranes (500 μ g protein) with [³H]-yohimbine (1.5 nM) in the presence or absence of a range of 12 concentrations of the competing ligand in a total volume of 500 μ l Tris assay buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C). Non-specific binding was defined as the concentration of bound ligand in the presence of 10 μ M phentolamine. Specific binding represented 60–70% of total binding at 1.5 nM [³H]-yohimbine. Following equilibrium (30 min at 25°C), bound radioactivity was separated from free by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel cell harvester and determined by liquid scintillation spectrometry. Saturation assays were performed under similar conditions using a range of concentrations of [³H]-yohimbine (0.1–15 nM).

[³H]-idazoxan binding to rat cerebral membranes Experiments were performed under identical conditions to [³H]-yohimbine binding assays, using a Tris HCl buffer system (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C). Membranes were incubated with [³H]-idazoxan (1 nM) to equilibrium (15 min) at 25°C in a final volume of 1 ml. Specific binding was determined using 3 μ M phentolamine and represented 50–57% of total binding at 1 nM [³H]-idazoxan (total d.p.m. 1975; non-specific d.p.m. 900). Saturation experiments were carried out under identical conditions with a range of concentrations of [³H]-idazoxan (0.1–12 nM). Membranes were collected through Whatman GF/B filters using a Brandel cell harvester and the radioactivity determined as described above. [³H]-idazoxan binding to non-adrenoceptor imidazoline binding sites was performed as described previously for the binding of [³H]-idazoxan with the exception that 3 μ M yohimbine was included to block binding to α_2 -adrenoceptors. In these assays non-specific binding was determined by use of 0.3 μ M cirazoline. In the presence of 3 μ M yohimbine total d.p.m. was reduced from 1975 to approximately 1000 d.p.m. and non-specific to 150 d.p.m.

Data analysis

Saturation analysis Fitting of data to the appropriate models was achieved with the Scafit non-linear regression analysis programme for LIGAND (Munson & Rodbard, 1980). Comparisons between different binding models were made using the extra sum of the squares principle as outlined by Munson & Rodbard (1980), and shown in equation 1.

$$F = \frac{(SS_1 - SS_2)/(d.f._1 - d.f._2)}{(SS_2/d.f._2)} \quad (1)$$

SS and d.f. refer to the residual sum of the squares and degrees of freedom associated with the two fits being compared. The subscript 1 is designated to the fit with highest number of degrees of freedom (i.e. the most complicated model of

analysis). Statistical significance of the F value was determined from a table of the percentage of the F distribution using d.f.₁ – d.f.₂ and d.f.₁ degrees of freedom as the numerator and denominator respectively.

Competition analysis The inhibition of the radioligands by competing ligands was analysed to estimate the IC₅₀ (concentration of competitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme (Michel & Whiting, 1988) specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness. When Hill coefficients were not significantly different from unity the concentration of competitor displacing 50% of specific binding (IC₅₀) was converted to an affinity constant (*K*_i) using the expression derived by Cheng & Prusoff (1973) and shown in equation 2. In this expression [L] and *K*_d represent the radioligand concentration and dissociation constant respectively.

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)} \quad (2)$$

All data were initially analysed assuming a one site model of radioligand binding. The data, with Hill coefficients less than unity, were then analysed assuming a two site model, and the results of the curves fitting were statistically compared with those of the one site fit by an F test. The two site model was accepted if the observed fit was significantly better (*P* < 0.05) than the one site fit.

Protein was determined by the method of Lowry with bovine serum albumin used as the standard (Lowry *et al.*, 1951).

Drugs

Reagents used were of the highest analytical grade. Compounds were kindly donated by their manufacturers, synthesized at Syntex (Palo Alto) (indicated by an asterisk) or purchased. The following compounds were used: [³H]-idazoxan (60 Ci mmol^{−1}) Amersham, U.K.; [³H]-yohimbine (89 Ci mmol^{−1}) Du Pont, U.K.; amiloride hydrochloride (Sigma); p-aminoclonidine hydrochloride (Research Biochemicals); BDF 6143 (4-chloro-2-[2-imidazoline-2-ylamino]-isoindoline) hydrochloride*; clonidine hydrochloride (Sigma); cirazoline*; 8-OH-DPAT (8-hydroxy-2 (di n-propylamino)-tetralin hydrobromide (Research Biochemicals); guanabenz acetate (Wyeth); guanfacine hydrochloride (Sandoz); 5-HT creatinine sulphate (Sigma); idazoxan hydrochloride (Reckitt & Colman); imiloxan hydrochloride (Syntex); methysergide bimeleate (Sandoz); naphazoline hydrochloride (Sigma); (−)-noradrenaline bitartrate (Sigma); phentolamine mesylate (Ciba); prazosin hydrochloride (Pfizer); rauwolscine hydrochloride (Carl Roth); RU 24969 (5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole (Roussel); WB4101 (2-[2,6-dimethoxy-phenoxyethyl]aminomethyl-1,4-benzodioxan dihydrochloride (Ward Blenkinsop); WY 26703 (N-methyl-N-(1,3,4,6,7,11b-hexahydro-2H-benzo- $\alpha\beta$ -quinolizin-2-yl)-s-butane-sulphonamide hydrochloride (Wyeth); yohimbine hydrochloride (Sigma).

Results

Saturation experiments carried out in the presence of 3 μ M or 10 μ M phentolamine, respectively, to define non-specific binding, were consistent with [³H]-idazoxan and [³H]-yohimbine each labelling a single population of high affinity sites in rat cortex and hippocampus (Table 1). Significant differences were observed in each tissue between the maximum number of [³H]-idazoxan and [³H]-yohimbine binding sites. The density of α_2 -adrenoceptors was higher with [³H]-yohimbine in each tissue. The affinity of each ligand however, did not differ between tissues. Saturation experiments repeated in

Table 1 Specific binding of [³H]-yohimbine and [³H]-idazoxan to rat cerebral cortex and rat hippocampus membranes

	[³ H]-yohimbine		[³ H]-idazoxan	
	K _d	B _{max}	K _d	B _{max}
Cortex	5.2 ± 0.9	121 ± 10	4.1 ± 0.9	87 ± 8
			3.6 ± 0.7	110 ± 21*
Hippocampus	5.8 ± 0.7	72 ± 6	3.5 ± 0.5	36 ± 6

Assays were performed as described in the methods section. Non-specific binding was defined with 10 μM phentolamine for [³H]-yohimbine binding and 3 μM phentolamine for [³H]-idazoxan binding to α₂-adrenoceptors. To determine [³H]-idazoxan binding to the imidazoline-like binding site, *saturation experiments were carried out in the presence of 3 μM yohimbine and non-specific binding at each ligand concentration defined with 0.3 μM cirazoline. Values shown were obtained using the non-linear iterative curve programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.mean of at least three experiments.

the presence of 3 μM yohimbine to block [³H]-idazoxan binding to α₂-adrenoceptors, revealed a second component of binding in the rat cortex, that was saturable, reversible and of high affinity (Table 1). The density of α₂-adrenoceptors and imidazoline-like binding sites labelled by [³H]-idazoxan in the rat cerebral cortex was not significantly different.

Displacement of [³H]-idazoxan binding from cortical and hippocampal membranes by prazosin was of low affinity and with Hill slopes close to unity (Figure 1). In contrast, inhibition of [³H]-yohimbine binding by prazosin was complex, with Hill slopes considerably less than unity (Figure 2). Computer analysis of the displacement curves indicated that prazosin displaced [³H]-yohimbine binding to hippocampal membranes with high (α_{2B}; pK_i 8.10 ± 0.3) and low (α_{2A}; pK_i 5.94 ± 0.08) affinity. The failure of prazosin to differentiate [³H]-idazoxan binding is thus unlikely to be due to the binding conditions employed but most likely represents an inherent difference in the two ligands.

[³H]-idazoxan and [³H]-yohimbine binding to rat cortex

The α₂-adrenoceptor antagonists examined inhibited [³H]-idazoxan and [³H]-yohimbine binding to the rat cortex with Hill slopes not significantly different from unity. A comparison of the affinities of α₂-adrenoceptor antagonists indicated that yohimbine, rauwolscine, WY 26703 and BDF 6143 dis-

Figure 1 Inhibition studies of [³H]-idazoxan binding to rat hippocampal membranes were conducted over a concentration-range of 1 × 10⁻¹⁰–1 × 10⁻⁴ M prazosin. Incubations were performed for 15 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 3 μM phentolamine. The data describe a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a one site fit.

Figure 2 Inhibition studies of [³H]-yohimbine binding to rat hippocampal membranes were conducted over a concentration-range of 1 × 10⁻¹⁰–1 × 10⁻⁴ M prazosin. Incubations were performed for 30 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10 μM phentolamine. The data describe a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit, *P* < 0.001. (1 site, SS₁ 62882; 2 site, SS₂ 2965; *F* value 91.1).

played higher affinity if determined against [³H]-yohimbine than against [³H]-idazoxan (Table 2). The difference in affinity was less marked for idazoxan and imiloxan.

Displacement of [³H]-idazoxan binding to rat cortex by the 5-HT₁ ligands 8-OH-DPAT, RU 24969, methysergide and 5-HT, was not complex, having Hill slopes not significantly different from unity. Inhibition data indicated that each ligand had low affinity towards the [³H]-idazoxan binding site. This contrasts with the complex displacement curves observed for the displacement of [³H]-yohimbine binding. Methysergide, RU 24969 and 8-OH-DPAT but not 5-HT showed biphasic displacement curves with a greater than two log order difference in affinity between their high affinity and low affinity sites. The rank order of potency for the higher affinity sites was methysergide > RU 24969 > 8-OH-DPAT.

Noradrenaline showed moderate affinity for [³H]-idazoxan binding (pIC₅₀ 6.70) and [³H]-yohimbine binding at both sites, α_{2B} (pIC₅₀ 5.73) and α_{2A} (pIC₅₀ 6.58). In each case the Hill slopes were significantly less than unity.

In contrast to the high affinity displayed by yohimbine, rauwolscine, WY 26703 and BDF 6143 towards the α₂-adrenoceptor labelled by [³H]-yohimbine and [³H]-idazoxan, these compounds were weak at displacing [³H]-idazoxan from the imidazoline binding site in the rat cortex. The low affinity of noradrenaline, adrenaline and 5-HT suggests that this site is neither an adrenoceptor nor a 5-HT binding site. The imidazoline related compounds, cirazoline, guanabenz and naphazoline displaced [³H]-idazoxan from this site with high affinity whereas guanfacine, clonidine, *p*-aminoclonidine and amiloride possessed only modest affinity. Cirazoline and guanabenz, under these conditions, displayed approximately three and one log order selectivity for the imidazoline binding site over α₂-adrenoceptors. The pharmacological profile of the imidazoline binding site is thus distinct from either the α_{2A}- or α_{2B}-adrenoceptor subtypes identified with [³H]-yohimbine or the α_{2A}-subtype labelled by [³H]-idazoxan.

[³H]-idazoxan and [³H]-yohimbine binding to rat hippocampus

The overall pharmacological profile of the compounds in displacing [³H]-yohimbine and [³H]-idazoxan binding to α₂-adrenoceptors in rat hippocampus was similar to that obtained for the binding to rat cortex. The imidazoline α₂-adrenoceptor antagonists idazoxan, BDF 6143 and the imidazole, imiloxan, were more potent at displacing [³H]-idazoxan than [³H]-yohimbine, whereas the non-imidazoline antagonists displayed higher affinity if determined against [³H]-yohimbine than [³H]-idazoxan (Table 3). The 5-HT₁ ligands 8-OH-DPAT, RU 24969 and methysergide, although

Table 2 Inhibition of [³H]-yohimbine and [³H]-idazoxan binding to rat cortex

Postulated sites	[³ H]-yohimbine		pK _i		[³ H]-idazoxan	
	α _{2B} %	α _{2A} %	α _{2A}	Imidazoline		
Yohimbine	7.90 ± 0.04		7.25 ± 0.07	5.28 ± 0.20		
Rauwolscline	8.15 ± 0.08		7.09 ± 0.03	4.97 ± 0.15		
WY 26703	8.04 ± 0.07		7.53 ± 0.03	4.79 ± 0.20		
Idazoxan	8.04 ± 0.04		8.12 ± 0.05	8.37 ± 0.03		
Imiloxan	7.10 ± 0.10		6.65 ± 0.12	5.18 ± 0.10		
BDF 6143	9.22 ± 0.07		8.65 ± 0.03	5.59 ± 0.2		
Oxymetazoline	6.92 ± 0.13	8.25 ± 0.20	7.50 ± 0.05	4.45 ± 0.05		
	40	60				
Prazosin	7.30 ± 0.11	6.00 ± 0.12	6.40 ± 0.06	4.84 ± 0.13		
	50	50				
5-HT		<4	<4	<4		
8-OH-DPAT	6.43 ± 0.04	4.97 ± 0.04	6.07 ± 0.08	5.24 ± 0.13		
	40	60				
RU 24969	6.70 ± 0.03	5.20 ± 0.07	6.02 ± 0.05	5.75 ± 0.13		
	40	60				
Methysergide	7.17 ± 0.08	5.35 ± 0.11	5.13 ± 0.05	4.05 ± 0.09		
	45	55				
Adrenaline	7.12 ± 0.13*		7.49 ± 0.11*	4.47 ± 0.16		
Noradrenaline	5.73 ± 0.20*	6.58 ± 0.25*	6.70 ± 0.13*	<4		
Amiloride	4.80 ± 0.1		5.05 ± 0.2	6.25 ± 0.19*		
Guanfacine	6.95 ± 0.05		7.00 ± 0.15	5.57 ± 0.15*		
Guanabenz	7.55 ± 0.15		7.70 ± 0.1	8.21 ± 0.17		
Naphazoline	7.21 ± 0.05		7.50 ± 0.2	7.33 ± 0.1*		
Cirazoline	6.03 ± 0.06		6.30 ± 0.05	8.59 ± 0.10		
Clonidine	7.85 ± 0.01*		8.00 ± 0.08*	6.27 ± 0.23*		
p-Aminoclonidine	8.11 ± 0.06*		8.35 ± 0.08*	5.23 ± 0.16*		

* nH < 1
The affinity values were obtained for specific [³H]-yohimbine and [³H]-idazoxan binding to rat cortex. Experiments were performed using 1–2 nM [³H]-yohimbine or [³H]-idazoxan and the concentration of drug that inhibited specific binding by 50% (IC₅₀) was obtained and converted to a K_i value using the relationship $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] represents the free ligand concentration and K_d = the equilibrium dissociation constant of the radioligand. Values shown are the mean pK_i ± s.e.mean of at least three experiments performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described under methods. A two site fit was accepted only if it was statistically better than a one site fit.
Where high and low affinity sites are found they are placed in the α_{2A} and α_{2B} categories on the basis of 1. The affinity of prazosin, 2, whether binding of the compound survives occlusion of the high affinity site by 10⁻⁷ prazosin (Brown *et al.*, 1990).

failing to differentiate [³H]-idazoxan binding to the hippocampus, clearly gave rise to biphasic displacement of [³H]-yohimbine binding. The low density of [³H]-idazoxan binding in the rat hippocampus made it difficult to characterize the imidazoline binding site in this tissue.
The K_i values of the compounds derived from the displacement of [³H]-idazoxan were compared with the values obtained for the sites labelled by [³H]-yohimbine (Figure 3). A significant correlation was found between the affinity of the compounds at the [³H]-idazoxan site and the α_{2A}-adrenoceptor subtype in both the cortex (r = 0.837; P < 0.001) and hippocampus (r = 0.821; P < 0.01). In contrast, a poor correlation was found for [³H]-yohimbine binding to the α_{2B}- site and the [³H]-idazoxan binding site in the cortex (r = 0.550) and hippocampus (r = 0.614). These results suggest that [³H]-idazoxan labels a site consistent with the α_{2A}-adrenoceptor subtype.

Discussion

The present study examining the binding of [³H]-idazoxan and [³H]-yohimbine provides additional evidence for heterogeneity of α₂-adrenoceptors and the existence of an imidazoline-like binding site in rat brain. Initial studies in rat cortex and hippocampal membranes show that the pK_i values for displacing [³H]-idazoxan or [³H]-yohimbine binding differ between the non-imidazoline and imidazoline related α₂-adrenoceptor antagonists. The non-imidazoline compounds show higher affinity for [³H]-yohimbine binding whereas the imidazolines show higher affinity for [³H]-idazoxan binding, suggesting differences in the α₂-adrenoceptor populations labelled by the two radioligands. Before this is

Table 3 Inhibition of [³H]-yohimbine and [³H]-idazoxan binding to rat hippocampus

	K _i (nM)		[³ H]-idazoxan
	[³ H]-yohimbine α _{2B} %	α _{2A} %	
Yohimbine	7.87 ± 0.07		7.29 ± 0.07
Rauwolscline	7.86 ± 0.06		7.15 ± 0.08
WY 6703	7.94 ± 0.07		7.70 ± 0.05
Idazoxan	7.67 ± 0.06		8.70 ± 0.05
Imiloxan	6.34 ± 0.07		—
BDF 6143	8.96 ± 0.04		9.59 ± 0.07
Prazosin	8.10 ± 0.3	5.94 ± 0.08	6.59 ± 0.37
	50	50	
5-HT	<5		
8-OH-DPAT	6.68 ± 0.08	4.79 ± 0.09	5.83 ± 0.15
	55	45	
RU 24969	7.50 ± 0.13	5.13 ± 0.07	6.04 ± 0.08
	50	50	
Methysergide	7.15 ± 0.06	4.69 ± 0.20	4.95 ± 0.05
	50	50	

The affinity values were obtained for specific [³H]-yohimbine and [³H]-idazoxan binding to rat hippocampus. Experiments were performed using 1–2 nM [³H]-yohimbine or [³H]-idazoxan and the concentration of drug that inhibited specific binding by 50% (IC₅₀) was obtained and converted to a K_i value using the relationship $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] represents the free ligand concentration and K_d = the equilibrium dissociation constant of the radioligand. Values shown are the mean pK_i ± s.e.mean of at least three experiments performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described in methods. A two site fit was accepted only if it was significantly better than a one site fit.

Figure 3 Pharmacological profile of the α_2 -adrenoceptor subtypes. The pK_i values of a number of compounds for α_{2A} - and α_{2B} -adrenoceptor subtypes are compared with the values obtained for the displacement of [³H]-idazoxan: (1) yohimbine; (2) rauwolscine; (3) WY 26703; (4) idazoxan; (5) imiloxan; (6) BDF 6143; (7) oxymetazoline; (8) prazosin; (9) 8-OH-DPAT; (10) RU 24969; (11) methysergide; (12) adrenaline; (13) noradrenaline; (14) amiloride; (15) guanfacine; (16) guanabenz; (17) naphazoline; (18) cirazoline. Correlations between the pK_i values for [³H]-idazoxan and the subtypes are as follows; $r = 0.837$, $P < 0.001$ for α_{2A} in cortex (3a); $r = 0.550$ for α_{2B} in cortex (3b); $r = 0.821$, $P < 0.01$ for α_{2A} in hippocampus (3c); $r = 0.614$ for α_{2B} in hippocampus (3d).

accepted as evidence for multiple sites several factors must be considered.

The range of densities of α_2 -adrenoceptor binding sites in the rat cortex reported in the literature is wide (90–242 fmol mg⁻¹ protein, Bylund & U'Prichard, 1983; Lane *et al.*, 1983), a finding probably related to different assay conditions and to membrane preparations being purified to different extents. In order to compare the binding of [³H]-idazoxan and [³H]-yohimbine, identical conditions were employed with regard to both membrane preparations and ligand binding. Under the present conditions (crude membrane preparation and Tris HCl buffer system: 50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C) the specific binding of [³H]-yohimbine (1–15 nM) and [³H]-idazoxan (1–12 nM) to membranes from rat cerebrum was rapid, reversible, saturable and of high affinity. Extensive washing of the membrane preparation with EDTA was used to remove endogenous Mg²⁺ and other divalent cations which can reduce the affinity of the radioligand and complicate analysis of displacement curves generated by competing drugs (Cheung *et al.*, 1982).

The α_2 -adrenoceptor has been shown to exist in different affinity states modulated by the presence of mono- and divalent cations and guanine nucleotides. ³H-agonist radioligands are affected whereas ³H-antagonist binding is generally little affected by these modulators. Lane *et al.* (1983) suggested that the binding of the antagonist [³H]-idazoxan is increased markedly in the presence of a high concentration of sodium ions (physiological buffer containing 119.2 mM sodium). However, they used a crude synaptosomal P₂-fraction prepared in sucrose followed by resuspension in physiological buffer. Apparent maximal [³H]-yohimbine binding to cortical membranes prepared in a similar manner was also increased in the presence of Na⁺ ions (Woodcock & Murley, 1982). Further investigation of this membrane preparation (Cheung *et al.*, 1984) revealed it to be comprised of a high proportion of intact synaptosomes containing endogenous noradrenaline, which could interfere with the subsequent α_2 -adrenoceptor binding assay. Sensitivity of [³H]-yohimbine binding to guanine nucleotides and mono- and divalent ions is not as marked with membranes prepared in hypotonic buffers.

In rabbit cerebral cortex, [³H]-yohimbine has been reported to label almost twice as many binding sites as does [³H]-idazoxan yet both ligands are reported to label an ' α_2 -adrenoceptor' binding site (Hamilton *et al.*, 1988). In this study both yohimbine and idazoxan bind to sites classifiable as an α_2 -adrenoceptor. The values obtained for [³H]-idazoxan binding were similar to those described for a rat P₂ cerebral membrane (Lane *et al.*, 1983). All the competing antagonists for the displacement of [³H]-idazoxan showed monophasic inhibition with Hill slopes not significantly different from unity. Prazosin, methysergide, 8-OH-DPAT and RU 24969 all showed low affinity towards the α_2 -adrenoceptor labelled by [³H]-idazoxan. However, the saturation studies indicated that [³H]-idazoxan labelled fewer binding sites in the rat cortex and hippocampus than did [³H]-yohimbine. Differences in the density (B_{max}) of α_2 -adrenoceptors labelled by the two ligands has been cited as evidence for heterogeneity within this class of receptor. The difference in B_{max} values obtained with the radioligands is consistent with the view that idazoxan binds to only one of the two sites labelled by [³H]-yohimbine. A number of compounds have been identified that show biphasic inhibition curves for the displacement of [³H]-yohimbine binding. Interestingly these compounds display monophasic displacement curves and with the exception of oxymetazoline only displace [³H]-idazoxan binding with low affinity. Thus affinities of these compounds for inhibiting [³H]-idazoxan binding show a good correlation with [³H]-yohimbine binding to the α_{2A} -subtype. Displacement studies suggest that α_{2A} and α_{2B} -adrenoceptors exist in approximately equal proportions in the hippocampus and 60:40% in favour of the α_{2A} in the cerebral cortex. Saturation studies demonstrated that [³H]-yohimbine labels twice as many sites as [³H]-idazoxan in the hippocampus consistent with [³H]-yohimbine labelling α_{2A} - and α_{2B} -adrenoceptors and [³H]-idazoxan binding to only the α_{2A} -adrenoceptor subtype. Similarly, the number of sites labelled by [³H]-idazoxan relative to [³H]-yohimbine in the cerebral cortex is in broad agreement with [³H]-idazoxan labelling the α_{2A} -adrenoceptor subtype.

Although [^3H]-idazoxan labels only a subpopulation of high affinity α_2 -adrenoceptors, it also labels a second site that is saturable, reversible and pharmacologically distinct from α_2 -adrenoceptors. In competition experiments specific binding of 1 nM [^3H]-idazoxan to α_2 -adrenoceptors in rat cerebral cortex varied between 50–57%. The remaining binding, minus a small percentage of non-specific binding, represents binding to the imidazoline-like sites and should approximate the number of α_2 -adrenoceptors. The identification of more selective imidazoline-like antagonists with low affinity at α_2 -adrenoceptors and the reverse will be required if accurate determination of the density of these two sites is to be achieved.

The observation that [^3H]-idazoxan labels two distinct populations of binding sites in rat brain (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987) is often used as evidence for heterogeneity within α_2 -adrenoceptors. These studies were carried out using a Na^+/K^+ buffer system. A possible explanation which could account for the large percentage of [^3H]-idazoxan binding reported by Boyajian *et al.* (1987), is that, under the conditions used, [^3H]-idazoxan binds preferentially to a proposed imidazoline-like site, similar to that reported to exist in rabbit adipocytes (Langin & Lafontan, 1989), rabbit urethral tissue (Yablonsky *et al.*, 1988; Yablonsky & Dausse, 1989) and hamster adipocytes (MacKinnon *et al.*, 1989). The low affinity of clonidine and *p*-aminoclonidine at the imidazoline-like binding sites in rat cerebral cortex indicates that this site is distinct from the imidazoline-like site labelled by [^3H]-clonidine and [^3H]-*p*-aminoclonidine in human, rat and bovine brain stem (Ernsberger *et al.*, 1987; Bricca *et al.*, 1988; 1989). Thus it is unlikely that the endogenous imidazoline substance, clonidine displacing substance (CDS) considered to act through the imidazoline-like binding site in the brain stem (Atlas *et al.*, 1987; Bousquet & Feldman, 1987) will act at the imidazoline-like site labelled by [^3H]-idazoxan in rat cerebral cortex. The functional importance of the

imidazoline-like binding site identified by [^3H]-idazoxan has still to be determined. Doxey *et al.* (1983) demonstrated that idazoxan antagonized guanabenz-induced contraction of the rat anococcygeus muscle which was not blocked by phentolamine or piperoxan. Further studies will be required to determine if the anococcygeus muscle represents a functional model for this imidazoline site.

The results of this study support the view that α_2 -adrenoceptor binding sites are not homogeneous. [^3H]-yohimbine labels two sites in the rat brain both of which have the overall profile of an α_2 -adrenoceptor, although a number of significant differences were observed between the sites in the hippocampus and cortex. [^3H]-yohimbine binding to the α_{2A} -subtype correlates with the binding of [^3H]-idazoxan. The pharmacological profile obtained for the displacement of both ^3H -ligands to this site is similar; moderate affinity for NA, high affinity for the α_2 -adrenoceptor antagonists, yohimbine, rauwolsine and the imidazolines, idazoxan and imiloxan but low affinity for orazosin, 8-OH-DPAT, RU 24969 and methysergide.

The second binding site labelled by [^3H]-yohimbine, (α_{2B}), shows high affinity for the α_2 -adrenoceptor antagonists yohimbine and rauwolsine but lower affinity than the α_{2A} -subtype for NA although this is still within a range considered indicative of α_2 -adrenoceptors. The α_{2B} -subtype is further characterized as having high affinity towards 8-OH-DPAT, RU 24969 and methysergide. Recently 8-OH-DPAT has been shown to be a postsynaptic α_2 -adrenoceptor antagonist (Crist & Surprenant, 1987; pA_2 6.9–7.2).

Further investigations of the properties of α_2 -adrenoceptor subtypes in both membrane and soluble populations are needed. It will also be important to establish whether the existence of α_2 -adrenoceptor subtypes and the identification of a distinct non- α_2 -adrenoceptor imidazoline-like binding site in rat cortex are reflected by appropriate functional responses.

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