Biochemical Pharmacology, Vol. 38, No. 3, pp. 455–463, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

HIGH AFFINITY BINDING OF ^{3}H RAUWOLSCINE AND ^{3}H RX781094 TO α_2 ADRENERGIC RECEPTORS AND NON-STEREOSELECTIVE SITES IN HUMAN AND RABBIT BRAIN CORTEX MEMBRANES

ANDRE CONVENTS,* DANIEL CONVENTS,* JEAN-PAUL DE BACKER,*
JACQUES DE KEYSER† and GEORGES VAUQUELIN*

* Department of Protein Chemistry, Instituut voor Molekulaire Biologie and † Department of Neurology, Akademisch Ziekenhuis, Vrije Universiteit Brussel, Brussels, Belgium

(*Received* 29 *April* 1988; *accepted* 25 *August* 1988)

Abstract—The radiolabeled antagonists ^{3}H RX 781094 and ^{3}H rauwolscine bind with high affinity to α_2 adrenergic receptors as well as to non-receptor sites in human and rabbit brain cortex membranes. These non-receptor sites form an important contaminant of the specific binding when non-specific binding is determined in the presence of 10 μM phentolamine or more. While phentolamine is no suitable ligand to discriminate both sites, (−)-epinephrine displays a sufficient affinity ratio to separate radioligand binding to these sites. When 1 μM (−)-epinephrine is used for the determination of the non-specific binding, both radioligands bind specifically to α_2 receptors. Under these conditions, ^{3}H rauwolscine and ^{3}H RX 781094 bind to the same amount of non-cooperative sites; binding isotherms for human brain are B_{max} = 113 ± 15 fmol/mg protein and K_d = 22.8 ± 4.2 nM for ^{3}H RX781094 and B_{max} = 110 ± 17 fmol/mg protein and K_d = 4.7 ± 2.5 nM for ^{3}H rauwolscine.

Competition binding experiments show, for both radioligands and in both species, the typical pharmacological potency order of α_2 adrenergic receptors, i.e. phentolamine > yohimbine > prazosin for the antagonists and UK 14304 > *p*-aminoclonidine ≥ (−)-epinephrine > (+)-epinephrine > isoproterenol for the agonists. Whereas the α_2 receptor sites display high affinity and stereoselectivity towards (−)-epinephrine and (+)-epinephrine, the non-receptor sites bind both epinephrine isomers with equal low affinity. Specific binding of both radioligands to these sites can be determined when total binding is performed in the presence of 1 μM (−)-epinephrine and non-specific binding the presence of 1 mM phentolamine. ^{3}H rauwolscine binding to the non-stereoselective sites can be displaced with high affinity by 5-HT, suggesting binding to a 5-HT$_1$-receptor. The ^{3}H RX 781094 binding displays low affinity for most α adrenergic ligands and do not correspond to β adrenergic, dopaminergic or serotonergic receptors.

α_2 Adrenergic receptors are present in the central nervous system [1] as well as in a wide range of other tissues and blood platelets [2]. These receptors have been implicated in diverse physiological functions, including blood pressure [3], antinociception [4], locomotor activity [5], platelet aggregation [6], gastrointestinal motility and secretion [7], memory [8], anxiety [9] and sexual activity [10]. At the level of the cell membrane, these receptors have been demonstrated to mediate inhibition of the adenylate cyclase activity [1] and to stimulate Na^+/H^+-exchange [11].

Initial attempts to characterize these receptors directly by binding of the radiolabeled antagonist ^{3}H dihydroergocryptine were hampered by the lack of selectivity of this compound towards the α_1 and α_2 receptor subtypes [1]. More recently, successful characterization of the α_2 adrenergic receptors has been reported using more selective radiolabeled agonists such as ^{3}H clonidine [12] and ^{3}H UK 14304 [13] and antagonists such as ^{3}H yohimbine [14], ^{3}H rauwolscine [10] and ^{3}H RX 781094 [15]. Agonist saturation binding curves often display curvilinear Scatchard plots. This phenomenon is based on the agonist's ability to distinguish between two affinity states of the receptor: the receptor molecules that are coupled to the adenylate cyclase inhibitory protein (G_i) possess high affinity while the free receptors have low affinity. The complications inherent to agonist binding can be overcome by use of the α_2 selective antagonists since they bind with equal affinity to the total receptor population.

^{3}H Rauwolscine shows a 50-fold higher α_2/α_1 selectivity ratio than ^{3}H yohimbine so that the former radioligand appears to be more suitable for the specific labeling of α_2 receptors. Despite their quite different chemical structure ^{3}H rauwolscine and ^{3}H RX 781094 shows comparable affinity and a high α_2/α_1 selectivity. Nevertheless, a growing number of studies suggest that the α_2 adrenergic receptors may comprise subpopulations with different affinity for these radioligands [4, 7, 16, 17]. Recently, Boyajian *et al.* [18, 19] evidenced that the distribution in rat brain of α_2 receptors labeled by ^{3}H rauwolscine was distinct from that labeled by ^{3}H RX 781094. Moreover, these authors concluded that ^{3}H rauwolscine labeled only part of the ^{3}H RX 781094 binding sites. In this study we demonstrate that ^{3}H rauwolscine and ^{3}H RX 781094 bind to an equal number of α_2 adrenergic receptors in human and rabbit brain cortex membranes when non-specific binding is determined under appropriate conditions, i.e. in the presence of 1 μM (−)-epinephrine. However, when non-specific binding is measured in the presence of

Table 1. Saturation binding of ^{3}H rauwolscine and ^{3}H RX 781094 to phentolamine (10 μM) displaceable sites in human and rabbit brain cortex membranes

	Saturation binding characteristics for phentolamine displaceable binding:					
	^{3}H rauwolscine			^{3}H RX 781094		
Species	K_d	B_{max}	n_H	K_d	B_{max}	n_H
Human	7.5 ± 1.7	222 ± 17	0.96 ± 0.08	16.4 ± 5.6	164 ± 15	1.01 ± 0.03
Rabbit	10.0 ± 1.0	213 ± 29	1.00 ± 0.05	7.3 ± 2.9	160 ± 26	1.01 ± 0.05

Membranes were incubated with increasing concentrations of ^{3}H rauwolscine or ^{3}H RX 781094. Specific binding (total binding minus non-specific binding, determined in the presence of 10 μM phentolamine) was analysed by nonlinear least square fitting using LIGAND. The resulting K_d and B_{max} values are expressed in nM and fmol/mg of protein respectively, and n_H is the calculated Hill coefficient. The saturation binding data are expressed as means and SEM of three experiments.

10 μM phentolamine, a condition often reported in the literature, the specific binding of both radioligands also includes high affinity sites which are distinct from adrenergic receptors.

MATERIALS AND METHODS

Materials. ^{3}H2-(2-(1,4-benzodioxanyl)-2-imidazolin HCl, ^{3}H RX 781094 (i.e. ^{3}H idazoxan, 40 Ci/mmol) was obtained from Amersham (UK) and ^{3}H rauwolscine (74 Ci/mmol) from New England Nuclear (Boston, MA). (−)- and (+)-Epinephrine bitartrate, (−)-isoproterenol hydrochloride and (±)-propanolol hydrochloride were obtained from Sigma (St Louis, MO). Yohimbine hydrochloride was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). (+)-Butaclamol hydrochloride from Research Biochemical Inc. The following were obtained were obtained as generous gifts: phentolamine hydrochloride (Ciba Geigy, Switzerland), prazosin hydrochloride (Pfizer Central Research, U.S.A.), UK 14304 tartrate (Pfizer Central Research), mianserin hydrochloride (Organon, The Netherlands), p-aminoclonidine hydrochloride (Boehringer Mannheim, F.R.G.), RX 781094 hydrochloride (Reckitt and Colman, U.K.) and SCH 23390 maleate (Schering Corporation, Belgium).

Membrane preparation. Rabbit brains were obtained in a local slaughterhouse and kept in ice during transportation. Human brains were provided by the University Hospital. Brains from three individuals aged 65–74, who died suddenly from heart attacks were removed within 5 hr after death and immediately frozen at −30°. All manipulations were performed at 0–4°. The cerebral cortex area was dissected and homogenized with an ultraturax for 15 sec in 10 vol. of 10 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$/0.25 M sucrose (sucrose buffer). This suspension was further homogenized with a motor-driven Potter Elvehjem homogenizer (10 strokes at maximum speed). The homogenate was centrifuged at 2000 *g* for 15 min. The pellet was resuspended in sucrose buffer and centrifuged at 2000 *g*. All supernatants were pooled and centrifuged at 29,000 *g* for 20 min. The resulting pellets were washed three times by centrifugation as above, suspended in 50 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$ containing 10% (v/v) glycerol and stored in liquid nitrogen. Protein concentrations were determined according to Lowry *et al.* [20] using bovine serum albumin as standard.

Binding of ^{3}H *rauwolscine and* ^{3}H *RX* 781094. The binding was performed as described previously for calf retina membranes [17]. Briefly, membrane protein (1 mg/ml) was incubated with the indicated concentration of ^{3}H rauwolscine or ^{3}H RX 781094 for 15 min at 37° in 50 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$ in a final volume of 500 μl. At the end of the incubation, the samples were filtered under reduced pressure through glass fiber filter (Whatman GF/B) and rapidly washed four times with 4 ml of ice-cold buffer. The amount of radioligand remaining on the filters was determined by liquid scintillation counting. Non-specific binding was obtained as described in Results.

Data analysis. Binding isotherms were analysed by nonlinear least square curve fitting with the program "LIGAND" [21] to determine the maximum number of sites (B_{max}) and equilibrium dissociation constants (K_d or K_i).

RESULTS

Characterization of α_2 adrenergic receptors by binding of the radiolabeled antagonists ^{3}H RX 781094 and ^{3}H rauwolscine is often carried out using 10 μM phentolamine for the determination of non-specific binding [11, 22, 23]. Under these conditions, saturation binding of both radioligands occurs to one class of non-cooperative sites on membrane preparations from rabbit and human brain cortex (Table 1). However, the number of binding sites for ^{3}H rauwolscine exceeds those for ^{3}H RX 781094 by approximately 35% in both membrane preparations. This excess is significant in both preparations (*t*-tests yield $P < 0.001$ for human brain and $P < 0.05$ for rabbit brain). Competition binding experiments were performed to investigate this discrepancy.

The competition binding curves for phentolamine and for the agonists (−)- and (+)-epinephrine are similar for human and rabbit brain cortex membrane preparations. As illustrated in Fig. 1 for human brain, maximal displacement of ^{3}H rauwolscine and ^{3}H RX 781094 binding is only achieved in the presence of 1 mM phentolamine. Moreover, the competition binding curves of phentolamine are shallow ($n_H = 0.64$ for ^{3}H rauwolscine and $n_H = 0.47$ for ^{3}H RX 781094, non-specific binding being measured in the presence of 1 mM phentolamine), suggesting the presence of sites with different affinity for this α adrenergic antagonist. Nonlinear regression analysis

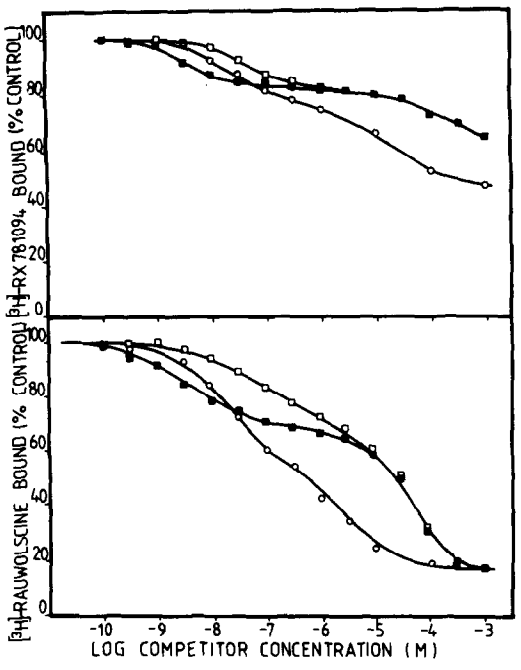

Fig. 1. Phentolamine and (−)- and (+)-epinephrine competition binding curves on human brain cortex. Membranes were incubated with 15 nM ^{3}H RX 781094 (upper panel) or 5 nM ^{3}H rauwolscine (lower panel) in the presence of increasing concentrations of phentolamine (○), (−)-epinephrine (■) and (+)-epinephrine (□). Binding shown corresponds to total binding and is expressed in percentage of control bindings, i.e. binding in presence of buffer only. Data shown are means of three experiments, each performed on a different membrane preparation and in duplicate. The mean standard deviations of the percentages averaged 3%.

indicates significant fitting of these curves to a two site model, both being present in about equal amount (Table 2). The competition binding curves of the agonist (−)-epinephrine and its enantiomer (+)-epinephrine are shallow as well (Fig. 1). Whereas epinephrine displays stereoselectivity at concentrations below 1 μM, both curves become superimposable at higher concentrations. Two site analysis of these curves reveals equal proportions of stereoselective and non-stereoselective components of the total displaceable binding (Table 2).

The stereoselective sites can be characterised using 1 μM (−)-epinephrine for the determination of non-specific binding since its competition curve displays a distinct plateau at this concentration. Alternatively, the phentolamine displaceable, non-stereoselective sites can be characterized by determination of the total binding in the presence of 1 μM (−)-epinephrine and non-specific binding in the presence of 1 mM phentolamine. Since stereoselectivity is a crucial property of the α_2 adrenergic receptors, the ^{3}H rauwolscine and ^{3}H RX 781094 saturation binding experiments were reassessed accordingly. As a typical example, Fig. 2 depicts the ^{3}H rauwolscine and ^{3}H RX 781094 saturation binding curves for human cortex membranes, determined either in buffer alone, in the presence of 1 μM (−)-epinephrine or in the presence of 1 mM phentolamine. Interestingly, binding in the presence of 1 μM (−)-epinephrine does not increase linearly with the radioligand concentration. Hence, the saturation binding parameters of the stereoselective sites can only be calculated adequately if the non-specific binding is determined for every radioligand concentration. The binding parameters of ^{3}H rauwolscine and ^{3}H RX 781094 for the stereoselective sites are given in Table 3. They behave as a single class of non-cooperative sites and the B_{max} values for both radioligands are now identical, i.e. approximately 110 fmol/mg protein in human cortex and 120 fmol/mg protein in rabbit cortex. In contrast to the saturation binding curve obtained in the presence of 1 μM (−)-epinephrine, binding in the presence of 1 mM phentolamine increases linearly with the radioligand concentration (Fig. 2). The binding parameters of ^{3}H rauwolscine and ^{3}H RX 781094 for the non-stereoselective sites are given in Table 4. The linear

Table 2. Phentolamine and (−)- and (+)-epinephrine competition binding parameters in human and rabbit brain cortex

	Binding parameters for competing with:					
	^{3}H rauwolscine			^{3}H RX 781094		
Compound	K_h (nM)	K_l (μM)	R_h (%)	K_h (nM)	K_l (μM)	R_h (%)
Human						
Phentolamine	4.9 ± 0.8	0.75 ± 0.05	53 ± 8	23 ± 2	17 ± 2	50 ± 7
(−)-Epinephrine	5.9 ± 1.1	55–20	44 ± 6	4.9 ± 2.5	68 ± 7	50 ± 3
(+)-Epinephrine	77 ± 23	60 ± 15	48 ± 4	34 ± 6	146 ± 75	46 ± 3
Rabbit						
Phentolamine	3.7 ± 0.3	1.3 ± 0.6	49 ± 6	8.2 ± 0.9	10 ± 2	31 ± 7
(−)-Epinephrine	5.3 ± 0.6	45 ± 6	43 ± 5	3.5 ± 0.6	200 ± 69	43 ± 6
(+)-Epinephrine	44 ± 13	114 ± 45	50 ± 6	21 ± 9	400 ± 100	45 ± 4

Competition binding experiments were performed as in Fig. 1 and analysed by nonlinear regression analysis with LIGAND. All curves could be analysed according to a two site model ($P < 0.01$) to yield the percentage of high affinity sites (R_h) and the K_l values for the high (K_h) and low (K_l) affinity sites. The values are means and SEM of three experiments.

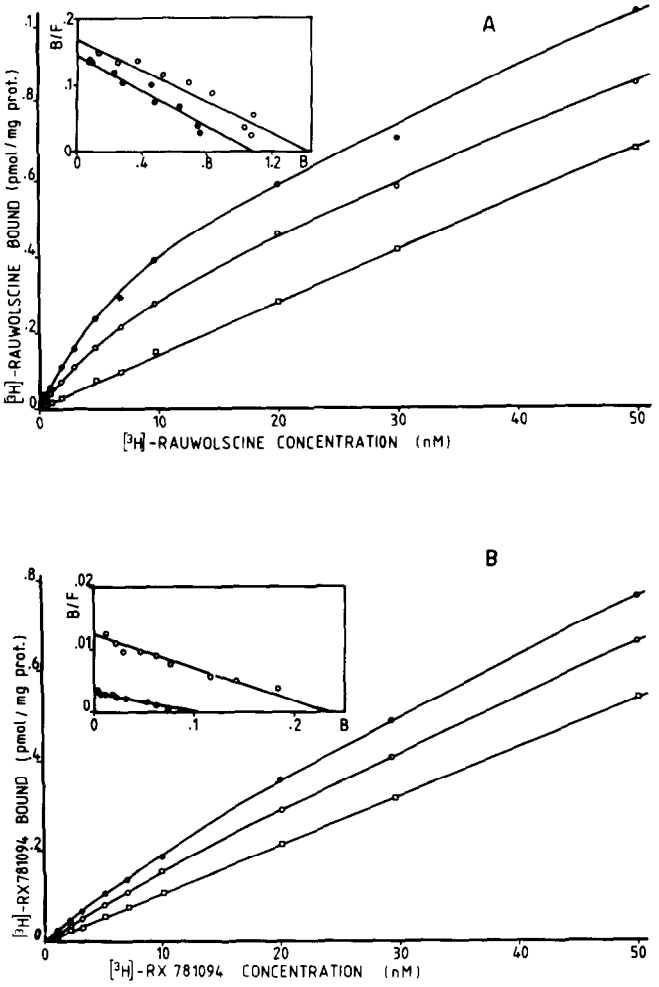

Fig. 2. Saturation binding curves for ^{3}H rauwolscine and ^{3}H RX 781094 on human brain cortex. Membranes were incubated with increasing concentrations of ^{3}H rauwolscine (A) or ^{3}H RX 781094 (B), either in buffer alone (●), in the presence of 1 μM (−)-epinephrine (○) or in the presence of 1 mM phentolamine (□). Insert: Scatchard plots of the saturation binding data in this figure. Specific binding of the radioligand (B, in pmol/mg protein) was calculated as follows: for the stereoselective sites (●): total binding minus binding in the presence of 1 μM (−)-epinephrine; for the non-stereoselective sites (○): binding in the presence of 1 μM (−)-epinephrine minus binding in the presence of 1 mM phentolamine). F is the concentration of free radioligand (in nM). The means ± SEM of the K_d and B_{max} values are given in Tables 3 and 4.

Table 3. Saturation binding of ^{3}H rauwolscine and ^{3}H RX 781094 to the stereoselective sites in human and rabbit brain cortex membranes.

Species	Saturation binding characteristics for the stereoselective sites (α_2 adrenergic receptors): ^{3}H rauwolscine			^{3}H RX 781094		
	K_d	B_{max}	n_H	K_d	B_{max}	n_H
Human	4.7 ± 2.5	110 ± 17	0.96 ± 0.05	22.8 ± 4.2	113 ± 15	1.00 ± 0.08
Rabbit	13.7 ± 2.0	120 ± 24	1.02 ± 0.03	18.5 ± 3.2	119 ± 16	0.96 ± 0.06

Binding was calculated as described in the legend of Fig. 2. The data were analysed according to the legend of Table 1. The resulting K_d and B_{max} values are expressed in nM and fmol/mg of protein respectively, and n_H is the calculated Hill coefficient. Values are means and SEM of three experiments.

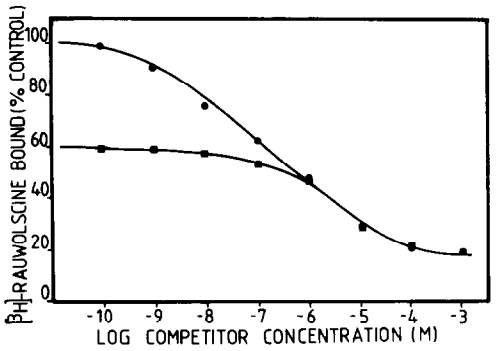

Fig. 3. Phentolamine ^{3}H rauwolscine competition binding in human cortex. Membranes were incubated with 5 nM ^{3}H rauwolscine and increasing concentrations of phentolamine either alone (●) or in the presence of 1 μM (−)-epinephrine (■). Binding shown corresponds to total binding and is expressed in percentage of control binding, i.e. binding in presence of buffer only. The standard deviations of the percentages averaged 4% (N = 3).

Scatchard plots ($r = 0.97$ and 0.98, respectively, Fig. 2) as well as the Hill coefficients ($n_H = 1.0$ and 1.1, respectively) are indicative for a single class of non-cooperative sites. The K_d values of ^{3}H rauwolscine and ^{3}H RX 781094 for the non-stereoselective sites are comparable to those for the stereoselective sites (Tables 3 and 4).

Competition binding studies were performed to characterize further the two populations of binding sites. Competition binding data for the stereoselective sites were calculated by subtracting the curves obtained in the presence of 1 μM (−)-epinephrine from those in the absence of the agonist. As an example, the phentolamine/^{3}H rauwolscine competition binding curve for the stereoselective sites in human cortex (Fig. 4A) is calculated from the original curves shown in Fig. 3. With the exception of the α_1 antagonist prazosin, the apparent K_i values of the investigated competitors were comparable, regardless of the radioligand and the species (Table 5). The K_i values for unlabeled RX 781094 and rauwolscine were similar to those obtained with their respective radioligands. The potency series for the adrenergic drugs are typical for α_2 adrenergic receptors, i.e. phentolamine > yohimbine > prazosin for the antagonists and UK 14304 > *p*-aminoclonidine ≥ (−)-epinephrine > (+)-epinephrine > isoproterenol for the agonists. The antagonist competition binding curves are steep with n_H values close to unity (Table 5). In contrast, agonist competition binding curves are shallow ($n_H < 1$). These data confirm that the stereoselective sites are identical for both radioligands and correspond to α_2 adrenergic receptors.

Binding properties for the non-stereoselective sites were evaluated by performing competition binding experiments in the presence of 1 μM (−)-epinephrine, non-specific binding being determined in the presence of 1 mM phentolamine. Typical examples of such competition binding curves are displayed in Fig. 4B and D. The K_i values determined with LIGAND, for most of the α adrenergic ligands (Table 6), are much higher than those expected for α_2 receptors. These data, along with the low affinity for prazosin, confirm the non-adrenergic character of the non-stereoselective sites in membranes from both human and rabbit brain cortex. Both radioligands, display for their respective cold ligands K_i values comparable to the K_d values obtained from saturation binding experiments. In contrast, the non-stereoselective sites of ^{3}H rauwolscine and ^{3}H RX 781094 show low affinity for RX 781094 and rauwolscine respectively. Moreover, the competition binding experiments with (±)-propranolol, Schering 23390, (+)-butaclamol and mianserin illustrate that the non-stereoselective sites do not correspond to β adrenergic, D_1 and D_2 dopaminergic or S_2 serotonergic receptors either (Table 6). Unlike ^{3}H RX 781094 binding, ^{3}H rauwolscine binding can be inhibited with nanomolar affinity by 5-HT, suggesting that the non-stereoselective sites correspond to 5-HT_1 receptors.

DISCUSSION

In this study we show that ^{3}H rauwolscine and ^{3}H RX 781094 bind with high affinity to α_2 adrenergic receptors as well as to non-adrenergic sites in membrane preparations from rabbit and human brain cortex. The agonist (−)-epinephrine displays a high affinity ratio for both sites (from about 8000 for ^{3}H rauwolscine in rabbit, to about 30,000 for ^{3}H RX 781094 in rabbit). (−)-Epinephrine ^{3}H rauwolscine as well as (−)-epinephrine/^{3}H RX 781094 competition binding curves are clearly biphasic with a plateau around 1 μM. When this concentration of agonist is used for the determination of non-specific binding, both radioligands can be demonstrated to specifically label the α_2 adrenergic receptors. These binding sites display affinity values and a phar-

Table 4. Saturation binding of ^{3}H rauwolscine and ^{3}H RX 781094 to the non-stereoselective sites in human and rabbit brain cortex membranes

	Saturation binding characteristics for the non-stereoselective sites: ^{3}H rauwolscine			^{3}H RX 781094		
Species	K_d	B_{max}	n_H	K_d	B_{max}	n_H
Human	9.9 ± 1.9	144 ± 30	1.11 ± 0.10	26.5 ± 3.4	236 ± 51	1.00 ± 0.05
Rabbit	9.0 ± 3.2	66 ± 14	0.98 ± 0.04	5.6 ± 1.4	105 ± 23	1.01 ± 0.03

Binding was calculated as described in the legend of Fig. 2. The data were analysed and according to the legend of Table 1. The resulting K_d and B_{max} values are expressed in nM and fmol/mg of protein respectively, and n_H is the calculated Hill coefficient. Values are means and SEM of three experiments.

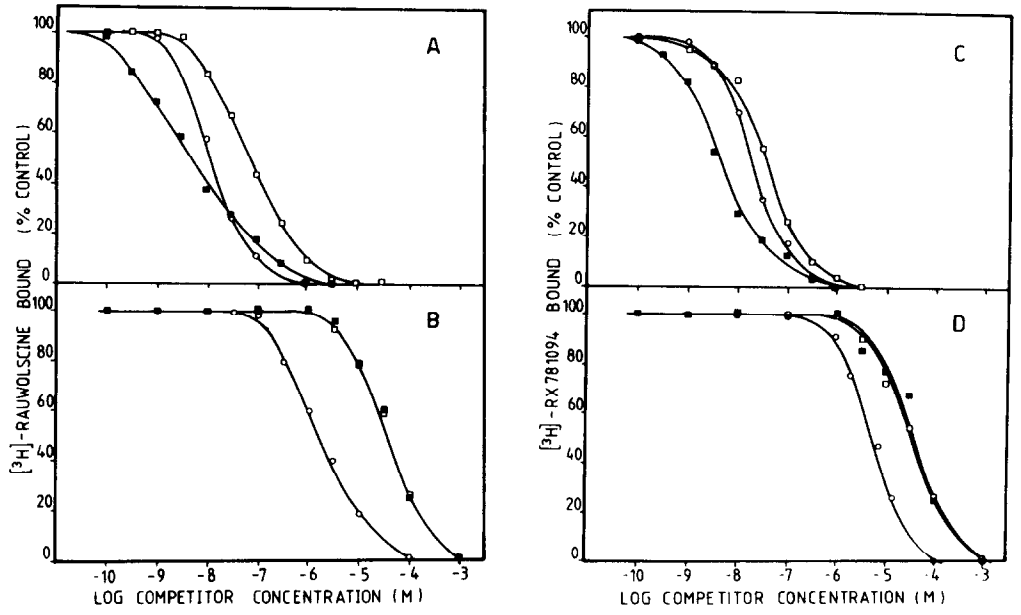

Fig. 4. Phentolamine and (−)- and (+)-epinephrine competition binding curves for the stereoselective and non-stereoselective sites on human brain cortex. Membranes were incubated with 5 nM ^{3}H rauwolscine (A and B) or 15 nM ^{3}H RX 781094 (C and D) and increasing concentrations of (−)-epinephrine (■), (+)-epinephrine (□) and phentolamine (○) either alone or in the presence of 1 μM (−)-epinephrine. A and C: Binding to the stereoselective sites (i.e. binding in the presence of competitor minus binding in the presence of the same concentration of competitor plus 1 μM (−)-epinephrine) is expressed in percentage of control binding, i.e. binding in presence of buffer only minus binding in the presence of 1 μM. (−)-Epinephrine. The competition binding parameters are given in Table 5. B and D: Binding to the non-stereoselective sites (i.e. binding in the presence of competitor and 1 μM (−)-epinephrine minus binding in the presence of the same concentration of competitor and 1 mM phentolamine) is expressed in percentage of control binding; i.e. binding in presence of 1 μM (−)-epinephrine minus binding in the presence of 1 mM phentolamine. The competition binding parameters are given in Table 6. The mean standard deviations of the percentages averaged 7% (N = 3).

macological rank order for different agonists and antagonists that is characteristic for α_2 receptors. These sites also show the required stereoselectivity for the epinephrine isomers. Whereas antagonist competition binding curves are steep, the agonist curves are shallow. This is a well described phenomenon for α_2 receptors, and can be interpreted by the ability of the receptors with high agonist affinity to

Table 5. Agonist and antagonist competition binding parameters for the stereoselective sites (α_2 adrenergic receptors) in human and rabbit brain cortex membranes

	Competition binding characteristics for the stereoselective sites (α_2 receptors) labeled with: ^{3}H rauwolscine				^{3}H RX 781094			
	Human		Rabbit		Human		Rabbit	
Compound	Apparent K_i	n_H	Apparent K_i	n_H	Apparent K_i	n_H	Apparent K_i	n_H
Rauwolscine	6.0	0.94	8.5	0.92	27	0.98	10	1.01
RX 781094	29	1.02	10	0.99	23	0.97	10	0.95
Phentolamine	4.4	1.04	3.5	0.93	20	1.19	3.6	0.93
Yohimbine	22	0.95	23	1.07	32	1.19	47	1.16
Prazosin	3700	1.02	9200	1.07	99,000	1.05	58,000	1.06
(−)-Epinephrine	5.1	0.73	5.7	0.65	3.2	0.77	3.1	0.74
(+)-Epinephrine	79	0.82	42	0.84	34	0.73	23	0.63
P-aminoclonidine	5.4	0.77	7.4	0.69	3.1	0.50	3.1	0.79
UK 14304	1.6	0.68	1.1	0.57	0.6	0.60	0.9	0.67
(−)-Isoproterenol	161	0.88	321	0.84	317	0.89	221	0.85

Competition binding data were calculated as described in the legend of Fig. 4. K_i (nM) values were determined by computer analysis with LIGAND. K_i values for the agonists are only apparent since curves deviate from the simple law of mass action. The values are means of two to three experiments.

Table 6. Agonist and antagonist competition binding parameters for the non-stereoselective sites in human and rabbit brain cortex membranes

	Competition binding characteristics for the non-stereoselective sites labeled with:			
	^{3}H rauwolscine (apparent K_i)		^{3}H RX 781094 (apparent K_i)	
Compound	Human	Rabbit	Human	Rabbit
Rauwolscine	12	8.5	>0.1 mM	>0.1 mM
RX 781094	2501	485	43	37
Phentolamine	680	840	5000	2300
Yohimbine	530	370	49,000	66,000
Prazosin	4400	9200	99,000	58,000
(−)-Epinephrine	41,000	69,000	51,000	>0.1 mM
(+)-Epinephrine	54,000	>0.1 mM	41,000	>0.1 mM
P-aminoclonidine	1600	2500	27,000	36,000
UK 14307	1700	1900	59,000	5,000
(−)-Isoproterenol	>0.1 mM	>0.1 mM	>0.1 mM	>0.1 mM
(±)-Propanolol	250	100	28,000	7,200
Schering 23390	290	460	85,000	2,000
(+)-Butaclamol	230	180	47,000	32,000
Mianserine	390	280	13,000	26,000
5-HT	1.9	2.6	>0.1 mM	>0.1 mM

Competition binding data were calculated as described in the legend of Fig. 4. K_i (nM) values were determined by computer analysis with LIGAND. Values are means from two to three experiments.

undergo functional coupling to the adenylate cyclase inhibitory component G_i while the low agonist affinity sites reflect the uncoupled receptors [24]. This hypothesis is strengthened by our observation that the entire receptor population displays low agonist affinity in the presence of guanine nucleotides (data not shown), known to provoke dissociation of the receptor G_i complex.

In both species, saturation binding studies with ^{3}H rauwolscine and ^{3}H RX 781094 yield the same number of α_2 adrenergic receptors. Since the non-adrenergic sites in rabbit and human cortex display high affinity for both radioligands as well, the non-specific binding determined in the presence of 1 μM (−)-epinephrine does not increase linearly with the radioligand concentration. Accordingly, adequate calculation of the saturation binding isotherms for the α_2 receptors requires the determination of non-specific binding, and its subtraction from total binding at every concentration of radioligand investigated. This implies also that computer assisted analysis of saturation binding curves with programs like "LIGAND" will provide incorrect binding isotherms if the non-specific binding is chosen by the computer as a linear function of the radioligand concentration. Because of its higher affinity and lower non-specific binding, ^{3}H rauwolscine appears to be more suitable than ^{3}H RX 781094 for the investigation of α_2 receptors in rabbit and human brain.

The non-adrenergic, high affinity sites for ^{3}H rauwolscine and ^{3}H RX 781094 can be characterized when total binding is measured in the presence of 1 μM (−)-epinephrine and non-specific binding in the presence of 1 mM phentolamine. Although the K_d values of both radioligands for these sites are comparable to those for the α_2 adrenergic receptors, they lack the desired stereoselectivity for epinephrine and display only low affinity for α adrenergic agonists and certain antagonists such as phentolamine. In both human and rabbit cortex membranes, the non adrenergic ^{3}H rauwolscine binding sites are in approximately 50% excess over those labeled with ^{3}H RX 781094 and both sites display marked differences in affinity for several of the drugs tested.

The non-stereoselective sites of ^{3}H rauwolscine might correspond to a 5-HT_1 receptor, as reflected by the nanomolar affinity by 5-HT [4]. 5-HT_1 Receptors are currently subdivided into four subclasses: 5-$HT_{1A\text{ to }D}$. Very recently Broadhurst *et al.* [16] suggested that ^{3}H rauwolscine binding to 5-HT_{1A} in rat brain interferes with the binding to α_2 adrenergic receptors. These findings were based on biphasic ^{3}H rauwolscine competition binding curves with spiroxatrine. On the other hand, rauwolscine has also been proposed to be a 5-HT_{1D} ligand [25], and affinity of spiroxatrine has not been tested for the 5-HT_{1D} subtype. Full characterization of binding properties of these non-stereoselective sites in human and rabbit brain will thus be required to determine the exact 5-HT_1 subtype of these sites (manuscript in preparation).

The non-stereoselective sites of ^{3}H RX 781094 do not correspond to any classical receptor and are distinct from the earlier described "imidazoline" binding sites [23] since imidazole-4-acetic acid failed to displace bound radioligand (data not shown). The nature of these sites still remains unclear, but due to the high affinity for ^{3}H RX 781094, it cannot be excluded that they might be responsible for some clinical effects produced by these classes of antagonists.

At least in human and rabbit cortex membrane preparations, α_2 adrenergic receptors cannot be investigated properly when the non-specific binding

is measured in the presence of 10 μM phentolamine, a condition often reported in the literature [14, 22, 26, 27]. Indeed, the K_i values of phentolamine for the α_2 receptors and the non-adrenergic sites are less than 10 μM. With an affinity ratio for both sites of less than a thousand, phentolamine does not provide an adequate discrimination at any other concentration either. Since our findings were similar in other buffer systems (no Mg^{2+}, data not shown), the inappropriate use of phentolamine for the determination of non-specific binding might also be the cause for some puzzling results. In this context, Boyajian *et al.* [18, 19] recently reported differences in the autoradiographic distribution of ^{3}H rauwolscine and ^{3}H RX 781094 binding sites in rat brain. Moreover, these workers concluded that ^{3}H rauwolscine only binds to a subclass of the α_2 adrenergic receptors, identified by binding of ^{3}H RX 781094. Since non-specific binding was measured in the presence of 10 μM phentolamine, the possibility arises that their specific binding also included non-adrenergic sites. Taking into account that ^{3}H rauwolscine is able to bind with high affinity to 5-HT_1 sites in human, rabbit and rat brain, these sites might be responsible for the observed differences in regional distribution of ^{3}H rauwolscine and ^{3}H RX 781094 binding.

In contrast, the use of phentolamine for the determination of non-specific binding might be appropriate in certain experimental conditions. This is well illustrated by a recent study on calf retina membranes [23]. In this tissue, both ^{3}H rauwolscine and ^{3}H RX 781094 specifically labeled α_2 adrenergic receptors when non-specific binding was determined in the presence of 10 μM phentolamine, whereas ^{3}H rauwolscine labeled additional sites as well when non-specific binding was recorded in the presence of (−)-epinephrine. These additional sites in calf retina membranes are clearly distinct from the non-adrenergic ^{3}H rauwolscine binding sites present on human or rabbit cortex membranes [17]. Taken together, these studies clearly stress the importance of a correct evaluation of the non-specific binding whenever a new tissue is taken for the investigation of α_2 receptors.

Dickinson *et al.* [22] recently reported that human α_2 receptors might differ from those present in tissues from other mammalian species, including the rabbit. In this study, however, we have failed to observe major pharmacological differences between the receptors present in human and rabbit cortex, by using both ^{3}H rauwolscine and ^{3}H RX 781094 as radioligands. Moreover, the number of receptor sites is about equal for both species. These findings indicate that the rabbit can be used as a suitable model system to investigate the interaction between α_2 adrenergic receptors and potential therapeutic drugs. The non-adrenergic sites, present in rabbit and in human brain cortex, also appear to be similar.

Acknowledgements—We are very grateful to Professor Ebinger of the University Hospital in Jette for his cooperation in obtaining human brains.

This work was supported by Astra-Nobelpharma, by the Solvay-Tournay Foundation for Medical Research, and by grants from the Fonds voor Geneeskundig en Wetenschappelijk Onderzoek and Lotto Belgium.

A.C. is a beneficiary of a Research Fellowship of the Instituut tot aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw, Belgium. G.V. in onderzoeksleider of the Nationaal Fonds voor Wetenschappelijk Onderzoek, Belgium.

REFERENCES

1. Timmermans PMWM and van Zwieten PA, α_2 Adrenoceptors: classification, localization, mechanisms and target for drugs. *J Med Chem* **25**: 1389–1401, 1982.
2. Michel T, Hoffman BB and Lefkowitz RJ, Differential regulation of the α_2 adrenergic receptor by sodium and guanine nucleotides. *Nature (Lond)* **288**: 709–711, 1980.
3. Fildes RD, Eisner GM, Alcagna PC and Jose PA, Renal adrenoceptors and sodium secretion in the dog. *Am J Physiol* **248**: F128–F133, 1985.
4. Jones SC and Gibhart GF, Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: mediation by spinal α_2 adrenoceptors. *Brain Res* **364**: 315–330, 1986.
5. Nomua Y, Oki K and Segawa T, Pharmacological characterization of control α adrenoceptor which mediate clonidine induced locomoter hyperactivity in the developing rat. *Naunyn-Schmiedberg's Arch Pharmacol* **311**: 41–44, 1980.
6. Kerry R, Scrutton MC and Wallis RB, Mammalian platelet adrenoceptors. *Br J Pharmacol* **81**: 91–102, 1984.
7. Chang EB, Field M and Miller RJ, α_2 Adrenergic receptor regulation of transport in rabbit ileum. *Am J Physiol* **242**: G237–G242, 1982.
8. Arnsten AF and Goldman-Rakic PS, α_2 Adrenergic mechanisms in prefrontal cortex is associated with cognitive decline in aged non human primates. *Science* **230**: 1273–1276, 1985.
9. Pellow S, Chopin P and File SE, Are the anxiogenic effects of yohimbine mediated by its action of benzodiazepine receptors? *Neurosci Lett* **55**: 5–9, 1985.
10. Clark JT, Smith CR and Davidson JM, Enhancement of sexual motivation in male rats by yohimbine. *Science* **225**: 847–849, 1984.
11. Nunnari JM, Repaske MG, Brandon S, Crague EJ and Limbird JE, Regulation of procine brain α_2 adrenergic receptors Na^+, H^+ and inhibitors of Na^+/H^+ exchange. *J Biol Chem* **262**: 12387–12392, 1987.
12. Asakura M, Tsukamoto T, Inaguku J, Matsui H, Ina M and Hesegawa K, Quantitative analyses of rat brain α_2 receptors discriminated by ^{3}H clonidine on ^{3}H rauwolscine. *Eur J Pharmacol* **106**: 141–147, 1985.
13. Loftus D, Stolk J and U'Prichard DC, Binding of the imidazoline UK 14304, a putative full α_2 adrenoceptor agonist, to rat cerebral cortex membranes. *Life Sci* **35**: 63–69, 1984.
14. Latifpour J, Jones SB and Bylund DB, Characterization of ^{3}H yohimbine binding to putative α_2 adrenergic receptors in neonatal rat lung. *J Pharmacol Exp Ther* **223**: 606–611, 1982.
15. Pimoulc C, Scatton B and Longer SZ, ^{3}H RX 781094: a new antagonist ligand labels α_2 adrenoceptors in the rat brain cortex. *Eur J Pharmacol* **95**: 78–85, 1983.
16. Broadhurst AM, Alexander BS and Wood MD, Heterogenous ^{3}H rauwolscine binding sites in rat cortex: two α_2 adrenoceptor subtypes or an additional non-adrenergic interaction? *Life Sci* **43**: 83–93, 1988.
17. Convents A, De Backer J-P and Vauquelin G, Characterization of α_2 adrenergic receptors of calf retina membranes by ^{3}H rauwolscine and ^{3}H RX 781094 binding. *Biochem Pharmacol* **36**: 2497–2503, 1987.
18. Boyajian CC and Leslie FM, Pharmacological evidence for α_2 adrenoceptor heterogeneity: differential binding properties of ^{3}H rauwolscine and ^{3}H idazoxan in rat

brain. *J Pharmacol Exp Ther* **241**: 1092–1098, 1987.
19. Boyajian CC, Lorighlen SE and Leslie FM, Anatomical evidence for α_2 adrenoceptor heterogeneity: differential autoradiographic distribution of ^{3}H idazoxan in rat brain. *J Pharmacol Exp Ther* **241**: 1079–1091, 1987.
20. Lowry OH, Rosebrough NJ, Farr AC and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
21. Munson PJ and Rodbard D, LIGAND: a versatile computerised approach for characterization of ligand binding systems. *Anal Biochem* **107**: 220–239, 1980.
22. Dickinson KEJ, McKerman RM, Miles CMM, Leys KS and Sever PS, Heterogeneity of mammalian α_2 adrenoceptors delineated by ^{3}H yohimbine binding. *Eur J Pharmacol* **120**: 258–293, 1986.
23. Ernsberger P, Meley MP, Mann JJ and Reis DJ, Clonidine binds to imidazole binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. *Eur J Pharmacol* **134**: 1–13, 1987.
24. Convents A, De Backer J-P, Convents D and Vauquelin G, Tight agonist binding may prevent the correct interpretation of agonist competition binding curves for α_2 adrenergic receptors. *Mol Pharmacol* **32**: 65–72, 1987.
25. Hoyer D, Functional correlates of serotonin 5-HT$_1$ recognition sites. *J Receptor Res* **8**: 59–81, 1988.
26. Byland DB, Comparison of ^{3}H clonidine and ^{3}H yohimbine binding: Possible subtypes of α_2 adrenergic receptors. *Pharmacologist* **23**: 215–220, 1981.
27. Cheung YD, Barnett DB and Nahorski , ^{3}H rauwolscine and ^{3}H yohimbine binding to rat cerebral and human platelet membranes: Possible heterogeneity of α_2 adrenoceptors. *Eur J Pharmacol* **84**: 78–85, 1982.