0026-895X/87/050655-08$02.00/0

MOLECULAR PHARMACOLOGY, 32:655-662

Relationship between α_2-Adrenergic Receptor Binding Sites and the Functional Receptors Inhibiting Norepinephrine Release in Rat Cerebral Cortex[1]

ALI NASSERI and KENNETH P. MINNEMAN

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Received April 6, 1987; Accepted August 3, 1987

SUMMARY

The properties of α_2-adrenergic receptor binding sites and the receptors inhibiting [^{3}H]norepinephrine (^{3}H-NE) release from slices of cerebral cortex were compared. [^{3}H]RX 781094, an α_2-adrenergic receptor antagonist radioligand, labeled a single class of binding sites in membranes at 37° with the pharmacological specificity expected of α_2-adrenergic receptors. 5′-Guanylimidodiphosphate (Gpp(NH)p) and NaCl caused small increases in the potencies of antagonists at the ^{3}H-RX binding sites but decreased the potencies of agonists 4–22-fold. Antagonists blocked the inhibition of potassium-evoked ^{3}H-NE release caused by exogenous NE with potencies similar to those in competing for ^{3}H-RX binding sites. Partial receptor inactivation with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was used to determine whether there was a receptor reserve. EEDQ dose-dependently decreased both the density of ^{3}H-RX binding sites and the maximal inhibition of ^{3}H-NE release by different agonists. For most agonists, K_A values calculated after the receptor inactivation did not differ significantly from EC_{50} values; however, for epinephrine a small receptor reserve was apparent. The proportion of ^{3}H-RX binding sites lost was similar to the proportion of functional receptors lost after EEDQ treatment. The functional K_A values for agonists correlated most closely with K_D values for the low affinity binding state observed in the presence of Gpp(NH)p and NaCl. However, both epinephrine and NE still showed two-site binding curves under these conditions, and it was the high affinity subpopulation of sites observed in the presence of Gpp(NH)p and NaCl which resembled most closely the functional K_A values. These data suggest that ^{3}H-RX labels binding sites with properties similar to the α_2-adrenergic receptors inhibiting ^{3}H-NE release in cerebral cortex. There is little or no receptor reserve for this effect, and there appears to be a binding state for the natural catecholamine agonists which has an affinity lower than that for mediating this functional response.

There is general agreement that there are two subtypes of α-adrenergic receptors with distinct pharmacological properties (1). α_1-Adrenergic receptors are usually found postsynaptically and α_2-adrenergic receptors are usually found presynaptically but also exist on some postsynaptic cells (2). Activation of presynaptic α_2-adrenergic receptors decreases NE release from nerve terminals of both central and peripheral neurons (3), although the regulatory significance of this effect is disputed (4).

α_2-Adrenergic receptors inhibit adenylate cyclase through an inhibitory guanine nucleotide binding protein (G_i) (5). The binding of the receptor to the G_i protein induces a high affinity state for agonists which can be reversed by GTP and its nonhydrolyzable analogs (6). Thus this receptor, like other receptors which interact with G proteins, exhibits multiple affinity states in radioligand binding assays. It is difficult to know which of these states is relevant to the functional receptor activated by agonists in the intact tissue.

α_2-Adrenergic receptor activation decreases depolarization-evoked tritium release from slices of rat cerebral cortex preloaded with ^{3}H-NE (7–9). Although α_2-adrenergic receptor activation undoubtedly causes other effects in brain, this is a convenient method for examining responsiveness in this tissue.

We wanted to compare the multiple affinity states exhibited by α_2-adrenergic receptor binding sites in membrane preparations with the properties of the receptors linked to a functional response in rat brain. Specifically, we wanted to address the following questions:

1) Are the binding sites labeled by the high affinity antagonist radioligand ^{3}H-RX 781094 (10, 11) the same as the functional α_2-adrenergic receptors inhibiting ^{3}H-NE release in brain?

2) Are the EC_{50} values for agonists in inhibiting ^{3}H-NE release equivalent to their binding constants at the functional receptor, or is there a receptor reserve for this effect (12)?

[1] This work was supported by National Institutes of Health Grant NS 21325.

ABBREVIATIONS: ^{3}H-NE, norepinephrine; ^{3}H-RX: ^{3}H-RX 781094; Gpp(NH)p, 5′-guanylimidodiphosphate; EEDQ: *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

3) Do any of the multiple affinity states exhibited for agonists in binding studies correspond to their potencies in activating this functional response?

An understanding of these questions should give us more insight into the functional significance of the different affinity states which α_2-adrenergic receptors exhibit toward agonists.

Materials and Methods

^{3}H-RX binding. Male Sprague-Dawley rats were sacrificed by decapitation. A particulate fraction of cerebral cortex was prepared essentially as described by Cheung *et al.* (13). The tissue was homogenized with a Polytron in 20 volumes of cold 5 mM Tris-HCl buffer (pH 7.7) containing 5 mM EDTA and centrifuged for 10 min at 20,000 × *g* at 4°. The pellet was resuspended in the same buffer and centrifuged again. The pellet was finally resuspended in buffer containing 50 mM Tris-HCl (pH 7.7), 10 mM $MgSO_4$, 0.5 mM EDTA, and 1 mM mercaptoethanol to a concentration of 2–4 mg of protein/ml. Binding assays were initiated by addition of tissue (0.2–0.4 mg of protein) and carried out for 20 min at 37° in a final volume of 0.25 ml. In some cases parallel assays were carried out in the absence and presence of 10 μM Gpp(NH)p and 200 mM NaCl. Reaction was terminated by the addition of 10 ml of cold 10 mM Tris-HCl buffer (pH 7.4) followed by filtration over glass fiber filters (Schleicher & Schuell No. 32) under vacuum. Filters were washed with 10 ml of 10 mM Tris-HCl buffer, dried, and counted in a scintillation counter. Specific binding was defined as the difference between total binding and binding remaining in the presence of 100 μM *l*-NE.

Measurement of ^{3}H-NE release. Male Sprague-Dawley rats were sacrificed by decapitation. Brains were removed and cooled for about 5 min in cold "half-calcium Krebs" containing (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11; ascorbic acid, 0.06; and EDTA, 0.03. Cortices were dissected and chopped into 0.3 × 2-mm slices using a McIlwain tissue chopper. Slices were preloaded by incubating for 30 min at 37° under 95% O_2/5% CO_2 in half-calcium Krebs containing 0.06–0.07 μM ^{3}H-NE. Two to four slices were transferred to each chamber of a superfusion apparatus where they were held between two layers of nylon mesh (350 μm pore size). Slices were continuously superfused (0.25 ml/min) with half-calcium Krebs at 37° containing 10 μM desmethylimipramine and equilibrated with 95% O_2/5% CO_2 for the remainder of the experiment (9). After an initial washing period of 36 min, fractions of the perfusate were collected every 4 min for 100 min. Some slices were exposed to half-calcium Krebs containing 26 mM KCl (96.8 mM NaCl to maintain osmolarity) for the 100-min collection period. The amount of tritium released during each 4-min collection period was expressed as the percentage of the tritium remaining in the tissue at the beginning of that collection period.

EEDQ treatment. After preloading with ^{3}H-NE and extensive washing, cortical slices were incubated in half-calcium Krebs containing 1–10 μM EEDQ for 10 min at 37° under 95% O_2/5% CO_2. Slices were thoroughly washed before being transferred to the superfusion chambers. For determination of ^{3}H-RX binding, slices were treated identically except that ^{3}H-NE was omitted from the preloading step and slices were homogenized immediately after EEDQ treatment and washing.

Analysis of binding data. Scatchard plots were analyzed by unweighted linear regression. IC_{50} values and Hill coefficients from competition binding assays were calculated from Hill plots. K_D values were calculated from IC_{50} values (14). The best two-site fit for a binding curve was calculated by minimizing the sum of squares of the errors using nonlinear regression analysis. Two-site models were compared to one-site models to determine whether the increase of goodness of fit was significantly more than would be expected on the basis of chance alone (15) using a partial *F* test. *p* values less than 0.05 were considered significant.

Analysis of release data. For each 4-min time period, basal tritium release (*B*) occurring in normal potassium buffer was subtracted from total tritium release occurring in high potassium buffer for control (*C*) and drug-treated (*D*) samples. The fractional ratio of potassium-evoked release in the absence and presence of drug $[(D - B)/(C - B)]$ was calculated for each 4-min time period. Each drug concentration was continued for 12 min, and the average of the fractional ratio for each of these three fractions was determined. The effect of each drug concentration was normalized as the percentage of this averaged fractional ratio during the drug treatment period relative to a similar averaged fractional ratio calculated for a 12-min pre-drug period. EC_{50} values were usually calculated by linear regression of all points between 20 and 80% of the maximum response to that drug. Schild plots for multiple doses of antagonist were constructed as described by Arunlakshana and Schild (16). K_B values were calculated from the shift in the agonist dose-response curve caused by a single concentration of antagonist assuming a slope of 1 on a Schild plot. K_A and *q* values were calculated from double-reciprocal plots of equi-effective agonist concentrations in dose-response curves from control and EEDQ-treated slices using the method of Furchgott (17) as previously described (18).

Materials. The drugs used were obtained from the following sources; prazosin, HCl, and UK 14,304 tartrate (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline) from Pfizer Inc., Groton, CT; phentolamine mesylate from CIBA-GEIGY Corp. Summit, NJ; clonidine-HCl from Boehringer Ingelheim Ltd., Ridgefield, CT; oxymetazoline-HCl from Schering Corp., Bloomfield, NJ; desmethylimipramine-HCl from USV Pharmaceutical Corp. Tuckahoe, NY. Norepinephrine, epinephrine, yohimbine, EEDQ, and Gpp(NH)p were purchased from Sigma. ^{3}H-RX 781094 [2-(2-[1,4-benzodioxanyl])-2-imidazoline] (40 Ci/mmol) was purchased from Amersham Corp. and ^{3}H-NE (42 Ci/mmol) was purchased from New England Nuclear. All other chemicals were obtained from standard commercial sources.

Results

Binding of ^{3}H-RX. Most binding studies of ^{3}H-RX reported previously have been performed at low temperatures to slow association and dissociation kinetics (10, 11); however, we wanted to examine binding at the same temperature at which the functional experiments were carried out. In preliminary experiments we found that the apparent K_D for ^{3}H-RX binding was increased by about 4-fold by increasing the temperature from 4 to 37°; however, the B_{max} was unchanged (data not shown). Scatchard plots for the binding of ^{3}H-RX to a particulate fraction prepared from rat cerebral cortex at 37° are shown in Fig. 1. ^{3}H-RX labeled an apparently homogeneous class of binding sites with a mean K_D of 12.6 ± 1.4 nM ($n = 4$) and a mean B_{max} of 158 ± 14.3 fmol/mg of protein. The affinity of ^{3}H-RX increased 2-fold under conditions designed to promote low affinity agonist binding (adding 10 μM Gpp(NH)p and 200 mM NaCl) reflected by a decrease in the apparent K_D to 6.7 ± 1.1 nM ($n = 4$), with no change in B_{max} (154 ± 8.3 fmol/mg of protein).

Inhibition by agonists and antagonists. Inhibition of specific ^{3}H-RX binding by various drugs was tested both in the presence and in the absence of 10 μM Gpp(NH)p and 200 mM NaCl, conditions designed to promote low and high affinity agonist binding, respectively. The potencies of agonists and antagonists in competing for specific ^{3}H-RX binding sites were similar to those expected at α_2-adrenergic receptors (Table 1). In the presence of Gpp(NH)p and NaCl there was either no effect, or an increase in the potencies of antagonists in competing for the binding sites. The potencies of agonists, however, were markedly decreased by the inclusion of Gpp(NH)p and NaCl (Table 1, Fig. 2). This effect was much greater for the full agonists NE, epinephrine, and UK 14,304 than it was for

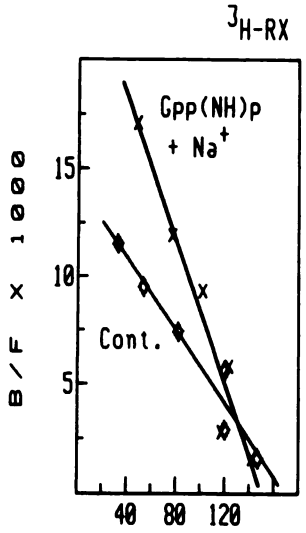

Fig. 1. Scatchard plot. ^{3}H-RX binding to particulate fractions of rat cerebral cortex was determined in the presence and absence of 10 μM Gpp(NH)p and 200 mM NaCl. Specific binding was defined as binding displaced by 100 μM *l*-NE. Each point is the mean ± SE of duplicate determinations from three experiments.

the partial agonists clonidine and oxymetazoline. In the absence of Gpp(NH)p and NaCl, the Hill coefficients for antagonists were close to 1.0, although for agonists these values were substantially less (Table 1). The Hill coefficients for agonists were increased in the presence of Gpp(NH)p and NaCl, although for NE and epinephrine they remained significantly less than 1.0. The inhibition curves for NE and epinephrine were analyzed by nonlinear regression analysis assuming two independent, non-interacting binding sites. A two-site model was a better fit ($p < 0.05$) than a one-site model for both agonists under both conditions (Table 2). The decrease in affinity caused by Gpp(NH)p and NaCl appeared to be due to both a decrease in the proportion of receptors in the high affinity state and a decrease in the apparent affinity of this binding state (Table 2). To ensure that optimal conditions were used to promote low affinity agonist binding, inhibition of ^{3}H-RX binding by norepinephrine was also determined in the presence of 200 mM NaCl and either 10 or 100 μM Gpp(NH)p. Analysis of the inhibition curves gave significantly ($p < 0.005$) better two-site fits under both conditions; however, the proportions and affinities of the sites did not differ significantly between the two conditions. There were 39 ± 2.8% and 27 ± 11.4% high affinity sites in the presence of 10 and 100 μM Gpp(NH)p, respectively. The $-\log K_D$ values were 6.30 ± 0.038 and 6.76 ± 0.305 for the high affinity state and 5.14 ± 0.029 and 5.19 ± 0.040 for the low affinity state in the presence of 10 and 100 μM Gpp(NH)p, respectively ($n = 3$).

Functional properties of α_2-adrenergic receptors inhibiting ^{3}H-NE release. We studied the functional effects of α_2-adrenergic receptor stimulation on the depolarization-evoked release of ^{3}H-NE from cerebral cortical slices (9). The pattern of release of tritium under control and continuous high KCl (26 mM) stimulation for the 100-min perfusion period is shown in Fig. 3. Effects of the agonists were examined in a cumulative fashion starting 28 min after the depolarizing stimulus was introduced. Inhibition of release was found to be maximal within 10 min of adding the agonist (data not shown), and each dose was continued for 12 min. Dose-response curves for NE, clonidine, and oxymetazoline in inhibiting potassium-evoked tritium release are shown in Fig. 4. The $-\log EC_{50}$ values and the maximal inhibition observed with each agonist are listed in Table 3. The full agonists NE, epinephrine, and UK 14,304 caused substantially more inhibition of release than did the partial agonists clonidine and oxymetazoline. However the maximal inhibition caused by epinephrine was significantly greater than the maximal inhibition caused by NE ($p < 0.05$) (Table 3).

Potencies of antagonists. Yohimbine, prazosin, and phentolamine were tested for their ability to block the effect of exogenous NE in inhibiting release. These drugs increased potassium-evoked tritium release to between 3.5 and 4.5% of remaining tissue tritium, probably by blocking the effects of endogenous NE. In the presence of each of these compounds,

TABLE 1

Inhibition of ^{3}H-RX binding by agonists and antagonists

IC_{50} and n_H values were determined from Hill plots constructed for the inhibition of specific ^{3}H-RX 781094 binding under control conditions or in the presence of 10 μM Gpp(NH)p and 200 mM NaCl. K_D values were determined by the method of Cheng and Prusoff (14). Each value is the mean ± SE of the number of observations listed.

Drug		N	$-\log K_D$	Antilog ratio	n_H
			M		
Agonists					
NE	Control	3	7.24 ± 0.018		0.53 ± 0.01
	Gpp(NH)p + NaCl	3	5.89 ± 0.038	22.4	0.80 ± 0.10
Epinephrine	Control	3	7.64 ± 0.176		0.59 ± 0.093
	Gpp(NH)p + NaCl	3	6.35 ± 0.145	19.5	0.80 ± 0.02
UK 14,304	Control	9	8.39 ± 0.064		0.78 ± 0.07
	Gpp(NH)p + NaCl	7	7.19 ± 0.069	15.9	1.25 ± 0.13
Clonidine	Control	6	8.09 ± 0.077		0.79 ± 0.07
	Gpp(NH)p + NaCl	3	7.63 ± 0.087	2.9	0.91 ± 0.14
Oxymetazoline	Control	5	8.23 ± 0.076		0.58 ± 0.07
	Gpp(NH)p + NaCl	5	7.51 ± 0.13	5.3	1.02 ± 0.15
Antagonists					
Phentolamine	Control	3	7.33 ± 0.106		0.80 ± 0.10
	Gpp(NH)p + NaCl	3	7.40 ± 0.105	0.9	1.08 ± 0.26
Yohimbine	Control	4	6.64 ± 0.111		0.92 ± 0.04
	Gpp(NH)p + NaCl	4	7.33 ± 0.053	0.2	1.23 ± 0.18
Prazosin	Control	3	5.22 ± 0.034		0.98 ± 0.05
	Gpp(NH)p + NaCl	3	5.82 ± 0.142	0.3	1.00 ± 0.02

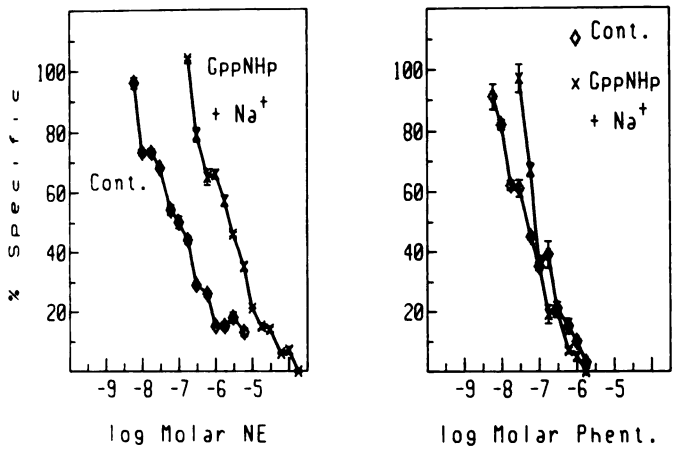

Fig. 2. Effect of Gpp(NH)p and NaCl on agonist and antagonist competition curves. Displacement of specific ^{3}H-RX 781094 binding by NE (*left*) and phentolamine (*right*) were examined in both the presence and absence of 10 μM Gpp(NH)p and 200 mM NaCl. Each point is the mean ± SE of duplicate determinations from three experiments.

TABLE 2

Two-site analysis of NE and epinephrine inhibition of ^{3}H-RX binding

Inhibition curves for NE and epinephrine inhibition of ^{3}H-RX binding were determined in the absence or presence of 10 μM Gpp(NH)p and 200 mM NaCl. Percent inhibition at each concentration of agonist was averaged for three different experiments, and this averaged data was computer fit to a two-site model (see Methods). K_D values for high (K_H) and low (K_L) affinity binding sites and the proportion of sites in the high (H) and low (L) affinity states were determined. A two-site fit was significantly better than a one-site fit ($p < 0.05$) using a partial F test for both agonists under both experimental conditions.

Agonist	−log K_H	−log K_L	Proportion H:L
	M	*M*	
NE			
Control	7.70	5.96	68:32
Gpp(NH)p + NaCl	6.48	5.46	47:53
Epinephrine			
Control	7.92	5.30	85:15
Gpp(NH)p + NaCl	6.80	5.70	63:37

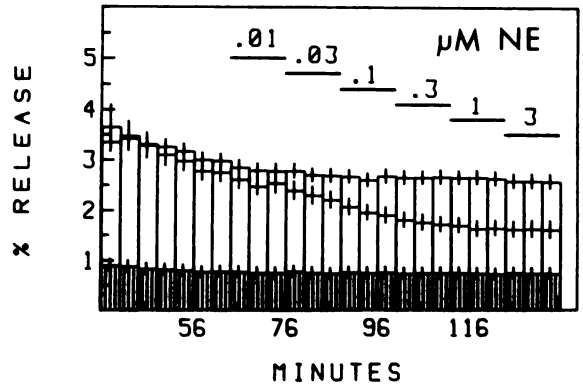

Fig. 3. Release of ^{3}H-NE. Slices were preloaded with ^{3}H-NE and tritium release examined during continuous perfusion with Krebs-Ringer bicarbonate buffer containing normal KCl (4.8 mM, *bottom curve*), or high KCl (26 mM) in the absence (*top curve*) or presence (*middle curve*) of increasing concentrations of exogenous NE. The *bars* indicate the length of perfusion with the concentration of NE indicated (μM). Each histogram is the mean ± SE of 24 (*bottom*), 8 (*middle*), or 30 (*top*) tissues.

the dose-response curve for NE was shifted to the right in a parallel manner with no change in maximum effect, indicative of competitive inhibition (Fig. 5). The apparent potencies of these drugs in blocking the functional receptors (K_B values)

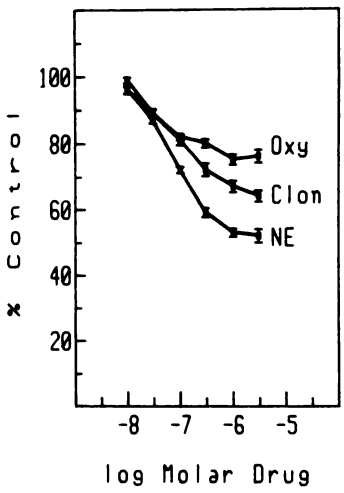

Fig. 4. Dose-response curves for inhibition of tritium release by NE, clonidine (*Clon*), and oxymetazoline (*Oxy*) were determined. Each point on the curves was expressed as percent of control prior to the addition of drug. Each point is the mean ± SE of eight (*NE*, *Oxy*) or six (*Clon*) determinations.

TABLE 3

Effects of agonists on ^{3}H-NE release

EC_{50} values and maximum effects on inhibition of ^{3}H-NE release were determined from dose-response curves to each agonist as described in Methods. Each value is the mean ± SE of the indicated number of observations.

Agonist	*n*	−log EC_{50}	Maximum inhibition
		M	%
NE	8	7.09 ± 0.056	49 ± 4.3
Epinephrine	8	7.58 ± 0.051	67 ± 1.8
UK 14,304	8	7.10 ± 0.097	57 ± 4.3
Clonidine	6	7.04 ± 0.062	36 ± 4.1
Oxymetazoline	7	7.26 ± 0.096	24 ± 4.1

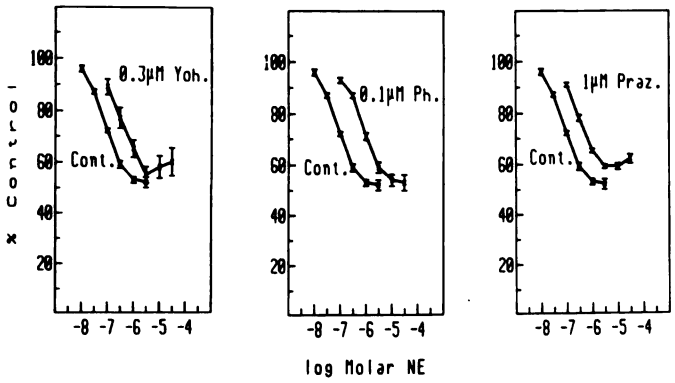

Fig. 5. Competitive antagonism. Blockade of the effects of exogenous NE on tritium release by 0.3 μM yohimbine (*Yoh*), 0.1 μM phentolamine (*Ph*), and 1 μM prazosin (*Praz*) were determined as described. Each point is the mean ± SE of eight observations.

were calculated from the decreases in NE potency (Fig. 5). −Log K_B values calculated for phentolamine, yohimbine, and prazosin were 7.90, 7.04, and 6.40, respectively. It was difficult to examine high doses of antagonists, since nonspecific effects on displacement of tritium were encountered when the NE dose-response curve was shifted far to the right. However, we did construct a Schild plot using three doses of yohimbine to block the response. These experiments yielded a slope of 0.92 and a pA_2 of 7.12 (data not shown), in agreement with our K_B

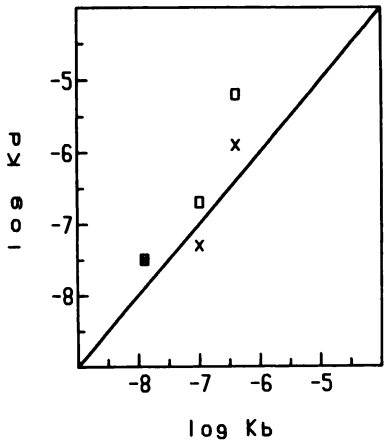

Fig. 6. Comparison of binding and functional potencies of antagonists. K_B values for blocking inhibition of tritium release by exogenous NE are compared to K_D values for inhibiting specific ^{3}H-RX binding by yohimbine, phentolamine, and prazosin. Binding values are plotted in the absence (□) or presence (×) of 10 μM Gpp(NH)p and 200 mM NaCl. The line of identity is shown for purposes of comparison.

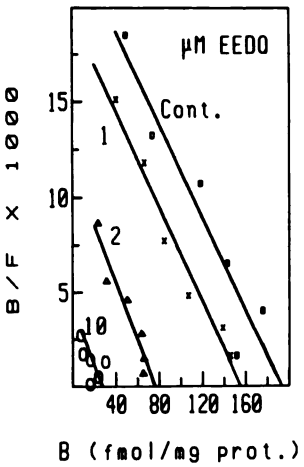

Fig. 7. Effect of EEDQ on ^{3}H-RX binding sites. Specific ^{3}H-RX 781094 binding was determined after treatment of rat cortical slices with 1–10 μM EEDQ as described in Methods. Each value is the mean of three experiments performed in duplicate.

measurement. The K_D values for the same drugs in competing for ^{3}H-RX binding sites under different experimental conditions are compared with their K_B values in Fig. 6.

Inactivation of ^{3}H-RX binding sites by EEDQ. EEDQ (19) was used to irreversibly inactivate α_2-adrenergic receptors in brain slices to determine the relationship between receptor occupancy and tissue response. Pretreatment of cortical slices for 10 min with EEDQ caused a dose-dependent decrease in the density of ^{3}H-RX binding sites in particulate preparations from the slices. The density of binding sites was decreased by 20, 60, and greater than 90% by 1, 2, and 10 μM EEDQ, respectively (Fig. 7), with no change in the apparent K_D for ^{3}H-RX following any of these EEDQ treatments.

Effects of EEDQ on release. Pretreatment of cortical slices with 10 μM EEDQ did not change basal tritium efflux, but caused a 60% increase in potassium-evoked release (Fig. 8), probably by releasing the slices from tonic inhibition by endogenous NE. EEDQ pretreatment also decreased receptor-mediated inhibition of tritium release. Pretreatment with 1, 2, and 10 μM EEDQ decreased the maximal response to NE and clonidine with little or no effect on EC_{50} values (Fig. 9). Similar

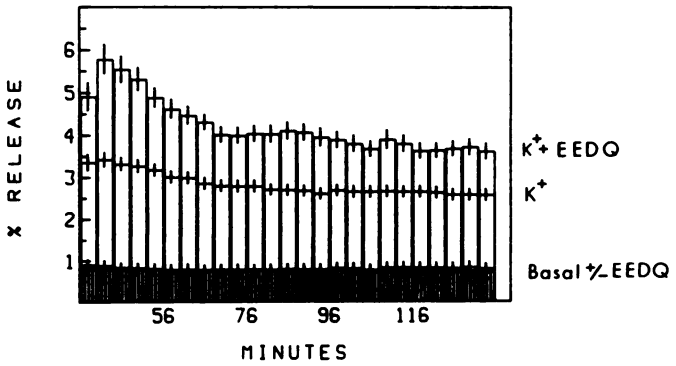

Fig. 8. Release of ^{3}H-NE following EEDQ treatment. After preloading with ^{3}H-NE, cortical slices were pretreated with 10 μM EEDQ as described. Each histogram is the mean ± SE of 24 (*Basal, bottom*), eight (*Basal + EEDQ, bottom*), 30 (K^+, *middle*) or eight (K^+ + EEDQ, *top*) determinations.

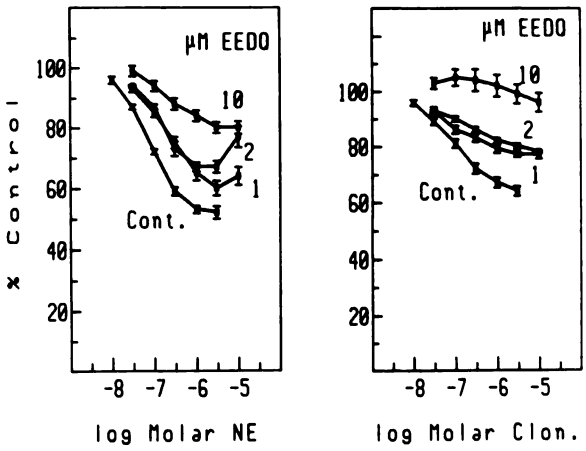

Fig. 9. Effect of EEDQ pretreatment on NE- and clonidine-induced inhibition of tritium release. Cortical slices were pretreated with 1–10 μM EEDQ as described in Methods. Inhibition of tritium release at each concentration of agonist was determined as described in Methods. All values are mean ± SE of six to eight observations.

TABLE 4

Agonist K_A values

K_A values (agonist affinity constants) were calculated (17) from dose-response curves for agonist inhibition of ^{3}H-NE release before and after partial receptor inactivation with 1–10 μM EEDQ. Each value is the mean ± SE of the indicated number of observations.

	NE	Epinephrine	UK 14,304	Clonidine
$-\log K_A$	7.11 ± 0.189 (n = 6)	7.04 ± 0.087 (n = 4)	6.84 ± 0.202 (n = 3)	6.85 ± 0.261 (n = 4)

results were obtained with the other agonists (data not shown). Binding constants at the functional receptors (K_A values) calculated from the dose-response curves before and after receptor inactivation (17), are listed in Table 4. For most agonists, the calculated K_A values did not differ significantly from the EC_{50} values of these drugs before receptor inactivation. However, the K_A value for epinephrine was 3.5-fold higher than its EC_{50} value ($p < 0.001$). K_A values for agonists were compared to K_D values for inhibiting ^{3}H-RX binding in the presence of Gpp(NH)p and NaCl in Fig. 10. Since NE and epinephrine were still best fit by a two-site model under these conditions, both K_D values for each of these agonists are shown. The K_A values most closely resemble the K_D values for the low affinity agonist binding state, but it is the subpopulation of sites with a higher affinity

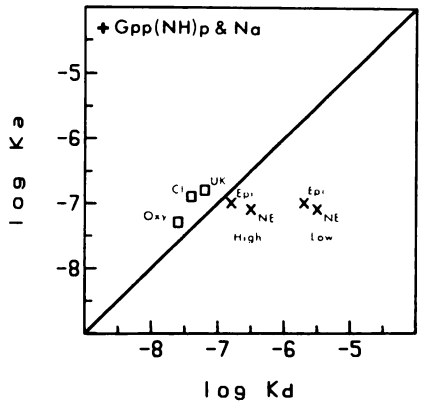

Fig. 10. Comparison of binding and functional potencies of agonists. K_A values for inhibiting tritium release are compared to K_D values for inhibiting specific ^{3}H-RX binding by agonists. Binding values are plotted in the presence of 10 μM Gpp(NH)p and NaCl. For NE and epinephrine, the K_H and K_L values from Table 2 are shown, since these curves were best fit by two-site models. The line shown is not the best fit line to the data points, but is simply the line of identity which is shown for the purpose of comparison.

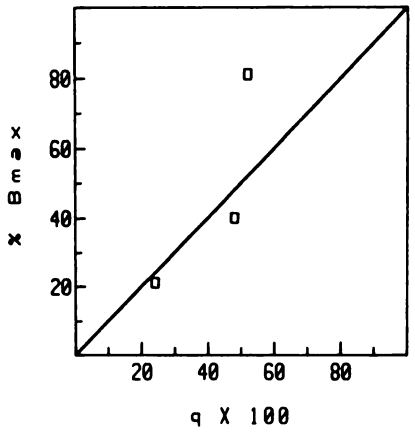

Fig. 11. B_{max} versus q values. Cortical slices were pretreated with EEDQ as described in Methods. B_{max} (density of ^{3}H-RX 781094 binding sites) were plotted versus q values (proportion of functional receptors remaining) after pretreatment of cortical slices with 1–10 μM EEDQ. The line shown is not the best fit line to the data points, but is simply the line of identity which is shown for the purpose of comparison.

for NE and epinephrine which resemble most closely the K_A values. In Fig. 11 the q values (the proportion of functional receptors remaining after inactivation) are compared to the density of ^{3}H-RX binding sites after inactivation with each concentration of EEDQ. The fairly good correlation supports the hypothesis that the binding sites are related to the functional receptors.

Discussion

High affinity antagonist radioligands have been shown to label binding sites with properties similar to α_2-adrenergic receptors mediating functional responses in a variety of tissues including kidney (20, 21), neuroblastoma-glioma cells (22), tracheal smooth muscle (23), and platelets (24). Our experiments show that ^{3}H-RX labels binding sites in rat cerebral cortex with drug specificities similar to the α_2-adrenergic receptors inhibiting ^{3}H-NE release in this tissue. If ^{3}H-RX labels these receptors, comparison of the potencies of agonists under different binding conditions with their potencies in activating this response should help elucidate the importance of the different binding states observed in membranes.

We examined ^{3}H-RX binding under conditions which have been shown to cause maximal changes in agonist affinities (25). Binding to washed particulate preparations was examined in the absence or presence of Gpp(NH)p and NaCl to promote "high" and "low" affinity agonist binding, respectively. As expected, the potencies of antagonists in competing for ^{3}H-RX binding were only slightly increased by the presence of Gpp(NH)p and NaCl, and were similar to their potencies in blocking the functional response. The potencies of agonists, however, were markedly decreased by inclusion of Gpp(NH)p and NaCl. Under "high affinity conditions" Hill coefficients for all agonists were significantly less than 1.0, consistent with the existence of binding site heterogeneity. Inclusion of Gpp(NH)p and NaCl increased these Hill coefficients, apparently by promoting the low affinity agonist binding state. However, the Hill coefficients for NE and epinephrine remained significantly less than 1.0, and the competition curves for these agonists remained best fit by a two-site model under "low affinity conditions." Although competition curves for these catecholamines were best fit by a two-site model under both low and high affinity conditions, the apparent decrease in affinity was caused by both a reduction in the proportion of high affinity sites and also a decrease in the affinity for these sites.

To compare the binding data with the potencies of agonists at the receptors inhibiting ^{3}H-NE release, it was important to determine whether there was a linear relationship between occupancy and response. In the presence of a receptor reserve, the EC_{50} values for agonists would not be the same as their equilibrium constants for binding to the functional receptor (12, 17, 26). Previous studies have shown a nonlinear relationship between α_2-adrenergic receptor occupancy and inhibition of adenylate cyclase activity in human platelets (27), and inhibition of ^{3}H-NE release from both rat heart (28) and cerebral cortex (29). However, we found little or no evidence for a receptor reserve in our experiments. Pretreatment of slices with increasing concentrations of the alkylating agent EEDQ (19) caused a progressive decrease in the density of ^{3}H-RX binding sites and also in the maximal effect of agonists in inhibiting ^{3}H-NE release. EEDQ pretreatment caused little or no change in the EC_{50} values for agonists in inhibiting release, however, and calculated K_A values did not usually differ significantly from EC_{50} values determined before receptor inactivation. The only agonist for which there was a significant difference between EC_{50} and K_A was epinephrine, and even this difference was small (3.5-fold).

EEDQ has been used to inactivate other types of receptors in addition to α_2-adrenergic receptors (30, 31). However, it is likely that the effects of EEDQ observed here are due to alkylation of α_2-adrenergic receptors. EEDQ was introduced as an α-adrenergic receptor alkylating agent (19) and it has been used successfully in previous studies to obtain K_A values for agonists at α_2-receptors (29). In addition, after pretreatment of cortical slices with EEDQ, the density of ^{3}H-RX binding sites decreased with almost exactly the same dose-dependence as the density of the functional α_2-receptors inhibiting ^{3}H-NE release (q values, Fig. 11). Finally, EEDQ pretreatment caused an increase in potassium-evoked ^{3}H-NE release that was almost identical to that caused by competitive α_2-receptor antagonist

drugs, suggesting that the increased release caused by this compound is caused by blockade of α_2-adrenergic receptors.

Adler *et al.* (29) reported evidence for a significant receptor reserve for UK 14,304 in inhibiting ^{3}H-NE release from cerebral cortex using a similar experimental protocol. This discrepancy may be due to minor differences in procedure, since Adler *et al.* (29) used a lower potassium concentration (20 versus 26 mM), and *in vivo* rather than *in vitro* EEDQ pretreatment. The depolarization in our experiments may have functionally antagonized the receptor reserve, or the additional incubation period required for *in vitro* EEDQ treatment may have been detrimental to the receptor coupling efficiency in our slices. The relationship between receptor occupancy and response depends on many different parameters, and variability in receptor reserve under different *in vitro* conditions is not necessarily surprising.

We were most interested in comparing the agonist binding properties of the receptors in membrane preparations with the state of the receptor activating the functional response. It is important to realize that drug concentrations and the microenvironment of the receptors might be slightly different in membrane and slice preparations, and therefore minor differences in drug potencies may not be surprising. For the full agonist UK 14,304 and the partial agonists clonidine and oxymetazoline, functional K_A values agreed most closely with the K_I values under low affinity conditions in the presence of Gpp(NH)p and NaCl. The K_A values were only 2–6-fold higher than the low affinity K_I, but 9–35-fold higher than the high affinity K_I. On the other hand, the K_A values for the natural catecholamine agonists NE and epinephrine were not similar to the low affinity K_I values, and correlated better with the K_I values determined under high affinity conditions. This was disturbing, since it would be surprising if different binding states of the receptor mediated responses to different agonists. Interestingly, only NE and epinephrine still exhibited low Hill coefficients and were best fit by two-site models under low affinity conditions, and the apparent high affinity site observed under these low affinity conditions was very similar to the K_A values for these agonists.

The high affinity agonist binding state of α_2-adrenergic receptors appears to be caused by formation of a stable ternary complex between agonist, receptor, and inhibitory G_i protein (32), which is destabilized by guanine nucleotides and sodium. It would not be surprising if agonists initially bind to the low affinity state of the receptor in the intact tissue, since this is the free receptor which is not complexed with G_i. We have made similar observations with β_2-adrenergic receptors in rat vas deferens, where the low affinity state not complexed with the stimulatory G_s protein appears to mediate the functional response (33). The similarities between the functional K_A values observed here and the K_I values for the entire population of binding sites for UK 14,304, clonidine, and oxymetazoline, and a high affinity subpopulation of binding sites for NE and epinephrine observed in the presence of Gpp(NH)p and NaCl, is consistent with this hypothesis.

The possible importance of the low affinity subpopulation of binding sites for NE and epinephrine observed in the presence of Gpp(NH)p and NaCl is intriguing. It is interesting that NE and epinephrine have a substantially lower affinity for binding to these sites than for activating the functional response. These sites might represent a low affinity nonfunctional state of the receptor which either exists *in vivo* or is an artifact of membrane preparation, although the absence of such a low affinity site for the synthetic full agonist UK 14,304 argues against this possibility. It is also possible that there are two subtypes of α_2-adrenergic receptors with different affinities for NE and epinephrine, and that only one subtype (with a high affinity for the natural agonists) is involved in inhibition of ^{3}H-NE release in cerebral cortex. Several authors have recently presented evidence for a heterogeneity in α_2-adrenergic receptor binding sites in different tissues (13, 34–37), although there is as yet no obvious relationship between these previous reports, which focused mainly on antagonists, and the two agonist affinity states reported here.

It is interesting to compare the functional K_A values we obtained for agonists with those calculated in previous reports. Using a similar system, Adler *et al.* (29) calculated the $-\log K_A$ for UK 14,304 in brain slices to be 6.04, slightly higher than our calculated value (6.84). The $-\log K_A$ for NE, however, has been reported to be 5.92 for inhibiting ^{3}H-NE release from rat heart (28) and 4.85 for inhibiting adenylate cyclase activity in platelets (27). These $-\log K_A$ values are substantially lower than our calculated $-\log K_A$ of 7.11, possibly supporting the hypothesis that different affinity states of the α_2-adrenergic receptor may mediate different functional responses. Interestingly, the $-\log K_A$ for oxymetazoline in rat heart was 7.26 (28), almost identical to the EC_{50} value we found for this partial agonist in brain. It will be interesting to determine the K_A values for these agonists in inhibiting cyclic AMP accumulation in brain slices (38).

In summary, we have presented evidence that the low affinity binding state of the α_2-adrenergic receptor induced by guanine nucleotides and sodium has pharmacological properties similar to those of the functional receptors inhibiting ^{3}H-NE release in this tissue. We interpret this data to suggest that this free form of the receptor, not complexed with G_i, is responsible for initially binding agonist. However, a subpopulation of these low affinity sites appears to have a substantially lower affinity for the natural catecholamine agonists NE and epinephrine, and may have some other function.

References

1. Berthelsen, B., and W. A. Pettinger. A functional basis for classification of α-adrenergic receptors. *Life Sci.* **21**:595–606 (1977).
2. Timmermans, P. B. M. W. M., and P. A. van Zwieten. Mini-review. The postsynaptic α_2-adrenoceptor. *J. Auton. Pharmacol.* **1**:171–183 (1981).
3. Starke, K. Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.* **77**:1–24 (1977).
4. Kalsner, S. Is there feedback regulation of neurotransmitter release by autoreceptors? *Biochem. Pharmacol.* **34**:4085–4097 (1985).
5. Bokoch, G. M., T. Katada, J. K. Northrup, M. Ui, and A. G. Gilman. Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.* **259**:3560–3567 (1984).
6. Gilman, A. G. G proteins and dual control of adenylate cyclase. *Cell* **36**:577–579 (1984).
7. Farnebo, L. O., and B. Hamberger. Drug-induced changes in the release of ^{3}H-monoamines from field stimulated brain slices. *Acta Physiol. Scand.* Suppl. **371**:35–44 (1971).
8. Starke, K., and H. Montel. Alpha-receptor-mediated modulation of transmitter release from central noradrenergic neurons. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **279**:53–60 (1973).
9. Frankhuyzen, A. L., and A. H. Mulder. A cumulative dose-response technique for the characterization of presynaptic receptors modulating ^{3}H-noradrenaline release from rat brain slices. *Eur. J. Pharmacol.* **78**:91–97 (1982).
10. Pimoule, C., B. Scatton, and S. Z. Langer. [^{3}H]RX 781094: A new antagonist ligand labels α_2-adrenoceptors in the rat brain cortex. *Eur J. Pharmacol.* **95**:79–85 (1983).
11. Lane, A. C., D. R. Howlett, and D. S. Walter. The effects of metal ions on the binding of a new alpha-2 adrenoceptor antagonist radioligand [^{3}H]RX 781094 in rat cerebral cortex. *Biochem. Pharmacol.* **32**:3122–3125 (1983).

12. Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol.* **11**:379–393 (1956).
13. Cheung, Y. D., D. B. Barnett, and S. R. Nahorski. Interactions of endogenous and exogenous norepinephrine with alpha-2 adrenoceptor binding sites in rat cerebral cortex. *Biochem. Pharmacol.* **33**:1293–1298 (1984).
14. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
15. Munson, P. J., and D. Rodbard. Ligand: A versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
16. Arunlakshana, O., and H. O. Schild. Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14**:48–58 (1959).
17. Furchgott, R. F. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes, in *Advances in Drug Research* (N. J. Harper and and A. B. Simmonds, eds.) Academic Press, London Vol. 3, pp 21–55 (1966).
18. Minneman, K. P., and P. W. Abel: "Spare" α_1-adrenergic receptors and the potency of agonists in rat vas deferens. *Mol. Pharmacol.* **25**:56–63 (1984).
19. Belleaue, B., R. Mortel, G. Lacasse, M. Henard, N. L. Weinberg, and Y. G. Petton. N-carboxylic acid esters of 1,2- and 1,4-dihydroquinolines. A new class of irreversible inactivators of the catecholamine alpha receptors and potent central nervous system depressants. *J. Am. Chem. Soc.* **90**:823–824 (1968).
20. Schmitz, J. M., R. M. Graham, A. Sagalowsky, and W. A. Pettinger. Renal α_1- and α_2-adrenergic receptors: Biochemical and pharmacological correlations. *J. Pharmacol. Exp. Ther.* **219**:400–406 (1981).
21. Woodcock, E. A., and C. I. Johnston. Characterization of adenylate cyclase-coupled α_2-adrenergic receptors in rat renal cortex using [^{3}H]yohimbine. *Mol. Pharmacol.* **22**:589–594 (1982).
22. Kahn, D. J., J. C. Mitrius, and D. C. U'Prichard. Alpha$_2$-adrenergic receptors in neuroblastoma × glioma hybrid cells. Characterization with agonist and antagonist radioligands and relationship to adenylate cyclase. *Mol. Pharmacol.* **21**:17–26 (1982).
23. Barnes, P. J., B. E. Skough, J. A. Nadil, and J. M. Roberts. Postsynaptic alpha$_2$-adrenoceptors predominate over alpha$_1$-adrenoceptors in canine tracheal smooth muscle and mediate neuronal and hormonal alpha-adrenergic contraction. *Mol. Pharmacol.* **23**:570–575 (1983).
24. Ferry, N., D. Henry, E. Buttais, A. Mary, C. Bonne, and J. Hanoune. Critical assessment of the platelet adenylate cyclase system as a potential model for testing alpha$_2$-adrenergic activity. *Biochem. Pharmacol.* **35**:1511–1516 (1986).
25. Cheung, Y. D., D. B. Barnett, and S. R. Nahorski. Heterogenous properties of alpha$_2$-adrenoceptors in particulate and soluble preparations of human platelet and rat and rabbit kidney. *Biochem. Pharmacol.* **35**:3767–3775 (1986).
26. Nickerson, M. Receptor occupancy and tissue response. *Nature* **178**: 697–698 (1956).
27. Lenox, R. H., J. Ellis, D. Van Riper, and Y. H. Ehrlich. Alpha$_2$-adrenergic receptor-mediated regulation of adenylate cyclase in the intact human platelet. Evidence for a receptor reserve. *Mol. Pharmacol.* **27**:1–9 (1985).
28. Fuder, H., H.-J. Braun, and R. Schimkus. Presynaptic alpha$_2$-adrenoceptor activation and coupling of the receptor-presynaptic effector system in the perfused rat heart: Affinity and efficacy of phenylethylamines and imidazoline derivatives. *J. Pharmacol. Exp. Ther.* **237**:237–245 (1986).
29. Adler, C. H., E. Meller, and M. Goldstein. Receptor reserve at the alpha$_2$-adrenergic receptor in the rat cerebral cortex. *J. Pharmacol. Exp. Ther.* **240**:508–515 (1987).
30. Chang, K. J., J. F. Moran, and D. J. Triggle. Mechanism of cholinergic antagonism by N-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline (EEDQ). *Pharmacol. Res. Commun.* **2**:63–66 (1970).
31. Battaglia, G., A. B. Norman, P. L. Newton, and I. Creese. *In vitro* and *in vivo* irreversible blockade of cortical S_2 serotonin receptors by N-ethoxy-carbonyl 2-ethoxy-1,2-dihydroquinoline. *Life Sci.* **32**:2247–2255 (1983).
32. Smith, S. K. and L. E. Limbird. Solubilization of human platelet α-adrenergic receptors: Evidence that agonist occupancy of the receptor stabilizes receptor-effector interactions. *Proc. Natl. Acad. Sci.* U. S. A. **78**:4026–4030 (1981).
33. May, J. M., P. W. Abel, and K. P. Minneman. Binding of agonists and antagonists to β-adrenoceptors in rat vas deferens: Relationship to functional response. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **331**:324–333 (1985).
34. Latifpour, J., S. B. Jones, and D. B. Bylund. Characterization of [^{3}H] yohimbine binding to putative alpha$_2$-adrenergic receptors in neonatal rat lung. *J. Pharmacol. Exp. Ther.* **223**:606–611 (1982).
35. Kawahara, R. S., and D. B. Bylund. Soubilization and characterization of putative α_2-adrenergic isoceptors from the human platelet and the rat cerebral cortex. *J. Pharmacol. Exp. Ther.* **233**:603–610 (1985).
36. Neylon, C. B., and R. J. Summers. [^{3}H]-Rauwolscine binding to α_2-adrenoceptors in the mammalian kidney: Apparent receptor heterogeneity between species. *Br. J. Pharmacol.* **85**:349–359 (1985).
37. Alabaster, V. A., R. F. Keir, and C. J. Peters. Comparison of potency of α_2-adrenoceptor antagonists *in vitro*: Evidence for heterogeneity of α_2-adrenoceptors. *Br. J. Pharmacol.* **88**:607–614 (1986).
38. Duman, R. S., and S. J. Enna. A procedure for measuring α_2-adrenergic receptor-mediated inhibition of cyclic AMP accumulation in rat brain slices. *Brain Res.* **384**:391–394 (1986).

Send reprint requests to: Dr. Ali Nasseri, Department of Pharmacology, Emory University Medical School, Atlanta, GA 30322.