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Alpha$_2$-Adrenergic Receptor-Mediated Regulation of Adenylate Cyclase in the Intact Human Platelet

Evidence for a Receptor Reserve[1]

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SUMMARY

The *alpha*$_2$-adrenergic receptor on the human platelet is known to mediate the inhibition of adenylate cyclase activity. A comparison of the binding and response properties of intact cells revealed that the full agonists norepinephrine and epinephrine inhibit cyclic AMP accumulation with apparently higher affinity than they exhibit in inhibiting the binding of [^{3}H]yohimbine. Additionally, Hill coefficients of the occupancy curves of the agonists were less than unity, suggesting the presence of a heterogeneous receptor population in intact platelets under conditions that permit robust inhibition of cyclic AMP accumulation. The partial agonist clonidine was found to possess the same affinity in the binding assay as in the response assay. These data are consistent with the presence of a receptor reserve in this system, a suggestion that was confirmed in experiments utilizing the irreversible *alpha*$_2$ antagonist phenoxybenzamine. The IC_{50} (100 nM) derived from the blockade of [^{3}H]yohimbine binding by phenoxybenzamine was significantly less than the IC_{50} (550 nM) for the corresponding reversal by phenoxybenzamine of the effects of norepinephrine on cyclic AMP accumulation. Further studies demonstrated a rightward shift in the dose-response curves for the inhibition by norepinephrine of cyclic AMP accumulation following pretreatment with increasing phenoxybenzamine concentration. These data consistently indicated that occupancy of approximately 10% of the *alpha*$_2$-adrenergic receptors by norepinephrine elicits a half-maximal adenylate cyclase response. The relationship of these findings to current models of receptor-effector coupling is discussed.

INTRODUCTION

The development of radioactive ligands of high specific activity has made it possible to measure the binding of ligands to receptors that mediate specific pharmacological responses. However, when the ligand under investigation is an agonist, these two approaches to the assessment of receptor affinity (that is, binding versus response) may yield discrepant results; due to possible nonlinear coupling of receptor occupation and measured response (1). When experimental conditions are such that a binding assay accurately reflects the degree of occupancy that exists in a corresponding response assay, the relationship between the results of the two assays may be used to investigate receptor-effector coupling processes.

Pharmacological responses are most commonly studied in intact tissues, while binding assays are usually conducted in cell-free systems. Intact dissociated cells represent ideal systems in which both assays may be performed. Because such a system minimizes the diffusional barriers of intact tissues, sophisticated binding assays become more feasible. It is worth noting that, in some systems wherein binding and response can be examined both in intact cells and in homogenates of those cells, the results obtained from the two preparations have been found to be markedly different (2, 3). These findings probably reflect the impossibility of simulating the extracellular environment and the environments of intracellular compartments at the same time.

The human platelet possesses adrenergic receptors that mediate the inhibition of the enzyme adenylate cyclase (4). Recent studies (4, 5) have demonstrated that [^{3}H]yohimbine labels *alpha*$_2$-adrenergic receptors on intact platelets with good specificity. Thus, intact platelets, which are readily obtained in very pure form from blood, are an ideal system in which to study the relationship

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between binding and response properties of *alpha*$_2$ receptors. MacFarlane and Stump (5) have recently reported a serial comparison of *alpha*$_2$-adrenergic binding and response in intact platelets. We report here that the comparison of parallel assays yields results that are consistent with their findings. That is, full *alpha*$_2$-adrenergic agonists achieve half-maximal inhibition of cyclic AMP accumulation in intact human platelets at concentrations which occupy much less than half of the *alpha*$_2$-adrenergic receptors defined by the binding of [^{3}H]yohimbine. Thus, the simplest model of receptor-effector coupling, i.e., the occupancy assumption (6), does not apply to this system. It is possible that the response might be mediated by a subpopulation of *alpha*$_2$-adrenergic receptors that possess high affinity for agonists, that a receptor reserve exists, or that nonlinear coupling of occupancy to response occurs by a mechanism that does not involve spare receptors. Some *beta*-adrenergic systems that exhibit such nonlinear coupling have been found to possess a receptor reserve (7, 8), while others have not (9, 10). In order to resolve this question, the present study has further evaluated the lack of a 1:1 correspondence between binding and response in this *alpha*$_2$-adrenergic system by the use of the irreversible antagonist phenoxybenzamine. Some of these data have been presented in a preliminary form (11).

MATERIALS AND METHODS

Platelet preparation. Blood was drawn from drug-free volunteers by venipuncture and collected in plastic centrifuge tubes containing 0.5 ml of 150 mM EDTA on ice. Platelets were isolated by centrifugation over Ficoll-Hypaque at 200 × *g* for 15 min at 4°.

Cyclic AMP accumulation. An aliquot of the platelet-rich plasma was incubated with [^{3}H]adenine (New England Nuclear, 17.0 Ci/mmol) for 1 hr at 37° at the ratio of 10 μCi of [^{3}H]adenine per 10^9 platelets. The platelets were then washed in cyclase wash buffer (2.5 mM KCl, 0.4 mM NaH_2PO_4, 6.6 mM EDTA, 35 mM Tris, 103.7 mM NaCl, 3.3 mM $NaHCO_3$, 5.0 mM glucose, pH 7.4) and resuspended in the same buffer containing 5 mM $MgSO_4$ and 5 mM theophylline (cyclase incubation buffer). The enzyme reaction was initiated by the addition of 1 μM PGI_2[3] (or 10 μM PGE_1) and terminated after 2 min (or 10 min) at 37° by the addition of 0.5 ml of 0.75 mM cyclic AMP and heating to 100° for 10 min. The reaction volume was 100 μl and the final platelet concentration was 10^9 platelets/ml. In studies which examined the effects of *alpha*$_2$ agonists, l-norepinephrine, l-epinephrine, or clonidine was added at the time of initiation. The [^{3}H]cyclic AMP formed was isolated using a modification of the sequential chromatography method of Salomon (12). The reaction products were centrifuged at 2500 × *g* for 10 min and the resulting supernatant was applied to 0.4 × 3.3-cm cation exchange columns (Bio-Rad AG 50W-X8) and the ^{3}H-labeled precursor nucleotides eluted with water. After eluting the contents into alumina columns, the [^{3}H]cyclic AMP was eluted with 0.1 M imidazole, pH 7.4. Aliquots of the alumina eluates and ^{3}H-labeled nucleotide precursor fractions were counted with 5 ml of scintillation cocktail. The results are expressed as per cent conversion of ^{3}H-labeled precursor to [^{3}H]cyclic AMP. Basal enzyme levels were determined in the absence of PGI_2 and column recovery was better than 70%.

Intact platelet-binding assays. An aliquot of the platelet-rich plasma was washed twice with cyclase wash buffer and the platelets were suspended in cyclase incubation buffer. Platelet-binding experiments were carried out in a volume of 300 μl for 10 min at 37°, conditions under which equilibrium values were achieved. Final platelet concentrations were approximately 6–8 × 10^8 platelets/ml. Reactions were stopped by the addition of 2 ml of cold rinse buffer (50 mM Tris, 5 mM EDTA, pH 6.9) and the contents of the tubes were immediately filtered over GF/B filters. The filters were then washed rapidly four times with 4 ml of cold buffer. The filters were placed in scintillation vials and counted with 5 ml of scintillation cocktail. Nonspecific binding was determined in the presence of 10 μM phentolamine. The binding of the unlabeled agonists was inferred from the concentration-dependent inhibition of the binding of [^{3}H]yohimbine.

Phenoxybenzamine experiments. Platelets in platelet-rich plasma were washed and suspended in cyclase wash buffer and incubated with phenoxybenzamine for 45 min at 37°. The platelets were then washed two times with buffer and split into two aliquots. The platelets for the cyclic AMP assay were incubated for 1 hr at 37° with [^{3}H]adenine in the presence of platelet-poor plasma; the platelets for the binding portion were incubated similarly, but without [^{3}H]adenine. The cyclic AMP assays were performed as above. The phenoxybenzamine-treated platelets for the binding studies were incubated with [^{3}H]yohimbine to determine the level of *alpha*$_2$ binding after phenoxybenzamine treatment. Platelets pretreated with phenoxybenzamine for membrane-binding studies were sonicated with a Branson cell disruptor. Binding assays were carried out in cyclase incubation buffer for 30 min at 25° followed by filtration with a Brandel cell harvester using GF/B filters. The filters were counted with 5 ml of scintillation fluid.

Data analysis. Curves representing concentration-dependent inhibition of cyclase activity were analyzed by a nonlinear four-parameter regression. Data from direct and competitive binding experiments were analyzed by a nonlinear curve-fitting program which has been extensively evaluated (13) and provides statistical measures of goodness-of-fit, comparisons of n to $n + 1$ receptor models, simultaneous analysis of several curves, and corrections for nonhomogeneity of variance and depletion of radioligand.

RESULTS

Studies of the binding of [^{3}H]yohimbine to intact platelets yielded results which were in agreement with previous reports (4). The ratio of off-rate (0.34 min^{-1}) to on-rate (0.083 min^{-1} nM^{-1}) is in good agreement with values obtained from equilibrium measurements (K_d = 3.1 nM, B_{max} = 153 sites/platelet; data not shown).

Comparison of binding and response characteristics of the *alpha*$_2$-adrenergic receptor on intact platelets (Fig. 1) revealed that the concentration curve for inhibition of cyclic AMP accumulation by norepinephrine lies to the left of the agonist competition curve for binding at the *alpha*$_2$ receptor site. These data demonstrate that the effects of norepinephrine exceed the levels of its receptor occupancy, i.e., the EC_{50} value is much lower than the corresponding $K_{I\,app}$ value.

In this study of the *alpha*$_2$ receptor, agonist-binding parameters were determined from the concentration-dependent competition for [^{3}H]yohimbine binding. Direct agonist-binding studies were not employed for several reasons. First, radiolabeled *alpha*$_2$ agonists generally label only high affinity *alpha*$_{2H}$ sites (14, 15) or label *alpha*$_{2H}$ sites plus a variable portion of low affinity *alpha*$_{2L}$ sites (16), which may add a significant amount of variability to direct binding studies. Additionally, the affinities of the agonists are markedly lower when assessed in intact cells with physiological buffers when compared with membranes resuspended in sodium-free buffers (4). Finally, the analysis of direct and competitive binding studies is simplest and most reliable when the binding of the radiolabeled ligand is adequately described

[3] The abbreviations used are: PGI_2 and PGE_1, prostaglandins I_2 and E_1; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

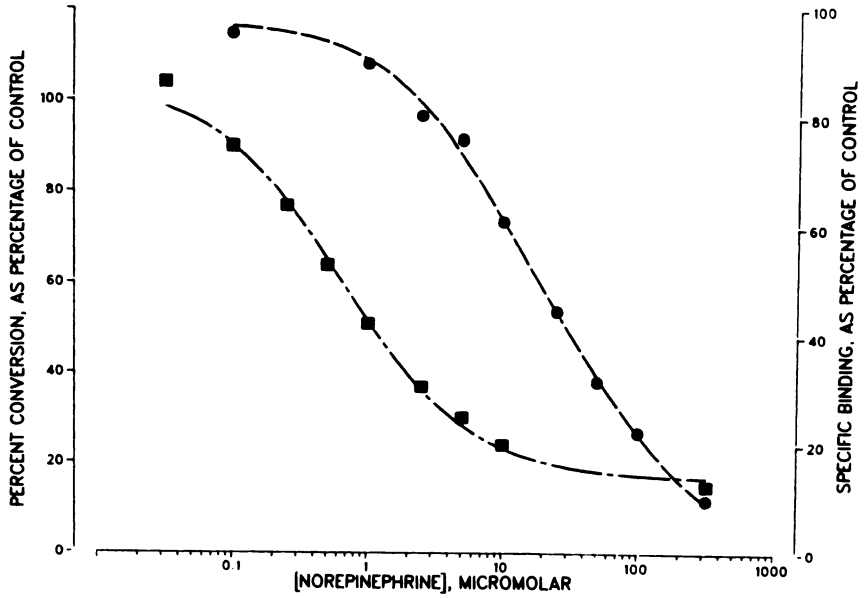

FIG. 1. *Comparison of cyclic AMP response and alpha$_2$-adrenergic receptor binding*

Norepinephrine inhibition of both cyclic AMP accumulation (■) and [^{3}H]yohimbine binding (●) was determined simultaneously using intact platelets obtained from the same individual (n = 3). For the adenylate cyclase assays, 100% corresponds to 3.6% conversion (range, 3.3–3.8). [^{3}H]Yohimbine was present at 2 nM. All points were determined in triplicate. Analysis of the binding data yielded high and low affinity dissociation constants for norepinephrine and epinephrine but only one dissociation constant for clonidine: norepinephrine, K_H = 5.6 ± 2.2 μM (59 ± 15%); K_L = 50 ± 27 μM; epinephrine, K_H = 0.16 ± 0.1 μM (31 ± 10%); K_L = 14 ± 5 μM; clonidine, K_D = 0.19 ± 0.02 μM. Values are mean ± standard error.

by a homogeneous receptor population. For these reasons, we believe that indirect competitive binding represents the only way to obtain data with the degree of precision required by the present study.

Epinephrine and norepinephrine inhibited approximately 80% of the PGI_2-stimulated cyclic AMP accumulation of intact human platelets (Table 1). Clonidine, however, achieved less than half of that level of inhibition even at saturating concentrations, in agreement with previous reports that it is a partial agonist in this system. As expected of a partial agonist, clonidine partially reversed the effects of the full agonist norepinephrine (Fig. 2). The *alpha$_2$* antagonist yohimbine, on the other hand, completely blocked the inhibition produced by epinephrine, norepinephrine, and clonidine (data not shown). As in the case of norepinephrine, the inhibitory effects of epinephrine on cyclic AMP accumulation exceeded the level of its receptor occupancy, with a significant discrepancy between EC_{50} and $K_{I\,app}$ values (Table 1). On the other hand, the EC_{50} and $K_{I\,app}$ values for the partial agonist, clonidine, were comparable, suggesting that receptor occupancy in this case may be linearly proportional to response.

TABLE 1

Comparison of binding and response characteristics of alpha$_2$-adrenergic agonists in intact platelets

Assays were performed as described in Materials and Methods. Results are expressed as the mean ± standard error.

Agonist	Cyclic AMP accumulation		Receptor binding	
	Maximal inhibition[a]	EC_{50}[b]	$K_{I\,app}$[c]	n_H[d]
	%	μM	μM	
Epinephrine	85 ± 2.0	0.24 ± 0.08	5.2 ± 1.0	0.63 ± 0.16
Clonidine	29 ± 1.6	0.16 ± 0.01	0.24 ± 0.04	0.86 ± 0.04
Norepinephrine	85 ± 1.6	0.65 ± 0.05	19 ± 5.1	0.73 ± 0.03

[a] Maximal inhibition refers to the extent of the reduction in per cent conversion of ^{3}H-labeled precursor to [^{3}H]cyclic AMP, at saturating levels of agonist; per cent conversion in the absence of *alpha$_2$*-adrenergic agonists was 3.5%.

[b] EC_{50} is the concentration of agonist producing half-maximal reduction in per cent conversion.

[c] $K_{I\,app}$ is the concentration of agonist which produces 50% reduction in specific binding of [^{3}H]yohimbine, corrected for the presence of [^{3}H]yohimbine, corresponding to 50% occupancy.

[d] n_H is the Hill coefficient, derived from the competitive binding assay.

Our data also indicate that the Hill coefficients for the agonists are consistently less than unity (Table 1). In platelet membranes, Hill coefficients for agonists are less than unity in the absence of sufficient levels of GTP or Gpp(NH)p; the presence of these guanyl nucleotides shifts agonist occupancy curves to the right and steepens them, so that n_H = 1 (17). Since the effectiveness of *alpha$_2$*-adrenergic agonists in inhibiting adenylate cyclase in cell-free assays is dependent on the presence of GTP (18, 19), it is likely that the intracellular environment of intact platelets satisfies this guanyl nucleotide requirement. At the same time, it appears that the intracellular level of GTP is not sufficient to promote the regulation of agonist binding and consequent shift to n_H = 1 that is observed in cell-free preparations. In agreement with U'Prichard *et al.* (20), we have not observed any effect of extracellular GTP on binding properties (data not shown). Thus, heterogeneity of agonist binding can be retained under conditions that permit robust inhibition of adenylate cyclase activity.

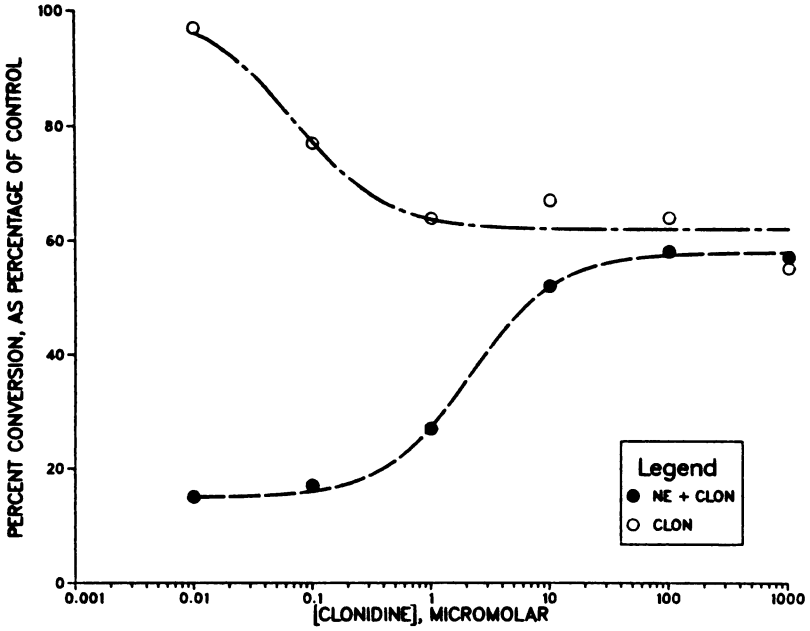

FIG. 2. *Agonist-antagonist properties of clonidine*

Platelets, prelabeled with [^{3}H]adenine, were stimulated with 1 μM PGI_2 in the presence of clonidine (*CLON*) or clonidine + 10 μM norepinephrine (*NE*). All points were determined in triplicate and the results are expressed as a percentage of the values obtained in the absence of both agents, which was 4.4% conversion.

It should be noted that the comparisons of binding and response shown in Table 1 were not conducted under exactly the same conditions. The binding assays did not contain PGI_2 and extended for 10 min while the adenylate cyclase assays did contain PGI_2 and persisted for only 2 min. It is possible that the duration of the assay or the presence of prostacyclin (and consequent rise in intracellular concentration of cyclic AMP) influenced the ratio of EC_{50} to $K_{I\,app}$ or the value of n_H. Since PGI_2 is substantially degraded in 10 min, PGE_1 was used to examine this possibility. Figure 3 shows the comparison of a 10-min cyclic AMP response system which included PGE_1 (10 μM) with a 10-min binding assay containing the same amount of PGE_1, along with the results of assays run under our standard conditions. These results indicate that there is a significant difference in the level of cyclic AMP accumulation achieved by PGI_2 as compared to PGE_1. This difference in level of cyclic AMP does not affect the sensitivity of the system to norepinephrine as evidenced by the similarity of the respective EC_{50} values. Moreover, it is clear that the presence or absence of the prostaglandin in the binding assay has no effect on the agonist occupancy curve. Our data demonstrate that the results of our standard assays comparing binding and response were not influenced by the differences in conditions that existed between the respective assays.

The nature of the interaction of phenoxybenzamine with platelet *alpha*$_2$ receptors was first investigated by assessing its effects on the binding of [^{3}H]yohimbine. Intact platelets were preincubated with different concentrations of phenoxybenzamine and the binding properties of these platelets were subsequently examined. Analysis of the curves shown in Fig. 4 indicated that phenoxybenzamine reduced the B_{max} for [^{3}H]yohimbine in a concentration-dependent manner without affecting the K_d, a result consistent with irreversible antagonism at the *alpha*$_2$ receptor site. The same conclusion was reached when similar binding assays were conducted with membranes prepared from such platelets (data not shown).

Competition by norepinephrine for [^{3}H]yohimbine binding to membranes prepared from platelets which had been preincubated with phenoxybenzamine was investigated in order to examine any possible effects of phenoxybenzamine on agonist interaction with the *alpha*$_2$ site. Preincubation with 100 and 200 nM phenoxybenzamine blocked approximately 40 and 60% of the *alpha*$_2$ sites, respectively, without affecting the affinity or the slope of the norepinephrine curve (Fig. 5). Computer analysis of the data (see Materials and Methods) confirmed that the agonist affinities of the subpopulations (*alpha*$_{2H}$ and *alpha*$_{2L}$) and the relative proportions of the subpopulations were not altered by the phenoxybenzamine preincubation, implying that phenoxybenzamine, like yohimbine, does not differentiate the agonist subpopulations of *alpha*$_2$ receptor.

In order to establish that the action of phenoxybenzamine is mediated by blockade of the *alpha*$_2$-adrenergic receptor, we examined both the binding of [^{3}H]yohimbine and the norepinephrine inhibition of cyclic AMP accumulation with intact platelets which had been preincubated with phenoxybenzamine alone or in the presence of yohimbine. The data shown in Fig. 6 demonstrate phenoxybenzamine's irreversible antagonism of the inhibitory effect of norepinephrine on cyclic AMP accumulation and blockade of [^{3}H]yohimbine binding. The presence of the reversible *alpha*$_2$ receptor antagonist yohimbine in the preincubation markedly reduces the

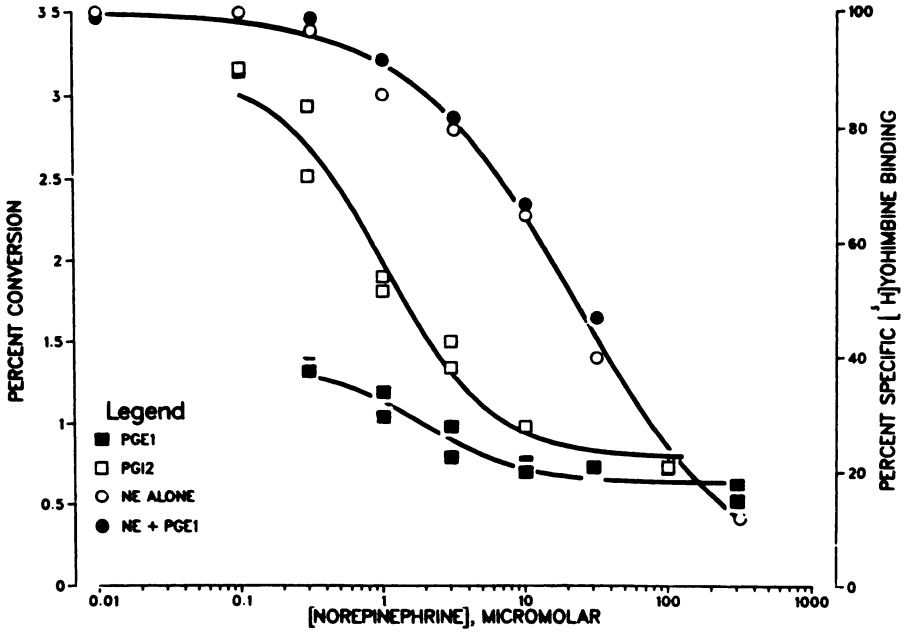

FIG. 3. *Norepinephrine-mediated inhibition of both cyclic AMP accumulation and [^{3}H]yohimbine binding under identical incubation conditions*
Determination of inhibition of cyclic AMP accumulation was carried out using either 10 μM PGE_1 for 10 min (■) or 1 μM PGI_2 for 2 min (□). Competition for [^{3}H]yohimbine binding (○) was done as described in Materials and Methods or in the presence of 10 μM PGE_1 (●), both for 10 min. PGI_2: EC_{50}, 1.03 μM; $K_{I\,app}$, 20 μM; n_H, 0.73. PGE_1: EC_{50}, 1.95 μM; $K_{I\,app}$, 25 μM; n_H, 0.74. *NE*, norepinephrine.

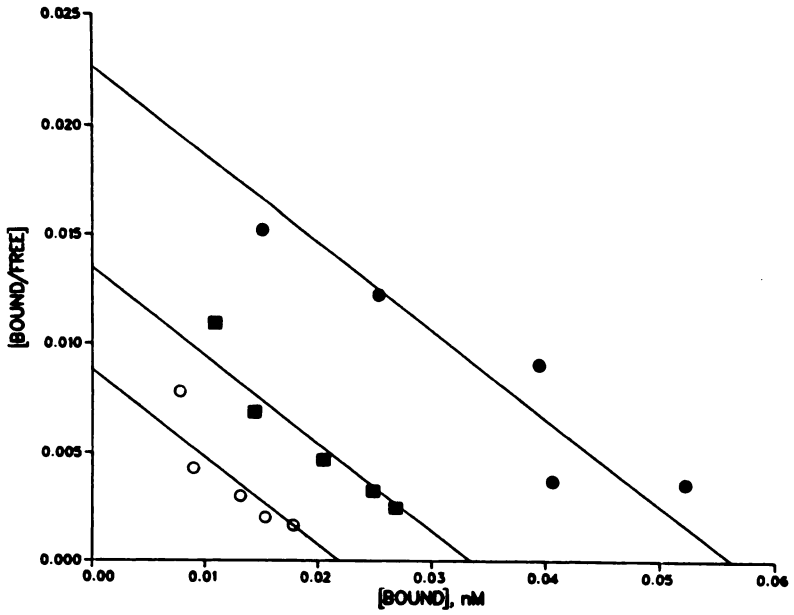

FIG. 4. *Binding of [^{3}H]yohimbine to intact platelets following preincubation with phenoxybenzamine*
Intact platelets were preincubated with 100 (●), 180 (■), or 315 nM (○) phenoxybenzamine, washed, and resuspended. The specific binding of [^{3}H]yohimbine was then assessed as described in Materials and Methods. The binding parameters were determined by nonlinear curve-fitting (see text) and then replotted in the Scatchard form for visual clarity. Control (without phenoxybenzamine; not shown because of scale): K_d = 2.5 nM, B_{max} = 0.22 nM; 100 nM phenoxybenzamine: K_d = 2.5 nM, B_{max} = 0.057 nM; 180 nM phenoxybenzamine: K_d = 2.3 nM, B_{max} = 0.032 nM; 315 nM phenoxybenzamine: K_d = 2.2 nM, B_{max} = 0.020 nM.

action of phenoxybenzamine in both binding and response assays, and further substantiates the *alpha*$_2$-adrenergic receptor as the site of action of phenoxybenzamine in this system.

The level of *alpha*$_2$-adrenergic activity remaining following varying degrees of receptor blockade was determined in intact platelets which had been preincubated with different concentrations of phenoxybenzamine (Fig. 7). The degree of blockade of the receptor population was assessed in a parallel assay in which the binding of 4 nM [^{3}H]yohimbine to membranes derived from the same cells was determined for each phenoxybenzamine concentration. The concentration-response curve for phenoxybenzamine's blockade of [^{3}H]yohimbine binding lies to the left of the corresponding curve for phenoxybenzamine's reversal of the effect of the norepinephrine

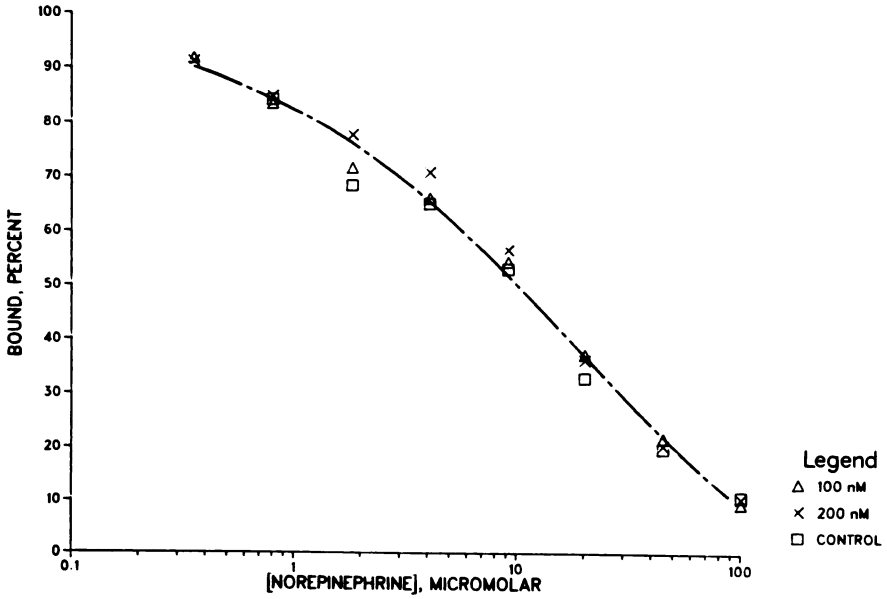

FIG. 5. *Effects of phenoxybenzamine on norepinephrine competition for [^{3}H]yohimbine binding to platelet membranes*

Intact platelets were treated with 100 or 200 nM phenoxybenzamine as above and then sonicated with a Branson cell disruptor. The resulting membranes were sedimented at 25,000 × *g* for 10 min and washed with cyclase wash buffer. Binding assays were carried out as previously described. The curves shown are a representative of three experiments, each point done in triplicate. The concentration of [^{3}H]yohimbine was 4 nM. Specific binding in the absence of norepinephrine (100%) corresponded to 1644 (□, control), 1023 (△, 100 nM phenoxybenzamine), and 597 cpm (×, 200 nM phenoxybenzamine).

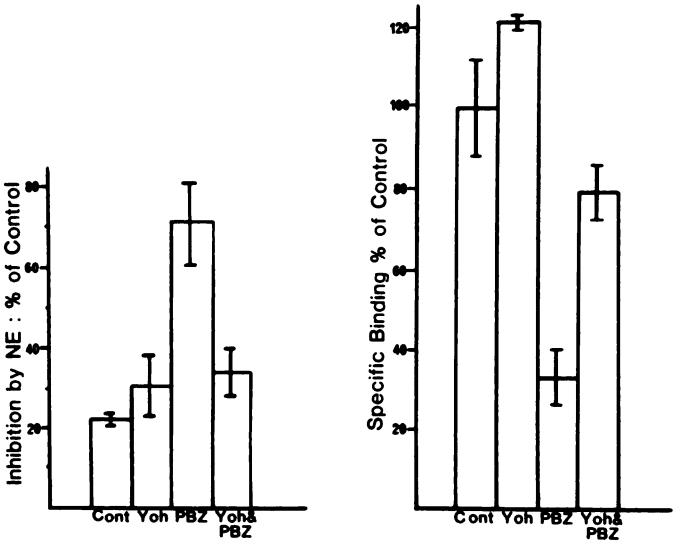

FIG. 6. *Protection of the alpha$_2$ site by yohimbine*

Platelets were prepared and preincubated with 315 nM phenoxybenzamine (*PBZ*) in the presence or absence of 25 μM yohimbine (*Yoh*). The effectiveness of norepinephrine (*NE*, 100 μM) in inhibiting the accumulation of cAMP was then determined and expressed as a percentage of control (*Cont*) values (preincubated without phenoxybenzamine or yohimbine, 4.0% conversion). The number of receptors remaining after the preincubation was determined from the specific binding of 4 nM [^{3}H]yohimbine to the intact platelets and expressed as a percentage of the binding found with untreated platelets (1281 cpm).

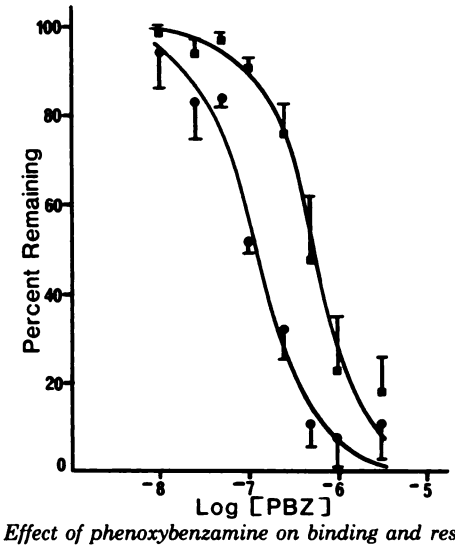

FIG. 7. *Effect of phenoxybenzamine on binding and response characteristics of the alpha$_2$ receptor*

Platelets were preincubated with the indicated concentrations of phenoxybenzamine (*PBZ*) and then split into two aliquots. The binding of 4 nM [^{3}H]yohimbine was determined in membranes derived from one aliquot (●), and the degree of inhibition of cyclic AMP accumulation by 100 μM norepinephrine was determined in intact cells of the other aliquot (■). The results are expressed as the percentage of the binding or of the degree of inhibition that was observed in tissue preincubated without phenoxybenzamine (951 ± 146 cpm and 79 ± 2% inhibition, respectively). The values shown are means ± standard error for four experiments.

on cyclic AMP accumulation (Fig. 7). Thus, 50% of the response remains under conditions that eliminate 90% of the binding sites.

Figure 8 displays concentration-response curves for the inhibition by norepinephrine of the accumulation of cyclic AMP in intact platelets that have been pretreated with 0, 125, or 250 nM phenoxybenzamine. Preincubation with phenoxybenzamine shifted the curves to the right to a statistically significant extent (see legend to Fig. 8), despite the fact that such preincubations did not alter receptor affinity for yohimbine (Fig. 4) or norepinephrine

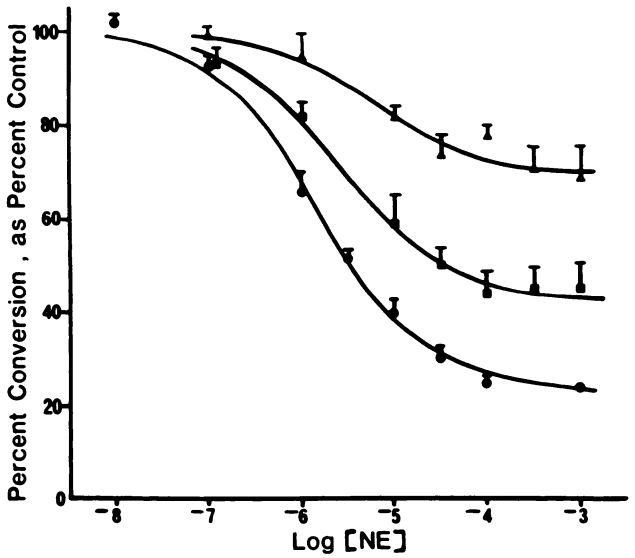

FIG. 8. *Effect of preincubation with phenoxybenzamine on the norepinephrine concentration-response curve*

Platelets were preincubated with phenoxybenzamine at concentrations of 125 (■) and 250 nM (▲), washed, and resuspended as described in Materials and Methods. Control platelets were preincubated with no phenoxybenzamine (●). Each curve represents three independent experiments. In the absence of norepinephrine (*NE*), conversion averaged 3.22%. The curves were analyzed in pairs by a nonlinear four-parameter regression function (described in Ref. 13). Each fit was conducted twice, once constraining the EC_{50} parameter (*C*, see Ref. 13) for the control and experimental curves to be equal and once allowing each EC_{50} value to "float" freely. The hypothesis that the differences in the unconstrained values were due to random variation was then tested by a partial *F* test. The control value for EC_{50} was 1.5 μM; after 125 nM phenoxybenzamine, EC_{50} = 2.6 μM ($\nu_2 = 9$, $F = 5.89$, $p < 0.05$); after 250 nM phenoxybenzamine, EC_{50} = 6.4 μM ($\nu_2 = 9$, $F = 9.79$, $p < 0.025$).

(Fig. 5). This finding is consistent with the concept that the apparent affinity (EC_{50}) of norepinephrine is approaching the true affinity (K_A) of norepinephrine for the site that mediates the response, as phenoxybenzamine progressively reduces the size of the receptor reserve. However, the size of the receptor reserve appears to be sufficiently modest that it is not possible to demonstrate a significant rightward shift without a loss of maximal response.

The data in Fig. 8 were analyzed by a modification of the method of Furchgott (see Ref. 21 and related references found therein) that rearranged the linearized form of the equation and employed nonlinear curve-fitting techniques to fit the curves. Our treatment avoids the complication of dealing with the statistical errors associated with reciprocal values. Thus, we employed the following equation:

$$A = \frac{qA'\,K_A}{K_A + A'\,(1 - q)} \qquad (1)$$

where A' is the concentration of agonist needed to produce a given level of response after inactivation of the fraction $(1 - q)$ of the receptors, A is the concentration that gave the level of response before inactivation, K_A is the true affinity of the agonist for the active site, and q is the fraction of receptors remaining active; these definitions are the same as in Ref. 21. The data points and best-fit curves are displayed in Fig. 9, along with details of how the analysis was performed. The conclusion from these fitting procedures is that the affinity of norepinephrine for the site that mediates the inhibition of cyclic AMP accumulation is approximately 14 μM. The half-maximal response is elicited in untreated platelets at 1.5 μM (Fig. 8), which corresponds to approximately 10% of the receptors being occupied by norepinephrine. This finding is in good agreement with the data shown in Fig. 7, which was derived by comparison of binding and response.

DISCUSSION

The findings presented here confirm previous reports that it is feasible to conduct *alpha*$_2$-adrenergic receptor-binding studies with intact platelets, that the degree of inhibition of cyclic AMP accumulation by *alpha*$_2$ agonists exceeds the level of occupancy, and that clonidine is a partial agonist in this system (4, 5, 22). In addition, we have provided data to indicate that full agonists bind to the *alpha*$_2$-adrenergic receptor of intact platelets in a manner that suggests heterogeneity of binding (Table 1). Most importantly, the use of the irreversible antagonist phenoxybenzamine has demonstrated the presence of a receptor reserve associated with *alpha*$_2$-adrenergic inhibition of cyclic AMP accumulation in human platelets (Fig. 7–9).

Many receptor systems display the property that the response of the system is not directly proportional to receptor occupancy (23). The concept of a receptor re-

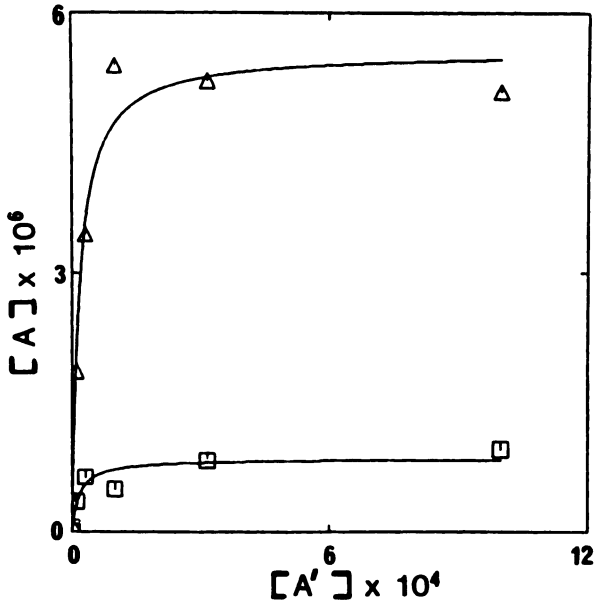

FIG. 9. *Determination of the true agonist affinity, K_A*

Values of A' were taken from the phenoxybenzamine-treated curves of Fig. 8. Corresponding values of A were interpolated according to the best fit parameters for the control curve in Fig. 8. The values of q and K_A (function [1], see text) then were adjusted to give the best paired values (A, A'). The best fit curves are shown with the data points. 250 nM phenoxybenzamine (□): $q = 5.0 \pm 2.4\%$, $K_A = 16 \pm 9$ μM; 125 nM phenoxybenzamine (△): $q = 32 \pm 7\%$, $K_A = 12 \pm 4$ μM.

serve (24) offers one explanation for EC_{50}/K_d ratios which differ from unity. It also accounts for the apparently reversible nature of receptor blockade by irreversible haloalkylamine antagonists (25). In studies of the *beta*-adrenergic system, Terasaki *et al.* (7) employed an essentially irreversible *beta*-adrenergic antagonist to confirm their previous finding (3) that maximal stimulation of adenylate cyclase activity in rat C6 glioma cells can be elicited by agonist occupation of less than 5% of the receptor population. Similar evidence for the presence of spare receptors has been presented for other *beta*-adrenergic systems (8). However, there are also systems in which all of the *beta* receptors need to be available to elicit a maximal response, even though EC_{50} is significantly less than K_d for full agonists (9, 10). Thus, the fact that EC_{50} values for full agonists at the *alpha*$_2$-adrenergic receptor are less than the corresponding $K_{I\,app}$ values (Table 1, Fig. 1) (5) is suggestive, but not conclusive, evidence for the presence of spare receptors. The equivalence of EC_{50} and $K_{I\,app}$ in the case of the partial agonist clonidine (Table 1) is similarly suggestive.

In order to evaluate more rigorously the possibility that there are spare *alpha*$_2$-adrenergic receptors in the intact platelet, two separate lines of experimentation were conducted using the irreversible antagonist phenoxybenzamine. The first approach (Fig. 7) found the inhibitory effect of norepinephrine to be disproportionately retained, compared to the number of receptor-binding sites, when the *alpha*$_2$-adrenergic receptors were occluded by phenoxybenzamine. In the second approach, concentration-response curves were generated for the inhibition by norepinephrine of cyclic AMP accumulation in platelets that had been preincubated with or without phenoxybenzamine (Fig. 8). The progressive rightward shift of these curves as the number of accessible receptors irreversibly declines is characteristic of systems in which there is a receptor reserve (25). Due to the technical limitation related to the distribution of active and occluded receptors across the population of cells in the assay (see Ref. 8), it is not yet possible to determine whether the size of the receptor reserve can account completely for the discrepancy between EC_{50} and $K_{I\,app}$ seen with the full agonists. There is, however, considerable agreement between the data of Figs. 1 and 7–9, that the half-maximal response is elicited when approximately 10% of the receptors are occupied by norepinephrine.

The receptor reserve of the *alpha*$_2$-adrenergic receptor in the platelet may offer some advantages related to physiological function. Circulating plasma levels of catecholamines are generally quite low relative to concentrations to which receptors are exposed within the synaptic cleft. A receptor reserve permits the presence of a larger number of receptor-binding sites, enhancing sensitivity to low agonist concentrations, since only a small proportion of binding sites need be occupied in order to achieve an effector response. Thus, the adenylate cyclase response of the *alpha*$_2$ receptor may occur at circulating plasma levels of epinephrine and/or norepinephrine, contributing to platelet aggregation *in vivo*. Moreover, regulation of the sensitivity of the receptor-mediated response may be achieved by changing the number of binding sites without changing the number of effector units.

Mechanistic models have been put forth that may account for the response properties of systems in which EC_{50} is less than K_d. The mobile receptor model (26) suggests that occupied receptors diffuse within the membrane to combine with and activate effectors. The collision coupling model (27) further stipulates that effectors remain activated after dissociation of the receptor-effector complex, so that multiple effectors may be activated by a single agonist-occupied receptor. MacFarlane (28) has recently presented an extension of the collision-coupling model of Tolkovsky and Levitzki (27) which accounts for both activation and inactivation of adenylate cyclase by stimulatory and inhibitory hormone receptors, respectively. The results of our experiments with phenoxybenzamine (Figs. 7–9) are consistent with the predictions of both this model and the mobile receptor model (26).

Theoretically, the presence of a *homogeneous* population of agonist receptor sites has been suggested as a criterion to reject the mobile receptor model in favor of the collision-coupling model (28). However, our data (Table 1 and Fig. 1) indicate that the binding curves of full agonists at *alpha*$_2$-adrenergic receptors of intact platelets deviate significantly from the Langmuir isotherm. There has been some controversy in the literature regarding the homogeneity versus heterogeneity of agonist binding to the *alpha*$_2$ receptor in the intact platelet. Thus, Motulsky *et al.* (4) and U'Prichard *et al.* (20) reported Hill coefficients that were less than unity, while MacFarlane and Stump (5) stated that the occupancy curve for epinephrine was adequately described by a single site. Hollister *et al.* (29) have recently found that elevated levels of epinephrine in the plasma of human subjects resulted in acute reduction (2 hr) of the affinity of agonists for intact platelet *alpha*$_2$-adrenergic receptors. The approximately 3-fold reduction in receptor affinity was accompanied by an increase in the Hill coefficient from 0.74 to 1.0. Their findings might explain the varying reports of heterogeneity observed in the binding of agonists to *alpha*$_2$ receptors of intact platelets. While a receptor reserve could be consistent with either concept of receptor coupling, the possible presence of multiple agonist affinity states in the intact cell does not permit a clear discrimination between the collision-coupling and mobile receptor models.

The value of K_A (about 14 μM) derived from analysis of the data in Fig. 9 is very near the geometric mean of K_H and K_L for norepinephrine, determined under identical conditions (see legend to Fig. 1). It is not possible, on the basis of the present data, to conclude whether *alpha*$_{2H}$, *alpha*$_{2L}$, or, indeed, both mediate the inhibitory response. However, the significance of the subpopulations of *alpha*$_2$-adrenergic receptors that are distinguished by agonists must be addressed by any theory that seeks to explain the mechanism by which these receptors are coupled to the inhibition of adenylate cyclase. The fact that the *alpha*$_{2H}$ form of the receptor does not exist under conditions that permit inhibition of

adenylate cyclase in cell-free preparations in the presence of physiological concentrations of GTP has led some investigators to suggest that the $alpha_{2H}$ form is inactive (15, 22). Others have speculated that the presence of agonist induces the excited $alpha_{2H}$ form, which in turn combines with GTP to mediate the response (30). Presumably, the fraction of receptors which exists as $alpha_{2L}$ in the presence of agonist but absence of GTP is considered inactive according to this theory. The intact platelet preparation described in the present report may allow a more direct approach to this question, since we have found that both $alpha_{2H}$ and $alpha_{2L}$ sites can be characterized under the same conditions employed in the study of the cyclic AMP response. Since $alpha_{2H}$ and $alpha_{2L}$ are necessarily defined by binding studies, the relationship between these subpopulations and the inhibition of adenylate cyclase will depend upon comparisons of binding and response. Phenomena that influence receptor-effector coupling, such as the receptor reserve reported here, must be recognized for such comparisons to be valid.

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