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Tight Agonist Binding May Prevent the Correct Interpretation of Agonist Competition Binding Curves for α_2-Adrenergic Receptors

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SUMMARY

α_2-Adrenergic receptors in calf retina membranes can be specifically labeled with the tritiated antagonist ^{3}H-RX 781094. Saturation binding occurs to a single class of noncooperative sites. The number of sites amounts to 1070 ± 243 and 935 ± 178 fmol/mg of protein, and the equilibrium dissociation constants equal 1.8 ± 0.4 and 3.8 ± 0.3 nM at 25° and 37°, respectively. Binding is rapid, equilibrium being reached within 5 min, and is reversible. At both temperatures, (−)-epinephrine competition binding curves are shallow in the presence of magnesium ions. The curves, obtained for incubation periods varying between 5 and 60 min, are superimposable at 37°. Computer-assisted analysis indicates that approximately 75% of the receptors (R_H sites) display high agonist affinity for (−)-epinephrine as well as for the other agonists tested: (−)-norepinephrine, clonidine, and UK 14304. However, the (−)-epinephrine competition curves display a time-dependent leftward shift at 25°. This can be attributed to an increase in agonist affinity for the R_H sites. Addition of 0.1 mM Gpp(NH)p causes a marked steepening and rightward shift of the curves, at both 25 and 37°. These curves are superimposable for all of the incubation times tested. The nonequilibrium of agonist competition binding at 25° can be attributed to slow dissociation of the agonist (i.e., tight binding) when the receptor is coupled to the regulatory component N_i. This dissociation rate can be measured by preincubation of the membranes with 10 μM (−)-epinephrine, followed by extensive washing and incubation with ^{3}H-RX 781094 for increasing lengths of time. The first order rate of agonist dissociation (i.e., receptor recovery) is appreciably faster at 37° than at 25°: i.e., 0.029 min^{-1} and 0.0044 min^{-1}, respectively. These findings are confirmed by kinetic experiments using the radiolabeled agonist ^{3}H-UK 14304. Slow agonist dissociating kinetics may prevent the correct evaluation of the agonist binding parameters by computerized analysis of competition binding curves when the incubation time is too short, especially at low temperature.

Agonist interactions with α_2-adrenergic receptors are converted into cellular responses by a mechanism which involves an adenylate cyclase inhibition and a resulting decrease in intracellular cAMP levels (1). Three membrane-bound components play an active role in this process: the α_2 receptor, a nucleotide-binding regulatory protein (N_i), and the adenylate cyclase enzyme (2). The agonist-receptor interaction is assumed to be immediately followed by functional coupling of the receptor to N_i (3), whereas the antagonist-bound receptor does not undergo such coupling.

During the past few years, α_2-adrenergic receptors have been intensively characterized by binding of radiolabeled agonists such as [^{3}H]clonidine (4) and ^{3}H-UK 14304 (5), as well as antagonists such as [^{3}H]rauwolscine (6) and ^{3}H-RX 781094 (7). As for many other adenylate cyclase-associated receptors, these studies have revealed that, when a radiolabeled antagonist is used as radioligand, agonists display shallow agonist competition binding curves, whereas those for antagonists are steep (8). Since guanine nucleotides provoke dissociation of the receptor-N_i complex and induce a rightward shift and steepening of the agonist competition curves, it is assumed that the receptors display high agonist affinity when functionally coupled to N_i. Accordingly, the shallow curves suggest an apparent heterogeneity of the receptors with regard to their ability to undergo functional coupling to N_i. Two major computer-assisted techniques have been developed for the quantitation of the proportion of the receptors displaying high and low agonist affinity (R_H and R_L) and their respective equilibrium dissociation constants for the agonist (K_H and K_L) (9, 10). These parameters have, however, only molecular significance if the receptor interaction with all ligands is reversible and if the competition binding curve reflects an equilibrium situation.

It has already been well documented that agonists become

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ABBREVIATIONS: ^{3}H-RX 781094, ^{3}H-(2-(2-(1,4-benzodioxanyl))-2-imidazolin HCl; N_i, guanine nucleotide-binding inhibitory component of adenylate cyclase; N_s, guanine nucleotide-binding stimulatory component of adenylate cyclase; Gpp(NH)p, guanylyl-imidodiphosphate; EDTA, ethylenediaminetetraacetic acid.

tightly bound to β-adrenergic receptors when these latter are functionally coupled to the regulatory component N_s. Moreover, Severne *et al.* (11) have recently demonstrated that this process of "tight binding" is responsible for a time-wise leftward shift of the agonist competition binding curves for β-adrenergic receptors in bovine trapezius muscle membranes. In this study, we demonstrate that calf retina α_2-adrenergic receptors are involved in tight binding as well. At 25°, this process is associated with a time-wise shift of the agonist competition curves. At 37°, however, agonists undergo much faster dissociation from the N_i-coupled receptor, and agonist competition binding curves are already at equilibrium after 5 min incubation. Calf retina membranes were taken as a model system because they contain a large amount of α_2-adrenergic receptors (1 pmol/mg of protein), 75 ± 5% of them being able to undergo functional coupling to N_i (12). Our findings indicate that, in order to evaluate agonist competition binding parameters correctly, care should be taken to ascertain that the competition curves are obtained under conditions of equilibrium binding for all of the ligands involved.

Materials and Methods

Chemicals. ^{3}H-RX 781094 (57 Ci/mmol) was obtained from Amersham UK and ^{3}H-UK 14304 (93.9 Ci/mmol) from New England Nuclear. (−)-Epinephrine bitartrate, clonidine hydrochloride, (−)-norepinephrine bitartrate, and phenoxybenzamine hydrochloride were purchased from Sigma, and Gpp(NH)p from Boehringer Mannheim. The following were obtained as generous gifts: phentolamine hydrochloride (Ciba Geigy) and UK 14304 tartrate (Pfizer Central Research).

Membrane preparation. Calf eyes were obtained and dissected in a local slaughterhouse. All subsequent steps were performed at 0–4°. Retinas were homogenized in 10 volumes 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 2,000 × *g* for 15 min. The pellet was homogenized in sucrose buffer and recentrifuged at 2,000 × *g*. All supernatants were pooled and centrifuged at 29,000 × *g* for 20 min. The resulting pellets were washed three times by centrifugation in 10 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$, suspended in the same buffer containing 10% (v/v) glycerol, and stored in liquid nitrogen. The protein concentrations were determined according to the method of Lowry *et al.* (13) using bovine serum albumin as the standard.

Preincubation with (−)-epinephrine. Membranes (2 mg/ml) were preincubated in Tris buffer (50 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$) in Eppendorf vials at 37° with 1 μM (−)-epinephrine for the indicated periods of time. The preincubation was terminated by centrifugation for 3 min in an Eppendorf centrifuge (15,000 × *g*) at 4°. The pelleted membranes were resuspended in 1 ml of Tris buffer containing 2% glycerol and the procedure was repeated twice. The final pellet was resuspended in 1.25 ml of Tris buffer.

Binding of ^{3}H-RX 781094 and ^{3}H-UK 14304. Membrane protein (0.25–0.5 mg/ml) was incubated with the indicated concentrations of ^{3}H-RX 781094 (0.1–50 nM for saturation binding, 5 nM for other experiments) or ^{3}H-UK 14304 (0.03–20 nM for saturation binding, 6 nM for other experiments) for the indicated periods of time in 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$, in a final volume of 500 μl. Experiments were carried out either at 25° or 37° as indicated in the text. At the end of the incubation, the samples were diluted in 4 ml of ice-cold buffer and filtered under reduced pressure through a 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 nonspecific binding (i.e., binding in the presence of 10 μM phentolamine) from the total binding. Nonspecific binding was 5–10% of total binding at 5 nM for ^{3}H-RX 781094 as well as for 6 nM ^{3}H-UK 14304.

Results

^{3}H-RX 781094 has previously been shown to label α_2-adrenergic receptors in calf retina membranes (12). Saturation binding of ^{3}H-RX 781094 to these membranes yields a linear Scatchard plot (Fig. 1) at 25° (r = 0.991) as well as at 37° (r = 0.994). Linear regression analysis of these plots yielded equilibrium dissociation constants (K_D) of 1.8 ± 0.4 (n_H = 0.997) and 3.8 ± 0.3 nM (n_H = 1.002), and B_{max} values of 1070 ± 243 and 935 ± 178 fmol/mg of protein at 25° and 37°, respectively. A typical example of four experiments is shown in Fig. 1.

The binding of ^{3}H-RX 781094 reaches equilibrium within 3 min at 25° and at 37° (Fig. 2). The calculated kinetic constants of association (k_{+1}) are 0.51 × 10^9 and 0.27 × 10^9 M^{-1} min^{-1}, respectively. At both temperatures ^{3}H-RX 781094 binding is totally reversible when 10 μM phentolamine is added to a preequilibrated mixture of membranes and radioligand. A k_{-1} of 0.39 and 1.76 min^{-1} was measured at 25° and 37°. Equilibrium dissociation constants of 1.4 and 3.5 nM, respectively, can be calculated from these kinetic constants. These values are in good agreement with the K_D values of the saturation binding data.

Competition binding curves with the agonist (−)-epinephrine are shallow, both at 25° and at 37° (Figs. 3 and 4). This phenomenon can be attributed to the existence of two affinity states of the receptor: the high affinity state (R_H) corresponds to receptors which are functionally coupled to the adenylate cyclase inhibitory protein (N_i), whereas free receptor only exhibits low agonist affinity (R_L). This interpretation is consistent with the ability of 0.1 mM Gpp(NH)p, known to induce

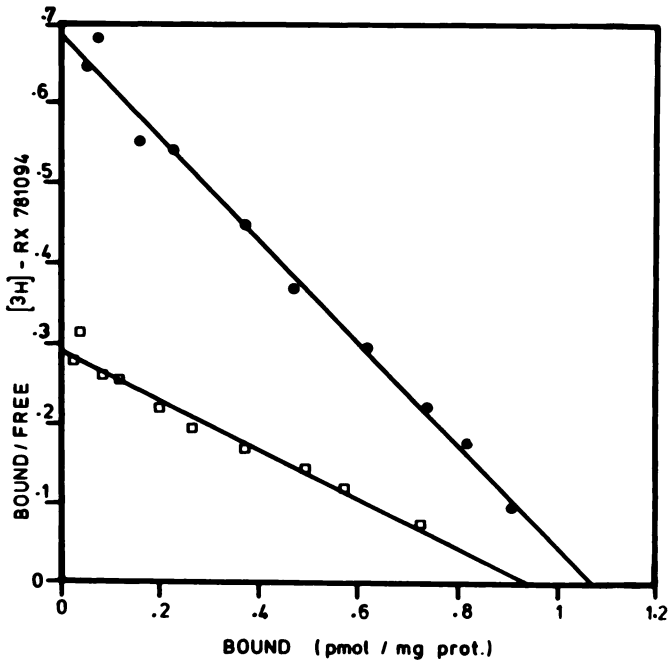

Fig. 1. Scatchard plots of ^{3}H-RX 781094 saturation binding to calf retina membranes at 25° (●) and 37° (□). Linear regression analysis of these plots yielded K_D values of 1.8 ± 0.4 and 3.8 ± 0.3 nM, and B_{max} values of 1070 ± 243 and 935 ± 178 fmol/mg of protein at 25° and 37°, respectively. The data shown are representative of those obtained in four similar experiments.

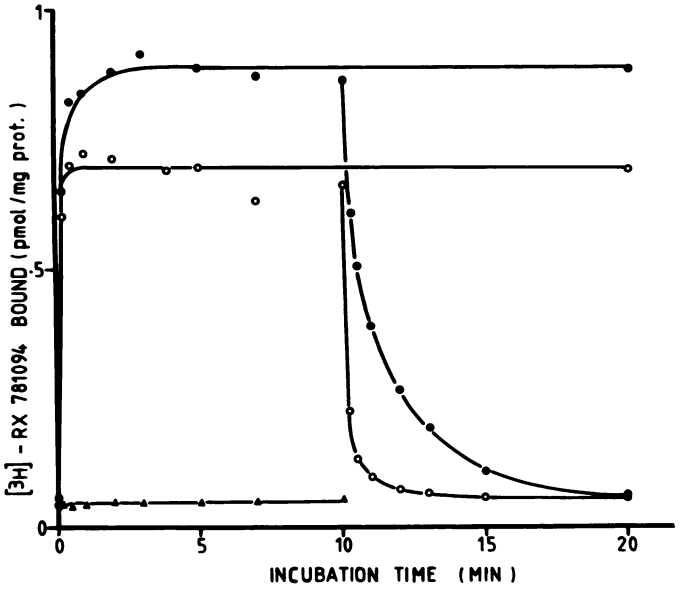

Fig. 2. Time course of ^{3}H-RX 781094 binding at 25° (●) and 37° (○). Membranes were incubated with 5 nM radioligand for increasing periods of time (*abscisa*) and total binding (●, ○) was measured (*ordinate*). Nonspecific binding (Δ) was determined in the presence of 10 μM phentolamine. Dissociation was measured by addition of 10 μM phentolamine to an equilibrated mixture of membranes and radioligand. Linear regression of the semilogarithmic plots yielded the k_{+1} (calculated from k_{ob}) and k_{-1} values noted in the text.

receptor-N_i dissociation, to provoke a rightward shift and with steepening of the curves at both temperatures investigated (Figs. 3 and 4).

At 37°, the (−)-epinephrine competition curves are superimposable for incubation times varying from 5 to 60 min, indicating that the agonist-R_H and agonist-R_L interactions are already at equilibrium after 5 min incubation. Computer-assisted analysis of these curves, following a two-site model as described by Minneman *et al.* (9), reveals that approximately 68% of the receptors display high affinity for the agonist (Table 1). When a competition binding is carried out at 25°, the curves undergo a leftward shift upon increasing the incubation time (Fig. 4). Computer-assisted analysis reveals that this shift is mainly due to a decrease in the equilibrium dissociation constants of (−)-epinephrine (K_H) for the R_H sites upon progressing incubation times. In contrast, the equilibrium dissociation constants (K_L) for the R_L sites remain constant over time (Table 1), indicating that the agonist-R_L interaction has already reached equilibrium at 5 min. These data are in agreement with the observations that the 5- and 60-min competition curves are superimposable in the presence of Gpp(NH)p (Fig. 4).

Motulsky and Mahan (14) have recently demonstrated that competition binding curves undergo a leftward shift if the receptor-competitor complex is slow dissociating. In this context, the time-dependent increase in agonist K_H values as a function of time at 25°, as compared to the steady state level at 37° (Table 1), might be attributed to the ability of the agonist to undergo much slower dissociation from R_H at 25° than at 37°. Kinetic experiments are in full agreement with this assumption. In the experiment shown in Fig. 5, calf retina membranes were preincubated with 1 μM (−)-epinephrine for 15 min at 37°, rapidly washed to remove free agonist, and finally incubated with 5 nM ^{3}H-RX 781094 for increasing lengths of time, either at 25° or at 37°. As shown in Fig. 5, binding of the radioligand is only 34 ± 3% of control binding after 5 min of incubation at 25°, and it increases slowly upon prolongation of the incubation time (50 ± 6% after 60 min). In contrast, binding undergoes much more rapid restoration at 37°: from 41 ± 5% after 3 min to 89 ± 14% after 60 min incubation. Saturation binding experiments, presented in Fig. 6, indicate that the decline in ^{3}H-RX 781094 binding in (−)-epinephrine-pretreated membranes is the result of a decrease in the number of receptor sites (B_{max} = 414 ± 43 fmol/mg of protein in (−)-epinephrine-pretreated membranes versus 1084 ± 134 fmol/mg of protein in control membranes) and that the affinity of the radioligand for the remaining sites is unchanged (1.9 ± 0.1 nM versus 2.0 ± 0.2 nM, respectively). No decrease in radioligand binding can be shown when 0.1 mM Gpp(NH)p is included in the preincubation medium (data not shown). Hence, the (−)-epinephrine-mediated decline in receptor number can be attributed to the disappearance of R_H sites. As shown in Fig. 5, these sites

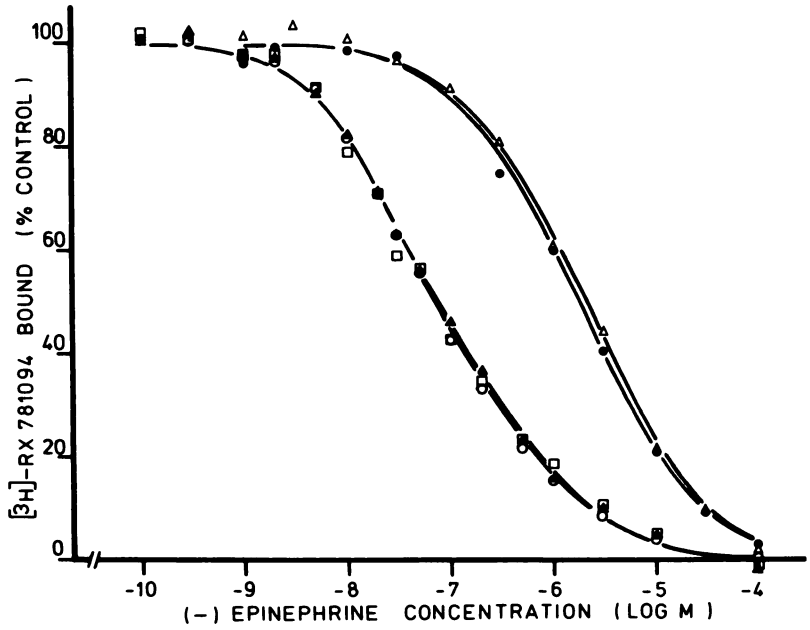

Fig. 3. (−)-Epinephrine/^{3}H-RX 781094 competition binding at 37° as a function of the incubation time. The membranes were incubated with 5 nM ^{3}H-RX 781094 and increasing concentrations of (−)-epinephrine for 5 (○), 20 (□), and 60 (▲) min. Control binding (100%) was measured in the presence of buffer only. Values are the mean of duplicate determinations in three independent experiments. The competition binding data were analyzed by the computerized iterative method described by Minneman *et al.* (9). The calculated parameters are listed in Table 1. The curves are computer-drawn using the data obtained by iteration. (−)-Epinephrine competition binding was also assessed in the presence of 0.1 mM Gpp(NH)p for 5 (Δ) and 60 (●) min.

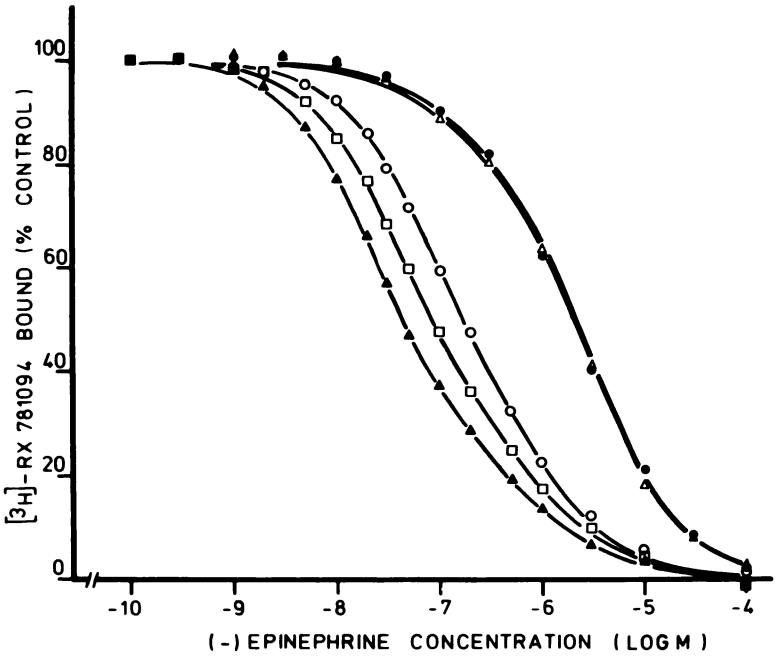

Fig. 4. (−)-Epinephrine/^{3}H-RX 781094 competition binding at 25° as a function of the incubation time. The membranes were incubated with 5 nM ^{3}H-RX 781094 and increasing concentrations of (−)-epinephrine for 5 (○), 20 (□), and 60 (▲) min, or for 5 (△) and 60 (●) min in the presence of 0.1 mM Gpp(NH)p. Values are the mean of duplicate determinations in three independent experiments. The competition binding data were analyzed as in Fig. 3.

TABLE 1

(−)-Epinephrine/^{3}H-RX 781094 competition binding as a function of the time at 25° and 37°

The competition binding data, shown in Figs. 3 and 4, were analyzed to yield the percentage of high affinity sites (% R_H) and the agonist IC_{50} value for the high and low affinity sites. The corresponding K_i values (i.e., K_H and K_L) were calculated according to the method of Cheng and Prusoff (23). Values are means of standard errors of three experiments.

Incubation time	Temperature	Calculated binding parameters for (−)-epinephrine		
		R_H	K_H	K_L
min		%	*nM*	*nM*
5	25°	60	67	884
20	25°	71	41	1069
60	25°	75	24	948
5	37°	68	29	820
20	37°	67	29	853
60	37°	61	25	664

reappear in a time-wise fashion. The pseudo-first order rate constants for receptor reappearance, obtained by linear regression analysis of the semilog plots shown in the *inset* of Fig. 5, are 0.0044 and 0.029 min^{-1} at 25° and 37°, respectively. Assuming that the restoration of R_H sites corresponds to the dissociation of (−)-epinephrine, it can be concluded that this process is approximately 7 times faster at 37° than at 25°.

Kinetic experiments also reveal that Mg^{2+} might be required for the stabilization of the slow agonist-dissociating state of the receptor. For this purpose, calf retina membranes were preincubated with (−)-epinephrine in the presence of Mg^{2+}, as described in Fig. 5, but were washed and incubated with ^{3}H-RX 781094 in the presence of 2 mM EDTA. Under these conditions, 77 ± 4% of the total radioligand binding is recovered after 5 min of incubation at 25° as compared to 34 ± 3% when Mg^{2+} is still present during washing and incubation.

Fig. 7 compares competition binding at 37° for the following α_2-adrenergic agonists: (−)-epinephrine, (−)-norepinephrine, clonidine, and UK 14304. Computer-assisted analysis of the curves reveals that, in the membrane preparation investigated, there are about 75% high affinity sites for all four compounds.

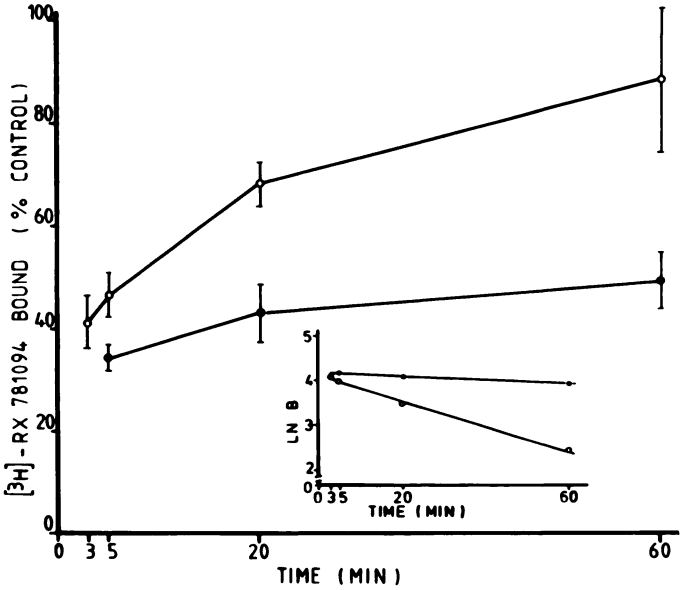

Fig. 5. Restoration of ^{3}H-RX 781094-binding sites with time at 37° (○) and 25° (●). Membranes were pretreated with 1 μM (−)-epinephrine for 15 min at 37°, washed three times, and incubated with 5 nM ^{3}H-RX 781094 for increasing lengths of time (*absicca*). Binding is expressed as percentage of control binding: i.e., binding of ^{3}H-RX 781094 to membranes pretreated with buffer only. Binding to membranes pretreated for 15 min with buffer only agrees with control binding within 10%. *Inset:* semilogarithmic representation of the same binding data. *B* represents the amount of the nonaccessible binding sites, i.e., 100× (control binding − binding at time *t*)/control binding. Values are means and standard errors of duplicate determinations in three independent experiments.

Although the absolute K_H and K_L values are different for each compound, the K_L/K_H ratios are nearly the same (Table 2), suggesting that all four compounds are full agonists in the retina. As shown in the *inset* of Fig. 7, the (−)-epinephrine competition binding characteristics are not affected when approximately half of the receptors are rendered inaccessible by 30 min preincubation of the membranes with a 5 nM concentration of the irreversible antagonist phenoxybenzamine.

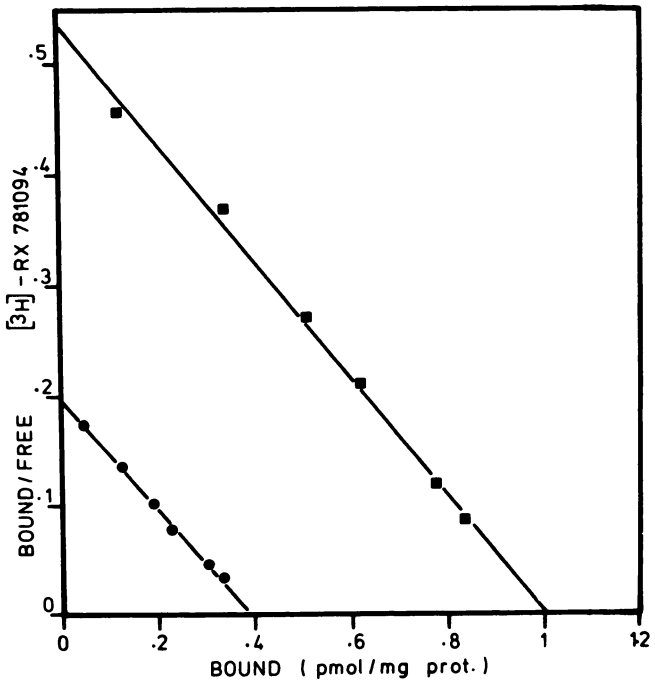

Fig. 6. Scatchard analysis of the specific binding of ^{3}H-RX 781094 to native and (−)-epinephrine-pretreated membranes. Membranes were preincubated for 15 min with 1 μM (−)-epinephrine (●) or buffer (■) only, washed, and subsequently incubated with increasing concentrations of ^{3}H-RX 781094 (0.1–10 nM) for 5 min. The data are representative of those obtained in three similar experiments.

Experiments with the radiolabeled agonist ^{3}H-UK 14304 confirm that the time required to reach equilibrium binding, as well as the rate of agonist dissociation from R_H sites, is very sensitive to temperature. ^{3}H-UK 14304 saturation binding experiments yield linear Scatchard plots at 25° and 37° ($r = 0.985$ and 0.997, respectively; Fig. 8). This linearity is in apparent contradiction with the existence of two agonist affinity states of the receptor. However, as discussed by Bürgisser (15), such resolution is probably impaired by the large percentage of high affinity sites. The K_D values are in close agreement with the K_H value calculated from UK 14304/^{3}H-RX 781094 competition binding experiments (Table 2). The B_{max} values correspond to the receptor concentration found with ^{3}H-RX 781094 in the same membrane preparation.

Kinetic studies at 37° and 25° confirm the heterogeneity of the ^{3}H-UK 14304-binding sites (Fig. 9). Nonlinear least squares analysis indicates that, at both temperatures, the kinetic data are best fit by a two-exponential model for both association and dissociation (Fig. 9B, *inset*). The reaction amplitudes and the corresponding rate constants are listed in Table 3. At 5 nM ^{3}H-UK 14304, the slow dissociating receptors account for 80 ± 2% and 84 ± 2% of the reaction amplitude at 25° and 37°, respectively. The rate of agonist dissociation from these sites is 7 times higher at 37° than at 25°. Fig. 9A also shows that the time required for the obtention of ^{3}H-UK 14304 equilibrium binding is considerably longer at 25° as compared to 37°.

Discussion

The biphasic association and dissociation kinetics for radiolabeled agonist binding as well as the shallow agonist/radiolabeled antagonist competition binding curves indicate the presence of two α_2-adrenergic receptor populations with different agonist affinity. The high affinity sites are assumed to be functionally coupled to the adenylate cyclase-inhibitory protein (N_i), whereas the low agonist affinity sites correspond to non-coupled receptors (16). The ability of guanine nucleotides, known to provoke dissociation of the receptor-N_i complex, to mediate a rightward shift of the competition curves is in full agreement with this assumption. The existence of two agonist affinity states can be explained by the ternary complex model, originally proposed by De Lean *et al.* (10) for β-adrenergic receptors. According to this model, all of the receptors are potentially capable of undergoing functional interaction with N_i, and the observed heterogeneity in agonist affinity implicates the presence of comparable amounts of receptor and N_i. However, Neubig *et al.* (17) have recently demonstrated that, in blood platelet membranes, the concentration of N_i is 23–112

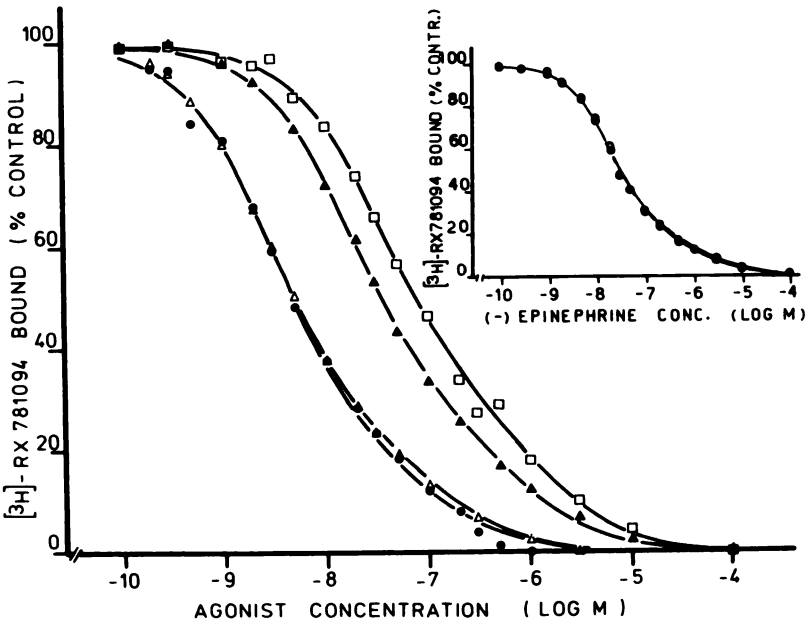

Fig. 7. Agonist competition binding for α_2 receptors at 37°. Membranes were incubated with 5 nM ^{3}H-RX 781094 for 15 min in the presence of increasing concentrations of (−)-epinephrine (□), clonidine (●), (−)-norepinephrine (▲), or UK 14304 (△). Binding shown was analyzed according to a two-site model described by Minneman *et al.* (9). The calculated parameters are listed in Table 2. Values are means of duplicate determinations in three independent experiments. *Inset:* (−)-epinephrine competition binding at 37° to native and phenoxybenzamine-treated membranes. Membranes were pretreated with 50 nM phenoxybenzamine and resulted in a 42% reduction (○) of the binding sites. (−)-Epinephrine competition curves to native (●) and phenoxybenzamine-pretreated membranes yielded K_H values of 19 and 20 nM, K_L values of 1022 and 1620 nM, and R_H values of 80 and 81%, respectively.

TABLE 2

Agonist binding parameters for α_2-adrenergic receptors at 37°

The competition binding data, shown in Fig. 7, were analyzed as described in the legend of Table 1.

Agonists	Binding parameters for agonists at 37°			
	R_H	K_H	K_L	K_L/K_H
	%	nM	nM	
Clonidine	80 ± 2	0.6 ± 0.2	24.5 ± 5.2	41
UK 14304	78 ± 2	0.8 ± 0.1	28.3 ± 5.5	35
(−)-Norepinephrine	74 ± 3	4.2 ± 0.5	170.2 ± 20.4	40
(−)-Epinephrine	74 ± 1	20.7 ± 6	993.2 ± 751.4	50

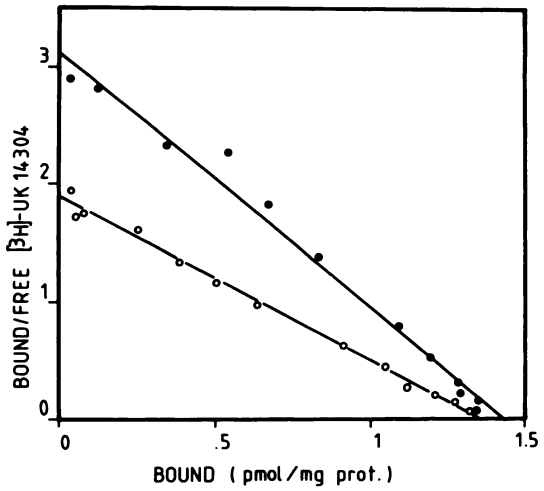

Fig. 8. Scatchard plots of ^{3}H-UK 14304 saturation binding to calf retina membranes at 37° (○) and at 25° (●). Linear regression of these plots yielded K_D values of 0.67 ± 0.7 nM and 0.47 ± 0.1 nM, and B_{max} values of 1282 ± 65 and 1401 ± 52 fmol/mg of protein at 37° and 25°, respectively. The data shown are representative of those obtained in four similar experiments.

times higher than that of α_2 receptors. Moreover, the agonist competition binding characteristics are not affected when the number of receptors is decreased by pretreatment of calf retina membranes with the irreversible antagonist phenoxybenzamine (Fig. 7, *inset*). These findings strongly suggest that the two agonist affinity states of the α_2 receptor do not result from a stoichiometric limitation of N_i. Hence, it is likely that the α_2-adrenergic receptors are composed of two subpopulations, only one of which is capable of undergoing functional coupling to N_i. The agonist-receptor interaction can then be described by the following model:

$$H + Rn \overset{K}{\rightleftharpoons} HRn$$

$$H + Rc + N_i \overset{K}{\rightleftharpoons} H\cdot Rc + N_i \overset{L}{\rightleftharpoons} HRcN_i$$

This model implies the following assumptions. 1) Two non-interconvertible receptor subpopulations are present, *Rc* and *Rn*. Only *Rc* can participate in a ternary complex. 2) The HRN_i complex is formed in two steps; the *H-Rc* interaction is followed by coupling of $H\cdot Rc$ to $N_i\cdot K$ and L are the "microscopic" equilibrium dissociation constants of both steps involved. 3) There is no stoichiometric limitation of N_i.

According to this model, agonist molecules will only bind with low affinity to the *Rn* sites: the "macroscopic" equilibrium dissociation constant equals K. When N_i is in large excess over *Rc*, the $H\cdot R\cdot N_i$ interaction can be described by a pseudo-first

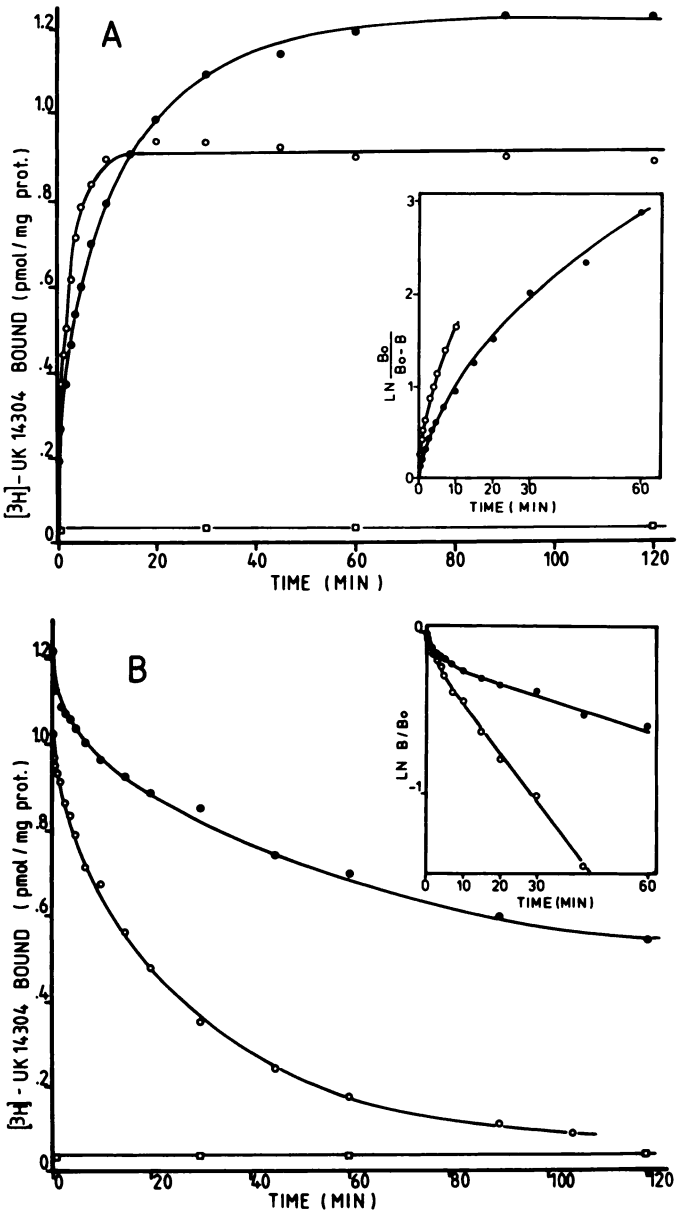

Fig. 9. Time course of ^{3}H-UK 14304 at 37° and 25°. A. Membranes were incubated with 6 nM radioligand for increasing periods of time (*abcissa*), at 25° (●) and at 37° (○) measured. Nonspecific binding (□) was determined in the presence of 10 μM phentolamine. B. Dissociation was measured by addition of 10 μM phentolamine to an equilibrated mixture of membranes and radioligand at 25° (Fig. 9B, ●) and at 37° (Fig. 9B, ○). *Insets:* semilogarithmic plots of the binding data. *B* corresponds to the binding at time *t* and B_0 is equilibrium binding. The kinetic constants derived from these curves are given in Table 3.

order reaction with the "microscopic" equilibrium dissociation constant $L' = L/N_i$. Under this condition, the agonist-*Rc* interaction can be described by a bimolecular reaction. The "macroscopic" equilibrium dissociation constant (Kc) for this interaction is defined by the following equation (18):

$$Kc = K \times L'/(L' + 1)$$

Provided that agonist competition binding curves reflect an equilibrium situation, they can be computer-analyzed in terms of two non-interconvertible sites with different agonist affinity (8). The high and low affinity sites (R_H and R_L) correspond to *Rc* and R_n, and the experimental equilibrium dissociation con-

TABLE 3

Kinetic constants of the ^{3}H-UK 14304 binding at 25° and 37°

The experiments were performed (n = 2) as described in the legend of Fig. 9. The pseudo-first order kinetic constants of association (k_{ob}) and dissociation (k_{-1}) as well as the reaction amplitudes (percentage) were determined by nonlinear least squares analysis of the curves according to a two-exponential model. The K_D values were calculated from the kinetic constants of association, k_{+1} [i.e., $k_{+1} = (k_{ob} - k_{-1})/[\text{UK-14304}]$, and dissociation ($k_{-1}$).

Temperature	Kinetic parameters for ^{3}H-UK 14304 binding					
	Dissociation		Association			K_D
	%	k_{-1}	k_{ob}	k_{+1}	%	
		min^{-1}		M^{-1} min^{-1}		nM
25°	20 ± 2	0.55	0.55	3.5×10^7	26 ± 4	15.7
25°	80 ± 2	0.0045	0.32	9.0×10^6	74 ± 4	0.6
37°	16 ± 2	0.83	1.30	7.9×10^7	26 ± 2	10.5
37°	84 ± 2	0.033	0.3	4.4×10^7	74 ± 2	0.8

stants (K_H and K_L) correspond to Kc and K, respectively. The 5- and 60-min (−)-epinephrine competition binding curves are superimposable when the experiments are carried out at 37° (Fig. 4). Hence, competition curves can be analyzed according to a two-state model when they have been obtained under these experimental conditions. Agonist competition binding experiments at 37° (Table 2) reveal that approximately 75% of the receptors display high agonist affinity and can thus be considered to be coupling prone. A similar proportion of coupling-prone receptors is found when membranes are preincubated with an excess of agonist at 37°, followed by washing and short-term ^{3}H-RX 781094 binding at 25° (Fig. 5). These methods are very useful for the quantification of the α_2 receptor subtypes. In contrast, radioligand binding with labeled ^{3}H-UK 14304 cannot be used for this purpose in retina, because saturation binding studies do not allow a statistical resolution of both affinity sites. Furthermore, the reaction amplitudes of kinetic studies with labeled agonist do not reflect the proportion of both receptor populations since these experiments are performed with subsaturating concentrations of agonist.

Competition binding studies show similar K_H/K_L ratios for clonidine, UK-14304, (−)-epinephrine, and (−)-norepinephrine, which suggests that all four compounds are full agonists in the retina. For the three latter compounds, this property is in agreement with observations in other tissues (19). However, clonidine has been reported to act as a full agonist in the central nervous system, but only as a partial agonist in blood platelets (20). In this context, the full agonist character of clonidine in the retina is not unexpected since the vertebrate retina is developed embryologically from the brain.

When (−)-epinephrine competition binding experiments are performed at 25° instead of 37°, the curves undergo a leftward shift when the incubation time increases from 5 to 60 min (Fig. 4). Two-site analysis reveals that a decrease in K_H is the main factor contributing to the time-dependent shift of these curves. In light of the recent theoretical study of Motulsky and Mahan (14), this nonequilibrium suggests that the ternary $H.Rc.N_s$ complex is quite stable at 25°. Three different observations support this assumption.

1) The (−)-epinephrine competition curve is already at equilibrium after 5 min when Gpp(NH)p, known to provoke rapid receptor-N_i dissociation, is included in the incubation medium (Figs. 3 and 4).

2) The kinetic data for association and dissociation of the radiolabeled agonist ^{3}H-UK 14304 can be interpreted in terms of two receptor populations (Fig. 9, Table 3). Dissociation of this agonist from the high affinity receptors is 7 times higher at 37° than at 25°.

3) The dissociation rate of the $H.Rc.N_i$ complexes can also be evaluated by measuring the recovery of ^{3}H-RX 781094-binding sites in membranes which have been preincubated with (−)-epinephrine. Agonist dissociation from the low affinity sites is very fast (Fig. 9), so that these sites can be assumed to be fully recovered, even at short-term incubation with ^{3}H-RX 781094. As shown in Fig. 5, the recovery of Rc sites is appreciably slower at 25° as compared to 37°: the rate constants are 0.0044 and 0.029 min^{-1}, respectively.

Despite the involvement of different guanine nucleotide-regulatory proteins, the agonist-binding properties of the α_2-adrenergic receptors are very similar to those previously described for β-adrenergic receptors. Only part of these receptors can undergo functional coupling to their guanine nucleotide-regulatory proteins, respectively, N_i and N_s. This functional heterogeneity cannot be attributed to a stoichiometric limitation of these regulatory proteins and is probably related to post-translational modifications of the receptors or to microheterogeneities in the membrane lipid environment (21). Bound agonists undergo much slower dissociation from the N_s- or N_i-coupled receptor as compared to the uncoupled receptor, since locking of the agonist in the coupled receptor, a phenomenon earlier referred to as "tight binding," appears to be Mg^{2+} dependent.

In this respect, "tight binding" of endogenous agonist molecules may interfere with the correct evaluation of α_2- and β-adrenergic receptor concentrations. Membrane preparations from brain and sympathetically innervated tissues such as the heart often still contain appreciable amounts of endogenous catecholamines. The ability of these catecholamines to undergo "tight binding" might result in an underevaluation of the actual receptor number. In this context, Nerme *et al.* (22) have recently shown that addition of GTP, which reverses tight agonist binding, causes a 4–35% increase in the β-adrenergic receptor number in membranes from control rats, whereas the nucleotide has no effect upon the receptor number in membranes from reserpinized animals. In a similar way, Cheung *et al.* (24) have shown that rat cerebral cortex membranes contain 10 nM (−)-epinephrine and less than 0.5 nM (−)-norepinephrine when prepared in isotonic or hypotonic buffer, respectively. Whereas the α_2 receptor concentration was 2 times higher in the hypotonic preparation, the receptor concentration in the hypertonic preparation became equally high when Gpp(NH)p and NaCl, both destabilizers of the HRN_i complex, were added to the incubation medium. These studies clearly show that "tight

binding" can be overcome in at least two ways: removal of the endogenous catecholamines or measurement of the receptor concentration in the presence of guanine nucleotides.

Finally, "tight binding" may also interfere with the correct evaluation of the agonist-binding properties for both α_2- and β-adrenergic receptors. This phenomenon can, indeed, be held responsible for the time-dependent shift of agonist competition binding curves towards higher affinities, observed by Severne *et al.* (11) for β_2-adrenergic receptors in bovine trapezius muscle membranes and, in this study, for the α_2-adrenergic receptors in calf retina membranes. The constants derived from agonist competition binding curves have only molecular significance if these curves reflect an equilibrium situation. For α_2-adrenergic receptors, this problem can be circumvented by performing the experiments at 37°. However, the very long incubation times which are probably required to reach equilibrium at 25° and below might seriously impair the thermodynamic analysis of the agonist-α_2 receptor interactions.

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References

1. Harwood, J. P., J. Moskowitz, and G. Krishna. Dynamic interaction of prostaglandin and norepinephrine in the formation of adenosine 3′,5′-monophosphate in human and rabbit platelets. *Biochim. Biophys. Acta* **261**:444–465 (1984).
2. Gilman, G. G proteins and dual control of adenylate cyclase. *Cell* **36**:577–579 (1984).
3. Cerione, R. A., J. W. Regan, H. Nakata, J. Codina, J. L. Benovic, P. Gierschik, R. L. Somers, A. M. Spiegel, L. Birnbaumer, R. J. Lefkowitz, and M. G. Caron. Functional reconstitution of the alpha 2 adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J. Biol. Chem.* **261**:3901–3909 (1986).
4. Nomura, Y., M. Kawai, K. Mita, and T. Segawa. Developmental changes of cerebral cortical ^{3}H clonidine binding in rats: influences of guanine nucleotides and cations. *J. Neurochem.* **42**:1240–1245 (1984).
5. Loftus, D., J. Stolk, and D. C. U'Prichard. Binding of the imidazoline UK 14304, a putative full alpha 2 adrenoceptor agonist, to rat cerebral cortex membranes. *Life Sci.* **35**:61–69 (1984).
6. Perry, B. D., and D. C. U'Prichard. ^{3}H Rauwolscine (alpha-yohimbine): a specific antagonist radioligand for brain alpha 2 adrenergic receptors. *Eur. J. Pharmacol.* **76**:461–464 (1981).
7. Pimoule, C., B. Scatton, and S. Z. Langer. ^{3}H RX 781094: a new antagonist ligand labels alpha 2 adrenoceptors in the rat brain cortex. *Eur. J. Pharmacol.* **95**:78–85 (1983).
8. Michel, T., B. B. Hoffman, and R. J. Lefkowitz. Differential regulation of the alpha 2 adrenergic receptor by sodium and guanine nucleotides. *Nature (Lond.)* **288**:709–711 (1980).
9. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. Simultaneous determination of beta 1 and beta 2 adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmacol.* **16**:34–46 (1979).
10. De Lean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
11. Severne, Y., A. IJzerman, V. Nerme, H. Timmerman, and G. Vauquelin. Shallow agonist competition binding curves for β-adrenergic receptors: the role of tight agonist binding. *Mol. Pharmacol.* **31**:69–73 (1987).
12. Convents, A., J.-P. De Backer, and G. Vauquelin. Characterization of alpha 2 adrenergic receptors of calf retina membranes by ^{3}H rauwolscine and ^{3}H RX 781094 binding. *Biochem. Pharmacol.*, in press.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
14. Motulsky, H. J., and L. C. Mahan. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* **25**:1–9 (1984).
15. Bürgisser, E. Radioligand-receptor binding studies: what's wrong with the Scatchard analysis? *Trends Pharmacol. Sci.* **4**:142–144 (1984).
16. Nomura, Y., Y. Kitamura, and T. Segawa. Decrease of clonidine binding affinity to alpha 2 adrenoceptor by ADP-ribosylation of 41,000-dalton proteins in rat cerebral cortical membranes by islet-activating protein. *J. Neurochem.* **44**:364–369 (1985).
17. Neubig, R. R., R. D. Gantzos, and R. S. Brasier. Agonist and antagonist binding to α_2-adrenergic receptors in purified membranes from human platelets. Implications of receptor-inhibitory nucleotide-binding protein stoichiometry. *Mol. Pharmacol.* **28**:475–486 (1985).
18. Vauquelin, G., S. Bottari, and A. D. Strosberg. Inactivation of beta-adrenergic receptors by *N*-ethylmaleimide: permissive role of beta-adrenergic agents in relation to adenylate cyclase activation. *Mol. Pharmacol.* **17**:163–171 (1980).
19. Timmerman, P. B. M. W. M., and P. A. van Zwieten. Alpha 2 adrenoceptors: classification, localisation, mechanisms and targets for drugs. *J. Med. Chem.* **25**:1389–1401 (1982).
20. Steer, M. L., and D. Atlas. Interaction of clonidine and clonidine analogs with human platelet alpha 2 adrenergic receptors. *Biochim. Biophys. Acta* **714**:389–394 (1982).
21. Severne, Y., D. Coppens, S. Bottari, M. Riviere, R. Kram, and G. Vauquelin. Influence of the beta adrenergic receptor concentration of functional coupling to the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **81**:4637–4641 (1984).
22. Nerme, V., Y. Severne, T. Abrahamsson, and G. Vauquelin. Endogenous noradrenaline masks beta adrenergic receptors in rat heart membranes via tight agonist binding. *Biochem. Pharmacol.* **34**:2917–2922 (1985).
23. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
24. Cheung, Y. D., D. B. Barnett, and S. R. Nahorski. Interactions of endogeneous and exogeneous norepinephrine with alpha 2 adrenoceptor binding sites in rat cerebral cortex. *Biochem. Pharmacol.* **33**:1293–1298 (1984).

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