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CHARACTERIZATION OF ALPHA$_2$-ADRENERGIC RECEPTORS OF CALF RETINA MEMBRANES BY [^{3}H]-RAUWOLSCINE AND [^{3}H]-RX 781094 BINDING

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Abstract—Alpha$_2$-adrenergic receptors were identified in calf retina membranes by binding of the radiolabelled antagonists [^{3}H]-RX 781094 and [^{3}H]-rauwolscine. When 10 μM phentolamine was used to determine the non-specific binding, both radioligands labelled a single class of non-cooperative sites: B_{max} = 1051 ± 252 fmol/mg protein, K_d = 5.1 ± 1.5 nM for [^{3}H]-RX 78104 and B_{max} = 1167 ± 449 fmol/mg protein, K_d = 21.0 ± 4.1 nM for [^{3}H]-rauwolscine. Competition binding experiments showed the typical pharmacological potency order of alpha$_2$-adrenergic receptors, i.e. phentolamine > yohimbine > prazosin. Agonist competition binding curves revealed the presence of two receptor populations, having respectively high affinity (70% of the total receptor population) and low affinity for agonists, but with the same affinity for the antagonists. The high affinity sites could be converted into low affinity sites by guanine nucleotides.

The non-specific binding of [^{3}H]-RX 781094 was the same if 0.1 mM (−)-epinephrine was used instead of phentolamine. In contrast, the non-specific binding of [^{3}H]-rauwolscine was markedly lower with (−)-epinephrine than with phentolamine. Under this condition, the Scatchard plot of [^{3}H]-rauwolscine saturation binding was curvilinear, indicating the presence of low affinity sites for the radioligand in addition to alpha$_2$-adrenergic receptors. Competition binding experiments revealed that these low affinity sites were distinct from adrenergic receptors: the catecholamine agonists (−)- and (−)-epinephrine, (−)-norepinephrine, (−)-isoproterenol and dopamine competed with similar K_i values (μM range) whereas clonidine did not interact. Furthermore, these sites bound reserpine and the alpha$_2$-adrenergic antagonists yohimbine and rauwolscine but not phentolamine.

The vertebrate retina is developed embryologically from the brain and offers considerable advantages as a model system for the investigation of the morphology, physiology and biochemistry of specific neuronal systems [1]. Receptors for acetylcholine [2], GABA [3], dopamine [4] and epinephrine [5] have already been detected to be present in the bovine retina. Radioligand binding studies, performed with the tritiated drugs phentolamine and clonidine, revealed that the receptors for epinephrine were of the alpha$_2$-adrenergic subtype [6, 7]. However, phentolamine is a non-selective antagonist, capable of binding with similar affinity to alpha$_1$- and alpha$_2$-receptors [8]. On the other hand, the Scatchard plots of clonidine saturation binding experiments are often curvilinear since this partial agonist can distinguish between two affinity states of the alpha$_2$-adrenergic receptor: the receptor molecules that are coupled to the adenylate cyclase inhibitory protein (Ni) display high affinity while the free receptors have low affinity [9].

The complications inherent to binding of radiolabelled phentolamine and clonidine might be overcome by use of the recently introduced alpha$_2$-selective antagonists [^{3}H]-yohimbine [10], [^{3}H]-rauwolscine [11] and [^{3}H]-RX 781094 [12].† [^{3}H]-rauwolscine shows a 50-fold higher alpha$_2$/alpha$_1$ selectivity so that this radioligand appears to be more suitable for the specific labelling of alpha$_2$-receptors. Despite their quite different chemical structure, [^{3}H]-rauwolscine and [^{3}H]-RX 781094 show comparable affinity and selectivity towards alpha$_2$-receptors.

In this study, we demonstrate that [^{3}H]-rauwolscine and [^{3}H]-RX 781094 display high affinity towards alpha$_2$-adrenergic receptors in bovine retina membranes if 10 μM phentolamine is used for the determination of non-specific binding. However, when 0.1 mM (−)-epinephrine is used instead of phentolamine, [^{3}H]-rauwolscine will also label low affinity sites which are distinct from alpha$_2$-receptors.

MATERIALS AND METHODS

Materials. [^{3}H]-2-(2-(1,4-benzodioxanyl))-2-imidazolin HCl, [^{3}H]-idazoxan or [^{3}H]-RX 781094 (60 Ci/mmol) was obtained from Amersham, U.K. and [^{3}H]-rauwolscine (88.7 Ci/mmol) from New England Nuclear. (−)- and (+)-epinephrine bitartrate, (−)-norepinephrine bitartrate, clonidine hydrochloride, dopamine hydrochloride, (−)-isoproterenol hydrochloride, (−)-propanolol hydrochloride and reserpine hydrochloride were obtained from Sigma. Yohimbine hydrochloride was purchased from Aldrich Chemical Company Inc., rau-

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† Abbreviations used: RX 781094, 2-(2-(1,4-benzodioxanyl))-2-imidazolin HCl; Gpp(NH)p, guanylylimidodiphosphate.

wolscine hydrochloride from Carl Roth KG., (+)-butaclamol hydrochloride from Research Biochemical Inc. and guanylylimidodiphosphate from Boehringer Mannheim. The following were obtained as generous gifts: phentolamine hydrochloride (Ciba Geigy), prazosin hydrochloride (Pfizer Central Research), ketanserin (Janssen Pharmaceutica) and SCH 23390 (Schering Corporation).

Membrane preparation. Calf eyes were obtained and dissected in a local slaughterhouse. All subsequent steps were performed at 0–4°. Retinae were homogenized in 10 vol. of 10 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$/0.25 M sucrose 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 homogenized in sucrose buffer and re-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 at a protein concentration of approximately 10 mg/ml. Protein concentrations were determined according to Lowry *et al.* [13] using bovine serum albumin as the standard.

Binding of [^{3}H]-*rauwolscine and* [^{3}H]-*RX* 781094. Membrane protein (0.25–0.5 mg/ml) was incubated with the indicated concentrations of [^{3}H]-rauwolscine (1–100 nM for saturation binding, 20 nM for competition binding) or [^{3}H]-RX 781094 (0.1 to 20 nM, 5 nM for competition binding) for 15 min at 37° in 50 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$ in a final volume of 500 μl. Under these conditions, binding of 20 nM [^{3}H]-rauwolscine and 5 nM [^{3}H]-RX 781094 reached equilibrium within respectively 10 and 3 min. At the end of the incubation, the samples were diluted in 4 ml of ice-cold buffer, and filtered under reduced pressure through glass fiber filter (Whatman GF/B, 2.5 cm diameter). Filters were washed rapidly four times with 4 ml of ice-cold buffer, placed in 20 ml polyethylene scintillation vials with 1 ml of 0.1 N NaOH and 8 ml of scintillation fluid (Picofluor 15 from Packard) and counted in a Packard liquid scintillation spectrometer. Determinations were performed in duplicate for each experiment. Specific binding was obtained by subtracting the non-specific binding (i.e. binding in the presence of 10 μM unlabelled phentolamine or 0.1 mM (−)-epinephrine) from total binding. The radiochemical purity of both radioligands was checked on thin layer chromatography (silica gel 60 F254 from Merck), using the solvent systems chloroform–diethylamine (9:1) and methylene chloride–methanol (9:1) for [^{3}H]-rauwolscine, and chloroform–methanol–ammonium (91:8:1) for [^{3}H]-RX 781094. The batches with detectable impurities were discarded.

RESULTS

Alpha$_2$-adrenergic receptors can be characterized on calf retina membranes by binding of the radiolabelled antagonists [^{3}H]-RX 781094 and [^{3}H]-rauwolscine, if non-specific binding is determined under appropriate conditions. For [^{3}H]-RX 781094, the non-specific binding is identical when determined in the presence of 10 μM phentolamine and 0.1 mM (−)-epinephrine (Table 1). The specific binding occurs to one class of non-cooperative sites. The Scatchard plot is linear ($r = 0.998$) and the Hill coefficient equals unity (nH = 1.0). Scatchard analysis of [^{3}H]-RX 781094 saturation binding data yields an equilibrium dissociation constant of 5.1 ± 1.5 nM and the total number of binding sites (B_{max}) is 1051 ± 252 fmol/mg protein (five experiments, a typical example is illustrated in Fig. 1).

In contrast, (−)-epinephrine yielded appreciably lower non-specific binding than phentolamine if [^{3}H]-rauwolscine was used as radioligand (Fig. 2). Since binding of 20 nM [^{3}H]-rauwolscine in the presence of 0.1 mM (−)-epinephrine plus 10 μM phentolamine is the same as in the presence of the agonist alone (Table 1), the (−)-epinephrine displaceable sites can be divided into two components: 70% of the sites (at 20 nM [^{3}H]-rauwolscine) are also displaceable by phentolamine (i.e. E/P-sites) and the remaining sites are displaceable by the agonist only (i.e. E-sites). These E-sites are present in the calf retina membranes, since agonist and antagonists do not compete with the radioligand for binding to the filter (Table 1).

The [^{3}H]-rauwolscine binding characteristics for the E/P-sites can be determined by saturation binding experiments wherein non-specific binding is measured in the presence of 10 μM phentolamine (Fig. 2). The Scatchard plot is linear ($r = 0.994$), which is indicative for a single class of non coop-

Table 1. [^{3}H]-Rauwolscine and [^{3}H]-RX 781094 displacement by (−)-epinephrine and phentolamine

Compound added	Radioligand binding (c.p.m.) [^{3}H]-rauwolscine Total	[^{3}H]-rauwolscine Filter*	[^{3}H]-RX 781094 Total
None	5653 ± 29	897 ± 147	3860 ± 8
Phentolamine (10 μM)	2281 ± 31	8834 ± 105	492 ± 10
(−)-Epinephrine (0.1 mM)	1149 ± 51	790 ± 77	489 ± 25
Phentolamine (10 μM) + (−)-epinephrine (0.1 mM)	1143 ± 47	920 ± 113	487 ± 26

Membranes were incubated with 20 nM [^{3}H]-rauwolscine or 5 nM [^{3}H]-RX 781094 either alone or in the presence of the indicated concentrations of phentolamine (−)-epinephrine or a mixture of both. Values in c.p.m. are expressed as the mean and SEM of 3 experiments.

* Values for the filter refer to experiments wherein membranes were omitted from the incubation medium.

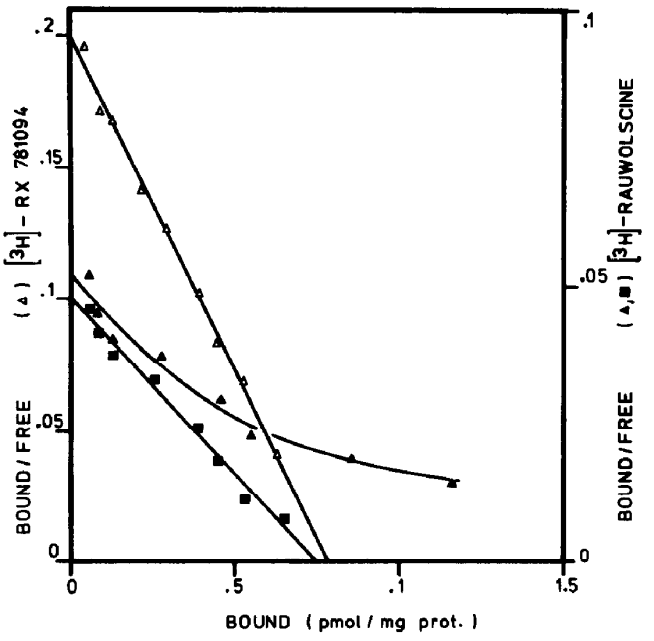

Fig. 1. Scatchard plots of [^{3}H]-RX 781094 and [^{3}H]-rauwolscine saturation binding to calf retina membranes. Membranes were incubated with increasing concentrations of radioligand as described in Materials and Methods. Specific binding of the radioligand (B, in pmol/mg protein) was calculated by subtracting non-specific binding (obtained in the presence of 10 μM phentolamine (■) or 0.1 mM (−)-epinephrine (▲) for [^{3}H]-rauwolscine and in the presence of 10 μM phentolamine for [^{3}H]-RX 781094) from total binding. F is the concentration of free radioligand (in nM). The Scatchard plot was linear for both radioligands if non-specific binding was measured in the presence of phentolamine. Linear regression analysis yielded equilibrium dissociation constants (K_d) of 21.0 ± 4.1 nM and 5.1 ± 1.5 nM and B_{max} values of 1167 ± 9 and 1051 ± 252 fmol/mg protein for [^{3}H]-rauwolscine and [^{3}H]-RX 781094, respectively. Data shown were obtained with the same membrane preparation. Mean data obtained in four to five similar experiments, each on a different membrane preparation, are in the text.

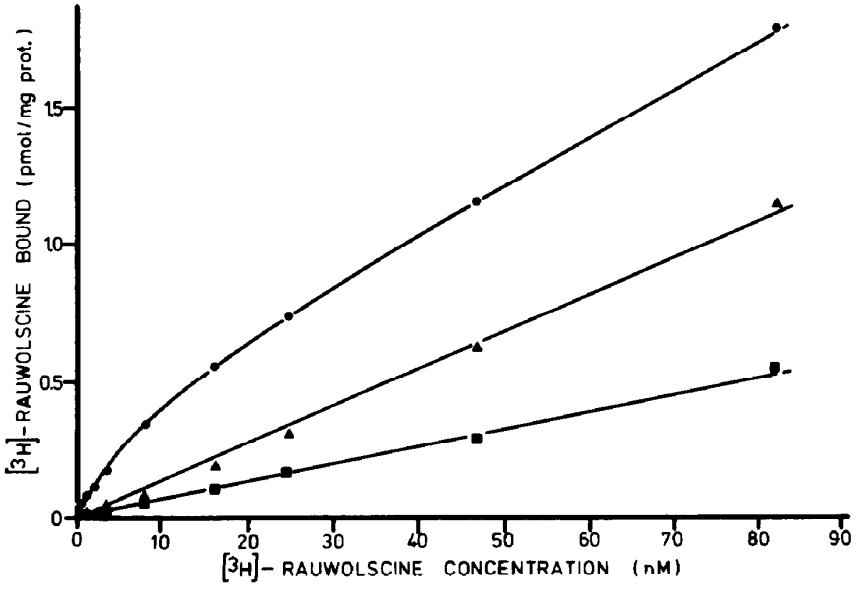

Fig. 2. Saturation binding of [^{3}H]-rauwolscine to calf retina membranes. Membranes were incubated with increasing concentrations of [^{3}H]-rauwolscine (0.7–100 nM) as described in Materials and Methods ●, total binding; ▲, "non-specific" binding measured in the presence of 10 μM phentolamine, ■, "non-specific" binding in the presence of 0.1 mM (−)-epinephrine. Data shown are means of three experiments.

erative sites. The equilibrium dissociation constant is 21.0 ± 4.1 nM and B_{max} is 1167 ± 449 fmol/mg protein. This latter value is close to the number of specific [^{3}H]-RX 781094 binding sites. Competition binding experiments confirm that the E/P-sites for [^{3}H]-rauwolscine and the specific [^{3}H]-RX 781094 binding sites are identical, and correspond to alpha$_2$-adrenergic receptors.

Competition binding data for the E/P-sites can be calculated by subtracting the [^{3}H]-rauwolscine competition binding curves, obtained in the presence of 10 μM phentolamine from those in the absence of phentolamine. As an example, the (−)-epinephrine competition binding curve for the E/P-sites (Fig. 4, ◇) is calculated from the original curves shown in Fig. 3. The potency series for adrenergic drugs are the same for the E/P-sites of [^{3}H]-rauwolscine and for the specific [^{3}H]-RX 781094 sites, and are typical for alpha$_2$-receptors [11, 12], i.e. phentolamine > yohimbine > prazosin for the antagonists (Table 2) and clonidine > (−)-epinephrine > (−)-isoproterenol for the agonists (Fig. 4, Table 2). Competition binding studies with [^{3}H]-RX 781094 and [^{3}H]-rauwolscine yield identical apparent K_i values for the alpha$_2$-selective drugs phentolamine, yohimbine, clonidine and (−)-epinephrine and only slightly different values for the alpha$_1$-antagonist prazosin and the beta-agonist (−)-isoproterenol (Table 2). Whereas the antagonist competition binding curves are steep, agonist curves are shallow (Fig. 4). Computer assisted analysis of these shallow curves, following a two-site model, using a program derived from Minneman *et al.* [14], showed comparable amounts of high affinity sites and K_is for both radioligands. (−)-Epinephrine and clonidine bind with high affinity to almost 70% of the sites (Table 3). Addition of 0.1 mM Gpp(NH)p to the incubation mixture converts high affinity sites into low affinity sites, resulting in a rightward shift of the agonist competition curves (Fig. 4, Table 3).

The [^{3}H]-rauwolscine binding characteristics for the E-sites can be determined by subtracting saturation binding in the presence of 0.1 mM (−)-epinephrine from those obtained in the presence of 10 μM phentolamine (Fig. 2). Both curves are linearly proportional to the [^{3}H]-rauwolscine concentration (r = 0.997 and r = 0.997, for concentrations between 1 and 80 nM, Fig. 2), suggesting that the E-sites possess low affinity and high capacity for the radioligand. Competition binding experiments with unlabelled rauwolscine are in agreement with this assumption.

Binding properties for the E-sites can be evaluated by performing competition binding in the presence of 10 μM phentolamine, non-specific binding being determined in the presence of 0.1 mM (−)-epinephrine. Under these conditions, specific binding still corresponds to about 1000 cpm of [^{3}H]-rauwolscine (Table 1). As shown in Table 4, the IC_{50} of unlabelled rauwolscine for the E-sites is 17 μM. Assuming that the K_d value for [^{3}H]-rauwolscine corresponds to the K_i value of the unlabelled compound, the equation of Cheng and Prusoff [15] can be simplified to the form: $K_d = IC_{50}$-L. Since the [^{3}H]-rauwolscine concentration (L = 20 nM) is well below the IC_{50} (17 μM), this latter value might be a good approximation for the K_d of the radioligand.

Catecholamine agonists such as (−)-epinephrine, (+)-epinephrine, (−)-isoproterenol, (−)-norepine-

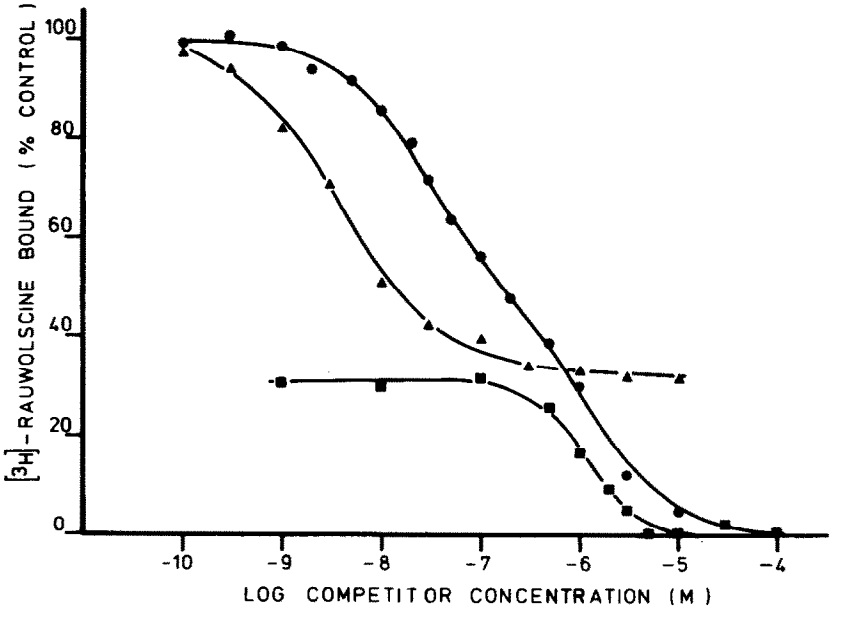

Fig. 3. Phentolamine- and (−)-epinephrine/[^{3}H]-rauwolscine competition binding. Membranes were incubated with 20 nM [^{3}H]-rauwolscine in the presence of increasing concentrations of phentolamine (▲), (−)-epinephrine (●) and (−)-epinephrine in the presence of 10 μM phentolamine (■). Binding shown corresponds to the difference between total binding and "non-specific" binding obtained in the presence of 0.1 mM (−)-epinephrine. Control binding (100%) was measured in the presence of buffer only. Data shown are means of three experiments.

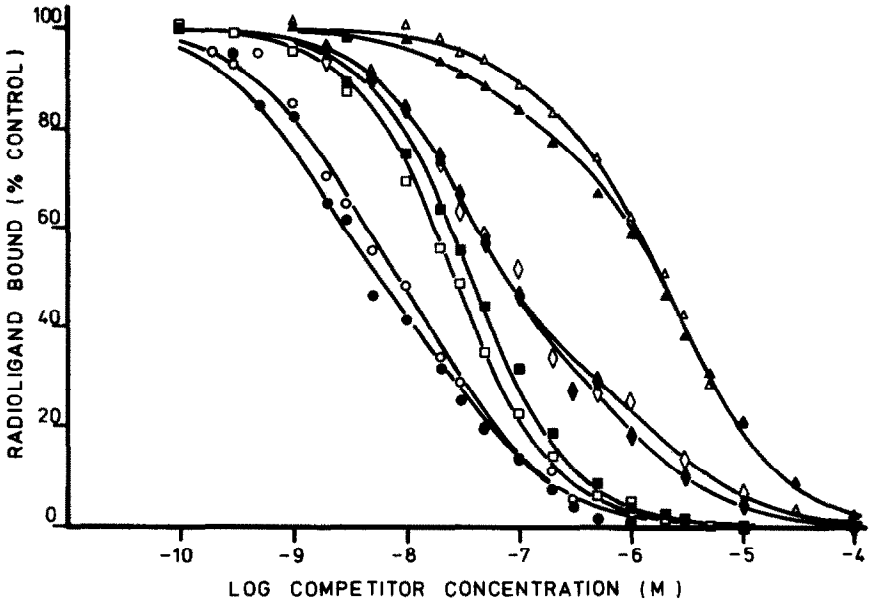

Fig. 4. Agonist competition binding for alpha$_2$-adrenergic receptors. Membranes were incubated with 20 nM [^{3}H]-rauwolscine (open symbols) or 5 nM [^{3}H]-RX 781094 (closed symbols) in the presence of increasing concentrations of (−)-epinephrine (◊, ♦), (−)-epinephrine plus 0.1 mM Gpp(NH)p (△, ▲), clonidine (○, ●) and clonidine plus 0.1 mM Gpp(NH)p (□, ■). Binding shown corresponds to total binding minus "non-specific" binding obtained in the presence of 10 μM phentolamine. For [^{3}H]-rauwolscine, "non-specific" binding was determined for each concentration of competitor and subtracted from total binding in the presence of the corresponding concentration of competitor. Control binding (100%) was measured in the presence of buffer only. Data shown are means of three experiments. Agonist binding parameters are given in Table 3.

phrine and dopamine yield steep competition binding curves, show comparable affinities for the E-sites and the presence of Gpp(NH)p does not affect their affinity (Table 3). [^{3}H]-rauwolscine binding cannot be displaced by (−)-propanolol, butaclamol, ketanserin, phentolamine, SCH 23390 and doxepine, so that the E-sites cannot be catalogued as alpha$_1$-, alpha$_2$- and beta-adrenergic, D_1- and D_2-dopaminergic and S_2-serotonergic receptors. On the other hand, reserpine which blocks the catecholamine uptake in granular storage vesicles [16], shows a similar IC_{50} value as the catecholamine agonists for competing with [^{3}H]-rauwolscine binding to the E-sites, i.e. $IC_{50} = 2.7\ \mu M$.

DISCUSSION

The radiolabelled alpha$_2$-adrenergic antagonists [^{3}H]-RX 781094 and [^{3}H]-rauwolscine bind to a single class of high affinity sites in calf retina membranes if

Table 2. Agonist and antagonist K_i values for alpha$_2$-adrenergic receptors

Compound	Competition binding characteristics for alpha$_2$-receptors labelled with: [^{3}H]-rauwolscine apparent K_i (nH)		[^{3}H]-RX 781094 apparent K_i (nH)	
Antagonists				
Phentolamine	4.4	0.91	5.1	1.10
Yohimbine	340	0.95	350	0.98
Prazosin	10,000	1.09	40,000	0.99
Agonists				
Clonidine	6.6	0.72	5.3	0.80
(−)-epinephrine	89	0.61	100	0.85
(−)-isoproterenol	20,000	0.71	6,200	0.62

Membranes were incubated with 20 nM [^{3}H]-rauwolscine or 5 nM [^{3}H]-RX 781094 in the presence of increasing concentrations of competitor. Specific binding to the alpha$_2$-receptors (E/P-sites for [^{3}H]-rauwolscine) was calculated as described in the legend of Fig. 4. K_i values were calculated from the competitor's IC_{50} values according to the method of Cheng and Prusoff [17]. K_i values for the agonists are only apparent since curves deviate from the simple law of mass action (nH < 1).

Table 3. Agonist binding parameters for alpha$_2$-adrenergic receptors: effect of Gpp(NH)p

Agonist	Binding parameters for agonists for competing with: [^{3}H]-rauwolscine R_h (%)	K_h (nM)	K_l (nM)	[^{3}H]-RX 781094 R_h (%)	K_h (nM)	K_l (nM)
(−)-Epinephrine	73 ± 6	13 ± 2	1320 ± 270	74 ± 1	24 ± 7	1150 ± 870
(−)-Epinephrine + Gpp(NH)p	17 ± 4	19 ± 13	2000 ± 600	21 ± 1	27 ± 9	1600 ± 50
Clonidine	66 ± 8	1.8 ± 0.9	33 ± 2	80 ± 2	1.4 ± 0.5	57 ± 1.2 20 ± 2
Clonidine + Gpp(NH)p	—	—	14 ± 1	—	—	

The agonist/[^{3}H]-rauwolscine and /[^{3}H]-RX 781094 competition binding data, shown in Fig. 4 were analysed according to a two-site model by the computerized method of Minneman *et al.* [14] to yield the percentage of high affinity sites (% R_h) and the agonist IC_{50} values for the high- and low-affinity sites. The corresponding K_i values (i.e. K_h and K_l) were calculated according to Cheng and Prusoff [15]. Values are means and SEM of three experiments.

10 μM phentolamine is used for the determination of non-specific binding (Fig. 1). These sites display affinity ratios for agonists and antagonists which are typical for alpha$_2$-adrenergic receptors (Table 2). For both radioligands, the number of receptor sites is about 1 pmol/mg protein. This is twice the amount of receptors originally reported by Bittiger *et al.* [6], and might be attributed to differences in the membrane preparation. As already noted in other membrane preparations such as brain cortex [17] and blood platelets [10], the alpha$_2$-receptors display steep competition binding curves for antagonists and shallow curves for agonists such as (−)-epinephrine and clonidine (Figs. 4 and 5). Whereas the receptors behave as a single class of sites with respect to antagonist binding, the shallow agonist curves can be explained by the ternary complex model, originally proposed by Hoffman and Lefkowitz [18]. This model stipulates that the receptor can adopt two different affinity states for agonist: the receptors which are functionally coupled to the adenylate cyclase inhibitory protein (Ni) display high affinity while the non-coupled receptors have low affinity. Approximately 70% of the receptors display high affinity for (−)-epinephrine as well as clonidine (Table 3). This indicates a high degree of alpha$_2$-receptor Ni coupling in the retina as compared to most other tissues [18–20].

In studies describing radioligand binding to alpha$_2$-adrenergic receptors, an excess of agonist is very often used for the determination of non-specific binding [7, 11, 21]. In our study, however, use of the agonist (−)-epinephrine results in an inappropriate identification of alpha$_2$-adrenergic receptors by [^{3}H]-rauwolscine binding. This is due to the ability of (−)-epinephrine to compete with [^{3}H]-rauwolscine for binding to non alpha$_2$-receptors (E-sites) in addition to the alpha$_2$-adrenergic receptors (E/P-sites). The K_d of [^{3}H]-rauwolscine for the E-sites (17 μM) is 800 times higher than the K_d of the radioligand for the alpha$_2$-receptor. The presence of these low affinity E-sites is responsible for the curvilinear Scatchard plot of [^{3}H]-rauwolscine saturation binding if (−)-epinephrine is used instead of phentolamine for determination of non-specific binding. Curvilinear Scatchard plots of [^{3}H]-rauwolscine saturation binding have also been reported by others in various tissues [17, 21–23], and it was suggested that some of the alpha$_2$-receptors might have low affinity for this radioligand. The E-sites in our study display, however, agonist competition binding characteristics which are quite different from those expected for

Table 4. Agonist and antagonist K_i values for the E-sites

	Competition binding characteristics for the E-sites labelled by (^{3}H)-rauwolscine apparent K_i	nH
(−)-Epinephrine	3.0	0.96
(−)-Epinephrine + Gpp(NH)p	2.3	1.02
(+)-Epinephrine	6.5	0.98
(−)-Norepinephrine	2.8	0.93
Dopamine	1.0	1.06
(−)-Isoproterenol	2.0	1.08
Reserpine	2.7	1.11
Yohimbine	17.2	1.04
Rauwolscine	17.4	0.92

Membranes were incubated with 20 nM [^{3}H]-rauwolscine in the presence of 10 μM phentolamine and increasing concentrations of the compounds listed. Binding corresponds to the difference between total binding and "non-specific" binding obtained in the presence of 10 µM phentolamine plus 0.1 mM (−)-epinephrine. Since the [^{3}H]-rauwolscine concentration is well below its estimated K_d value (17 μM), the competitor's K_i and IC_{50} values are equal.

alpha$_2$-adrenergic receptors (Table 4). These sites do not interact with clonidine, have similar affinities for catecholamines and do not show stereospecificity or Gpp(NH)p-modulation of (−)-epinephrine binding.

Competition binding experiments with several neurotransmitter receptor antagonists indicated that the E-sites did not correspond to alpha-adrenergic, beta-adrenergic, dopaminergic, and S_2-serotonergic receptors as well. The tricyclic antidepressant doxepine also fails to displace [^{3}H]-rauwolscine binding to the E-sites. Interestingly, [^{3}H]-rauwolscine binding is displaced by reserpine. It is known that reserpine acts as an antihypertensive drug by blocking a monoamine translocation mechanism. However, reserpine has in chromaffin vesicles from adrenal medulla [16] and in synaptic vesicles from bovine caudate nucleus [24] an affinity of 10 nM for the monoamine translocator, whereas the K_i of reserpine for the E-sites is about a 100-fold higher (μM range). Competition by reserpine is not unexpected since reserpine, yohimbine and rauwolscine are alkaloids of the Rauwolfia Serpentia with a similar structure. In this context, the higher affinity of reserpine versus rauwolscine might be related to the higher degree of substitution of reserpine. Further experiments are needed to find out if these E-sites have some physiological relevance. Preliminary data indicate that, E-sites are also present in calf brain cortex membranes but not in calf basal arteries. At a [^{3}H]-rauwolscine concentration of 20 nM, the difference between the non-specific binding determined with (−)-epinephrine and phentolamine was not significant in basal arteries, about 10% of total binding in brain cortex and 20% in calf retina.

Finally, the presence of E-sites has consequences for obtaining the correct competition binding data for alpha$_2$-receptors when rauwolscine is used as radioligand. Indeed, non-specific binding values with phentolamine must be subtracted from total binding for every concentration of competitor. Failure to do so will give rise to competition curves which will go below non-specific binding, especially for agonists. These problems are not encountered with RX 781094, since no interaction with E-sites occurs. This, together with the four times higher affinity of [^{3}H]-RX 781094 to alpha$_2$-receptor sites as compared to [^{3}H]-rauwolscine, makes [^{3}H]-RX 781094 a far more suitable radioligand for the investigation of alpha$_2$-receptors in calf retina. The high concentrations of alpha$_2$-receptors as well as the high degree of coupling of these receptors to Ni makes calf retina a very elegant model system for the investigation of the pharmacological properties of alpha$_2$-receptors in the central nervous system, as well as regulation mechanisms of receptor number.

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