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Agonist and Antagonist Binding to α_2-Adrenergic Receptors in Purified Membranes from Human Platelets

Implications of Receptor-Inhibitory Nucleotide-Binding Protein Stoichiometry

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SUMMARY

The agonist- and antagonist-binding properties of the α_2-adrenergic receptor in a purified plasma membrane preparation from human platelets were determined both by direct binding of radiolabeled ligands and by competition with the labeled α_2-antagonist, [^{3}H] yohimbine. Binding of [^{3}H]yohimbine was characterized by a single high affinity binding site (K_d = 6.2 ± 1.4 nM, B_{max} = 507 ± 53 fmol/mg). In direct binding studies, the imidazoline full α_2-agonist, [^{3}H]-5-bromo-6-*N*(2-4,5-dihydroimidazolyl)quinoxaline ([^{3}H] UK 14,304), bound to only one quantifiable high affinity site (K_d = 0.88 ± 0.17 nM), representing 65 ± 6% of the number of [^{3}H]yohimbine sites. Binding of the partial agonist [^{3}H]-*p*-aminoclonidine (PAC) showed nonlinear Scatchard plots. Analysis according to a model of multiple independent binding sites showed the data to be consistent with two sites (K_{d1} = 0.62 ± 0.18 nM and K_{d2} = 7.9 ± 1.4 nM). The high affinity site corresponded to 15 ± 6% and the low affinity site corresponded to 39 ± 6% of the number of [^{3}H]yohimbine sites. Competition for binding of the α_2-antagonist, [^{3}H]yohimbine, with nonradiolabeled ligands revealed a single affinity for yohimbine. In contrast, competition for [^{3}H]yohimbine binding by the full agonist UK 14,304 and epinephrine is best fit by a model with two independent binding sites. The partial agonist PAC was best characterized by a model with three distinct binding sites.

The full agonists UK 14,304 and epinephrine inhibited adenylate cyclase approximately 30%, whereas PAC produced only 12% inhibition.

The inhibitory guanine nucleotide-binding protein (N_i) with M_r 40,700 was the sole pertussis toxin substrate in the purified membranes. It was quantitated by pertussis toxin-catalyzed [^{32}P]ADP ribosylation in cholate extracts. There is a 20- to 100-fold excess of N_i over α_2-adrenergic receptors. Comparisons made between the experimental data for agonist binding and theoretical predictions of the simple ternary complex model suggest that there is compartmentalization of N_i and/or that the α_2 receptors are heterogeneous.

INTRODUCTION

Adrenergic receptors can produce either activation of adenylate cyclase via β-receptors or inhibition via α_2-receptors (1). Activation of the enzyme by β-adrenergic receptors requires a guanine nucleotide-binding protein (N_s;[2] see Ref. 2 for review). There are significant parallels between the mechanisms of receptor-mediated activation and inhibition (1). The identification of a guanine nucleotide-binding protein (N_i) associated with inhibition of adenylate cyclase (3) led to studies of the interactions of the two guanine nucleotide-binding proteins with each other and with the catalytic subunit of adenylate cyclase (4, 5).

The guanine nucleotide proteins also interact with adrenergic receptors. This has been demonstrated indirectly by guanine nucleotide effects on agonist binding to β-adrenergic (6) and α_2-adrenergic receptors (7). In

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[2] The abbreviations used are: GppNHp, guanosine 5′-(β, imido)triphosphate; IAP, islet-activating protein, pertussis toxin; N_i, inhibitory nucleotide-binding protein; N_s, stimulatory nucleotide-binding protein; PAC, *p*-aminoclonidine; TED buffer, 20 mM Tris-Cl, 1 mM sodium ethylenediamine tetraacetic acid, 1 mM dithiothreitol, pH 8.0; TM buffer, 50 mM Tris-Cl, 10 mM $MgCl_2$, pH 7.6; TME, TM buffer containing 1 mM ethylene glycol tetraacetic acid (Tris salt); UK 14,304, 5-bromo-6-*N*-(2-4,5-dihydroimidazolyl)quinoxaline.

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the case of β-adrenergic receptors, direct evidence of an interaction between agonist-occupied receptors and N_s has been obtained (8). A model of agonist binding to turkey erythrocyte β-adrenergic receptors has been proposed that involves a ternary complex of agonist, β-adrenergic receptor, and a third component, presumably N_s (9). This model has been applied to α_2-adrenergic receptors as well. Additional evidence that α_2-adrenergic receptors interact with N_i, the inhibitory guanine nucleotide-binding protein, comes from recent observations that IAP reduces α_2-agonist binding affinity in neuroblastoma × glioma hybrids (3). In order to understand better the interactions of α_2-adrenergic receptors with the N_i protein, it would be desirable to be able to measure accurately agonist and antagonist binding to this receptor. Few direct comparisons of agonist and antagonist binding to a single membrane preparation are available (10, 11). This is due in part to difficulties in measurements of agonist binding. Agonist binding can be selectively affected by guanine nucleotides, sodium ion, or endogenous catecholamines (7).

Many investigators have used a washed crude membrane fraction from human platelets for α_2-receptor binding studies (10–12). Quantitative analysis of agonist binding in these preparations has been difficult because of low specific and high nonspecific binding. The availability of a purified plasma membrane preparation for the nicotinic cholinergic receptor system has facilitated biochemical and mechanistic studies of that receptor. Human platelets provide a ready source of α_2-adrenergic receptors (17, 18, 20), and methods for purifying plasma membranes from human platelets have been reported (13). One study of the binding of [^{3}H]PAC, a partial α_2-agonist, to a purified platelet plasma membrane preparation has been published (13). We have recently described a large scale preparation of human platelet plasma membranes which are essentially free of guanine nucleotides.[3] Also, a noncatechol α_2-adrenergic full agonist, UK 14,304, radiolabeled to high specific activity, has recently become available (15).

In this report we describe a quantitative characterization of the binding of the α_2-adrenergic full agonist, [^{3}H] UK 14,304, a partial agonist, [^{3}H]PAC, and an antagonist, [^{3}H]yohimbine, to these membranes. We have also quantitated the amount of N_i protein present in this purified membrane preparation by labeling with the IAP of *Bordetella pertussis* in order to investigate the relative stoichiometry of agonist and antagonist binding and N_i protein. The results described here suggest that the α_2-adrenergic receptor exhibits heterogeneity not explained by a *simple* ternary complex interaction with N_i.

MATERIALS AND METHODS

Radiochemicals. [^{3}H]PAC (40.0–40.5 Ci/mmol), [^{3}H]yohimbine (75.0–82.7 Ci/mmol), [^{3}H]UK 14,304 (84.0–88.0 Ci/mmol), and [^{32}P] NAD (21–22 Ci/mmol) were obtained from New England Nuclear. [^{3}H]-3′,5′-Cyclic AMP (41 Ci/mmol) and [α-^{32}P]ATP (15–43 Ci/mmol) were from Amersham Corp. Radiochemical purity was checked by the thin layer chromatography systems suggested by the manufacturers.

Drugs and Chemicals. Clonidine and PAC were gifts from Boehringer-Ingelheim, and oxymetazoline was a gift of Schering Corp. UK 14,304 was a gift of Pfizer Ltd. *d*-Epinephrine was a gift of Sterling-Winthrop. *l*-Epinephrine tartrate, yohimbine, phenylmethylsulfonyl fluoride, and guanosine 5′-triphosphate were from Sigma Chemical Co. GppNHp (tetralithium salt) was a gift from Dr. M. Gnegy. Forskolin was from Calbiochem-Behring. IAP was from List Biologicals. All other chemicals were reagent grade or better from standard suppliers.

Human platelet concentrates were obtained from the Detroit Red Cross and used within 24 hr of collection. Blood was anticoagulated with CPDA-I (2 g of glucose, 1.66 g of sodium citrate, 206 mg of citric acid, 140 mg of NaH_2PO_4, and 17.3 mg of adenine/per 450 ml of whole blood). The platelets were unsuitable for human use because of breaks in sterile technique, erythrocyte contamination, or positive antierythrocyte antibody screens.

Membrane preparation. Purified plasma membranes were prepared from washed, frozen human platelets as described.[3] In brief, frozen platelets were supplemented with phenylmethylsulfonyl fluoride (0.1 mM), thawed in a water bath at 15°, and immediately chilled on ice. Thawed platelets were sonicated twice with a Branson sonifier, layered on a discontinuous gradient containing 20 ml of 14.5% sucrose (w/w) and 20 ml of 34% sucrose (w/w), and centrifuged at 105,000 × *g* for 3 hr in a Beckman type 35 rotor. The clearly defined interface between 14.5 and 34% sucrose was collected, diluted with 2 vol of ice-cold distilled-deionized water, centrifuged at 105,000 × *g* for 60 min, and resuspended in buffer containing 50 mM Tris-Cl, 10 mM $MgCl_2$, 1 mM Tris-ethylene glycol tetraacetic acid, pH 7.6 (TME buffer). Aliquots were quick-frozen in a dry ice/ethanol bath and stored at −70° for up to 2 months until use. Bovine brain synaptic plasma membranes were a kind gift of Dr. T. Ueda (16).

[^{3}H]Yohimbine binding was measured in a final volume of 0.1 ml or 0.5 ml (as indicated) of TME buffer at room temperature (22–24°). The binding reaction was initiated by addition of membranes and stopped after 30–60 min by dilution with 3 ml of TM buffer (50 mM Tris-Cl, 10 mM $MgCl_2$, pH 7.6). This was immediately poured over Whatman GF/C filters, filtered under vacuum, and washed twice with 10 ml of TM buffer (room temperature). Filters were dried for 15 min under a heat lamp and counted in 4 ml of OCS (Amersham) at 32–42% efficiency. Nonspecific binding was determined in the presence of 10 μM yohimbine and was less than 7% of total binding.

[^{3}H]PAC and [^{3}H]UK 14,304 binding was measured in a final volume of 1 ml of TME buffer at room temperature (22–24°). Aliquots of membranes were diluted at least 20-fold in TME buffer and were warmed for 2–5 min in a 23° bath before being added to an equal volume of radioligand. For measurement of the time course of ligand binding, equal volumes of diluted membrane suspension and radioligand were rapidly mixed (<2 sec) using a manual two-syringe mixer and collected in a large test tube (17). One-milliliter aliquots were removed at the indicated times, diluted in 5 ml of TM buffer, filtered, and washed as above. Dissociation kinetics were initiated by addition of a small (<1%) volume of competing drug. One-milliliter aliquots were removed and filtered at the indicated times. For equilibrium determinations of [^{3}H]PAC binding, the reaction was stopped at exactly 20 min by addition of 5 ml of TM buffer followed by immediate filtration over Whatman GF/C filters. Incubations for binding of [^{3}H] UK 14,304 were for 60–90 min. The filters were washed twice with 10 ml of TM buffer and radioactivity was measured as above. Nonspecific binding was determined in the presence of 10 μM oxymetazoline and was less than 25% for 1 nM [^{3}H]PAC or [^{3}H]UK 14,304 and between 40 and 60% for the highest concentrations used (30 nM [^{3}H]PAC and 20 nM [^{3}H]UK 14,304).

Analysis of binding data. [^{3}H]Yohimbine-binding isotherms were analyzed by nonweighted linear least squares fits of Scatchard transformations of specific binding. [^{3}H]PAC and [^{3}H]UK 14,304 saturation binding curves and inhibition of [^{3}H]yohimbine binding by nonradioactive ligands were fit using LIGAND, a nonlinear least squares computer program (18). Time course data were fit using a nonlinear least

[3] R. R. Neubig and O. Szamraj, submitted for publication.

squares program, EXPFIT, for analyzing multiple exponential processes (19).

Adenylate cyclase assays. Measurement of adenylate cyclase activity and its inhibition by α_2-agonists was done in buffer containing (final concentrations) Tris-HCl, 25 mM, pH 7.6; NaCl, 100 mM; $MgCl_2$, 2.5 mM; EGTA, 1.5 mM; cAMP (Tris salt), 1 mM; isobutylmethylxanthine, 0.1 mM; GTP, 10 μM; phosphocreatine, 5 mM; creatine phosphokinase, 50 units/ml; propranolol 10 μM; and ATP, 0.2 mM (0.5 μCi of [α-^{32}P] ATP/tube) plus the indicated concentrations of drugs. Tubes containing epinephrine also contained 0.01% sodium ascorbate which was shown to have no effect in control experiments. [^{32}P]cAMP formed was measured by the method of Salomon *et al.* (20). Activity was linear to 20 min in the presence and absence of epinephrine. Assays were routinely conducted for 10–15 min at 30°.

Pertussis toxin-catalyzed ADP ribosylation of N_i protein in membrane extracts. Pertussis toxin labeling of cholate extracts of platelet and bovine brain membranes was performed essentially as described by Bokoch *et al.* (4). Briefly, membranes were pelleted and resuspended at 10 mg of protein/ml in buffer containing 20 mM Tris-Cl, 1 mM sodium ethylenediamine tetraacetic acid, 1 mM dithiothreitol, pH 8.0 (TED buffer). Twenty per cent sodium cholate in TED was added to bring the final concentration to 2% cholate, followed by shaking on ice for 60 min. Samples were centrifuged at 100,000 × *g* for 60 min and supernatants were immediately diluted 20-fold in TED containing 0.1%. Lubrol PX. Diluted extracts were either assayed immediately or frozen in dry ice-ethanol for up to 2 weeks prior to assays. Incubation of 10 μl of diluted cholate extract with [^{32}P]NAD (2 μM) and IAP (12.5 μg/ml) was exactly as described by Bokoch *et al.* (4). Reactions were stopped by addition of 2 vol of sodium dodecyl sulfate-containing electrophoresis sample buffer and aliquots were run on 10% polyacrylamide gels according to the method of Laemmli (21). Gels were stained with Coomassie blue and dried; then, autoradiography was performed with Kodak RP-Xomat film at −70° for 5–48 hr. The single labeled band was cut out of the dried gels and counted in 4 ml of OCS (Amersham).

Miscellaneous. Protein was determined by the method of Lowry *et al.* (22) with bovine serum albumin as standard. All binding measurements are means of triplicate determinations unless otherwise indicated, with values expressed as mean ± standard deviation.

RESULTS

Binding of [^{3}H]UK 14,304 to purified platelet plasma membranes. UK 14,304 is an imidazoline compound which exhibits full agonist activities at platelet α_2-receptors (Ref. 23; see also below). Binding of [^{3}H]UK 14,304 to this purified platelet membrane preparation required 60–90 min to reach steady state and was stable for an additional hour (Fig. 1A). Dissociation was slow, but more than 95% of specific binding dissociated within 3 hr after addition of competing ligands. Semilogarithmic transformations of the data were nonlinear for both association and dissociation (Fig. 1, A and B, *insets*). Nonlinear least squares analysis showed the data to be best fit by a two-exponential model for both association and dissociation. The half-times for association (1 nM) were 1.5 and 26 min, representing 66 ± 7 and 34 ± 7% of the reaction amplitude, respectively (n = 2). For the dissociation kinetics, the half-times were 2.4 and 31 min. The fast component accounted for 41 ± 6 and the slow component 57 ± 8% of the reaction (n = 5).

Binding of [^{3}H]UK 14,304 displayed the expected α_2-adrenergic pharmacologic specificity (Table 1). The amount of binding inhibited by 10 μM oxymetazoline, yohimbine, *l*-epinephrine, phentolamine, and clonidine was identical (data not shown). As reported by Loftus *et*

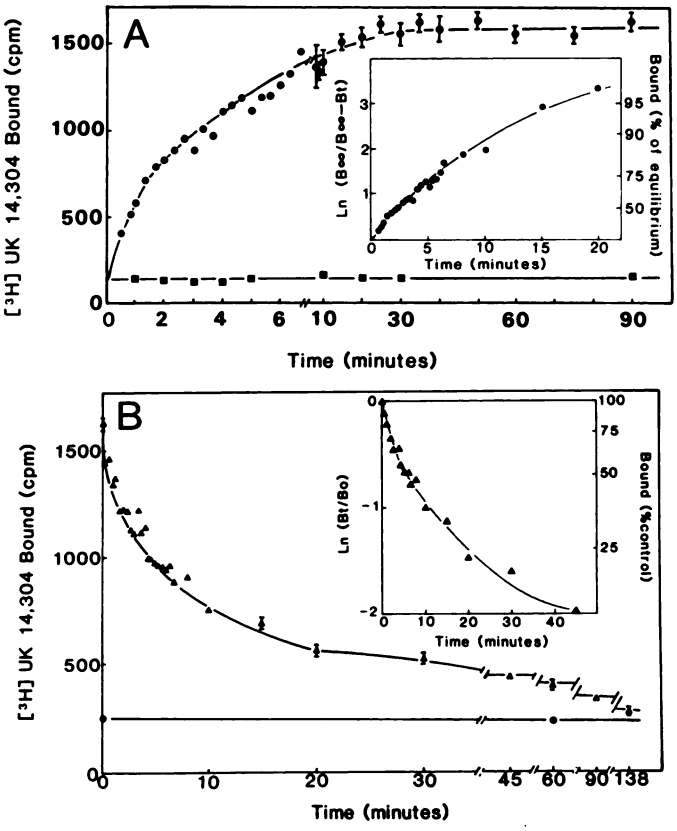

FIG. 1. *Time course of binding of [^{3}H]UK 14,304 to purified platelet plasma membranes*

A: Thirty milliliters of purified platelet membranes (0.344 mg/ml) were prewarmed in a shaking water bath for 3 min at 23°. They were then loaded in a manual two-syringe mixer (17) and mixed with an equal volume of 2 nM [^{3}H]UK 14,304. One-milliliter aliquots were removed at the indicated times, diluted with 5 ml of TM buffer, filtered, and counted as described in Materials and Methods (●). The time course of nonspecific binding (■) was determined by mixing, in a similar fashion, 10 ml of platelet membranes preincubated with 10 μM oxymetazoline with an equal volume of 2 nM [^{3}H]UK 14,304 also containing 10 μM oxymetazoline. Data points for 0–6 min are single determinations, and those for 10–90 min are the mean ± SD of triplicate determinations. Specific binding was calculated by subtracting a linear least squares estimate of nonspecific binding from total binding. A semilogarithmic plot (*inset*) of the specific binding data is not linear, indicating heterogeneity or complex association kinetics (see Results and Discussion). $B\infty$ is the equilibrium-specific binding determined as an average of the values from 60 to 90 min. *Bt* is specific binding at the indicated time. *B*: Platelet membranes (0.260 mg/ml) were incubated with 1 nM [^{3}H]UK 14,304 for 90 min at 23° in the absence (▲) or presence of 10 μM oxymetazoline (●). Triplicate 1-ml aliquots were diluted and filtered, and bound radioactivity was determined as above. Dissociation was then initiated by addition of a 1% volume of 1 mM oxymetazoline (▲; 10 μM final) or buffer (●), followed by dilution and filtration as above. Data from 0 to 10 min are single determinations and those for times greater than 10 min are mean ± SD of triplicate determinations. Specific binding was calculated by subtraction of the average nonspecific binding from total binding at each time. A semilogarithmic plot of specific binding is nonlinear (*inset*). *Bo* is specific binding just prior to initiation of dissociation and *Bt* is binding at the indicated times.

TABLE 1

Pharmacologic specificity of ligand binding to purified platelet plasma membranes

Binding of radiolabeled ligands in the presence of a range of concentrations of the indicated nonradioactive ligands was measured as described in Materials and Methods. The concentrations of radioligands used were 2 nM [^{3}H]UK 14,304, 3 nM [^{3}H]PAC, and 5 nM [^{3}H]yohimbine. Measurements of inhibition of [^{3}H]yohimbine binding was determined in 0.1 ml in the absence and presence of 10 μM GppNHp. IC_{50} values are the concentrations producing 50% displacement of specific binding of the labeled ligand. Values are arithmetic means of two to six determinations.

Competing Ligand	[^{3}H]UK 14,304 IC_{50}	[^{3}H]PAC IC_{50}	[^{3}H]Yoh IC_{50}		IC_{50} (Yoh/GppNHp)/ IC_{50} (UK)[a]	IC_{50} (Yoh/GppNHp)/ IC_{50} (Yoh)[b]
			−GppNHp	+GppNHp		
Agonists and parital agonists	*nM*		*nM*			
UK 14,304	4.8	3.2	40	74	15	1.9
l-Epinephrine	9.3	7.8	280	800	86	2.9
PAC	15.0	3.6	42	106	7.3	2.5
Oxymetazoline	20.0	4.4	50	66	3.3	1.3
Clonidine	ND[c]	21	52	88	ND	1.7
d-Epinephrine	260.0	ND	ND	ND	ND	ND
Antagonists						
Phentolamine	26.0	ND	ND	ND	ND	ND
Yohimbine	31.0	8.3	5.6	8.4	0.3	1.5
Prazosin	>10,000	ND	>10,000	ND	ND	ND
Propranolol	>10,000	ND	>10,000	ND	ND	ND

[a] The ratio of the IC_{50} for inhibition of [^{3}H]yohimbine binding in the presence of 10 μM GppNHp to the IC_{50} for inhibition of [^{3}H]UK 14,304 binding.

[b] The ratio of the IC_{50} for inhibition of [^{3}H]yohimbine binding in the presence of GppNHp to the IC_{50} for inhibition of [^{3}H]yohimbine binding in the absence of GppNHp.

[c] ND, not determined.

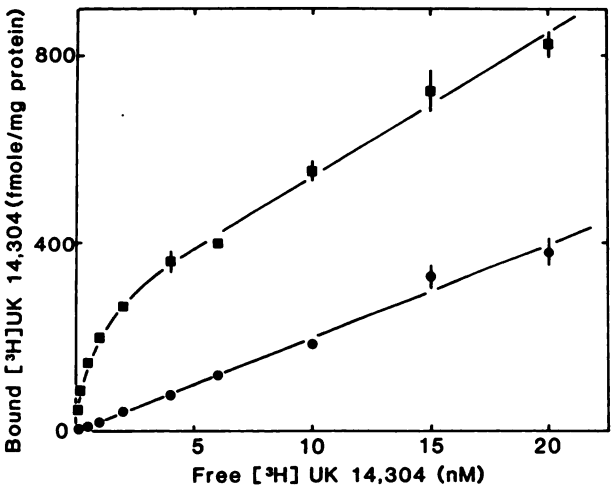

FIG. 2. *Equilibrium binding of the full α_2-agonist [^{3}H]UK 14,304*

Binding of the indicated concentrations of [^{3}H]UK 14,304 to purified membranes (0.172 mg/point) was measured after 60–90 min at 23°. Binding in the presence (●) and absence (■) of 10 μM oxymetazoline is shown. Data points are mean ± SD of triplicate determinations.

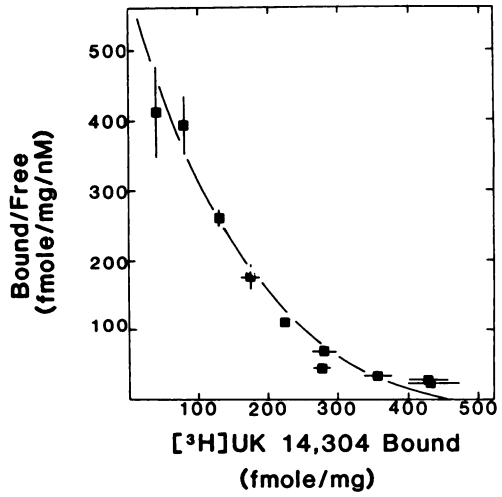

FIG. 3. *Scatchard plot of [^{3}H]UK 14,304 binding*

Specific [^{3}H]UK 14,304 binding was determined for the data in Fig. 2 by subtracting a linear least squares estimate of nonspecific binding from total binding. A Scatchard transformation of specific [^{3}H]UK 14,304 binding is shown. The *error bars* represent most probable error of specific binding (SD total + SD nonspecific)/$\sqrt{2}$. These data are from a single experiment which has been repeated five times with comparable results (see the text).

al. (15), UK 14,304 itself inhibited slightly more radioligand binding (5–15%) than did the other compounds. This could be attributed to binding of [^{3}H]UK 14,304 to the GF/C filters in the absence of platelet membranes. This effect was abolished by use of polyethyleneimine-treated filters (24, data not shown). Nonspecific binding was defined using 10 μM oxymetazoline because of its stability, availability, and α_2-selectivity.

Saturation isotherms for [^{3}H]UK 14,304 were determined for four different membrane preparations (six experiments). For each preparation, a [^{3}H]yohimbine binding curve was also measured to determine the B_{max} for yohimbine. Nonspecific binding represented 5–16% of total binding at 0.5 nM and 34–60% at 20 nM [^{3}H]UK 14,304 (Fig. 2). In four of the six experiments, there was curvature of the Scatchard plot at high concentrations, suggesting the existence of a low affinity binding component (Fig. 3). Nonlinear least squares analysis of the six binding curves revealed a high affinity K_d of 0.88 ± 0.17 nM and a B_{max} of 276 ± 18 fmol/mg of protein. The amount of high affinity [^{3}H]UK 14,304 binding corresponded to 65 ± 6% of the number of [^{3}H]yohimbine binding sites present in those preparations. Parameter

estimates for the apparent low affinity binding component could not be reliably obtained for data with UK 14,304 concentrations up to 20 nM (see competition binding experiments below).

Binding of [^{3}H]UK 14,304 was markedly reduced in the presence of 0.1 mM GppNHp. There was no high affinity binding, and nonspecific binding accounted for 80% of total binding at 20 nM [^{3}H]UK 14,304 (data not shown). Specific binding was consistent, with a single K_d of 20 nM. In competition experiments utilizing [^{3}H]yohimbine, an even higher K_i of ~40 nM was observed in the presence of 10 μM GppNHp. (Table 1).

Characterization of [^{3}H]PAC binding to purified membranes. As previously reported (20), [^{3}H]PAC binding to purified platelet membranes was characterized by markedly nonlinear Scatchard plots (Fig. 4). Nonlinear least squares analysis of full saturation isotherms for [^{3}H]yohimbine and [^{3}H]PAC binding to five different membrane preparations was done to obtain a direct comparison of the binding stoichiometry. There were 507 ± 53 fmol/mg of [^{3}H]yohimbine binding sites with a K_d of 6.2 ± 1.4 nM. The high affinity [^{3}H]PAC binding corresponded to 15 ± 6% of the total number of [^{3}H]yohimbine sites with a K_d of 0.61 ± 0.18 nM. The low affinity [^{3}H]PAC binding represented 39 ± 6% of the yohimbine sites with a K_d of 7.9 ± 1.4 nM. The total number of [^{3}H]PAC-binding sites as determined by the nonlinear least squares fits of the data represented 49 ± 8% of the total [^{3}H]yohimbine-binding sites.

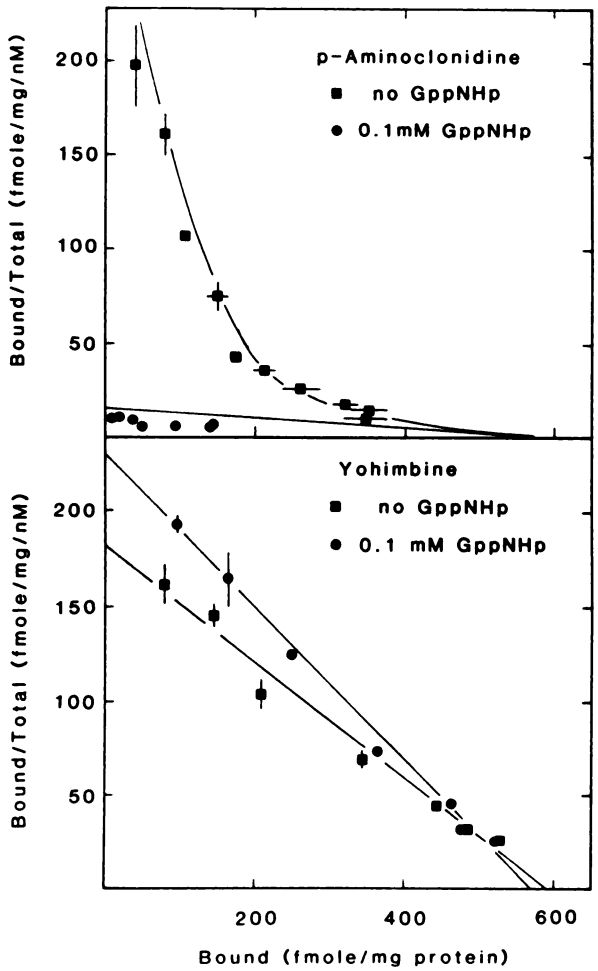

FIG. 4. *Effect of GppNHp on agonist and antagonist binding to purified platelet membranes*

Top: [^{3}H]PAC binding to platelet membranes (0.063 mg/point, 1 ml total volume) was measured in the presence (●) and absence (■) of 0.1 mM GppNHp (tetralithium salt). The GTP analog produced a marked decrease in [^{3}H]PAC binding. Data are expressed as in Fig. 3. *Solid lines* are nonlinear least squares fits of the data assuming an equal number of [^{3}H]yohimbine and [^{3}H]PAC binding (see the text). *Bottom:* [^{3}H]Yohimbine binding was determined using aliquots of the same membrane fraction used above (final volume, 0.1 ml). Data were analyzed as described in Materials and Methods. The same scale is used for the *abscissa* of both graphs.

As reported by others (11, 13), the high affinity [^{3}H]PAC binding is totally eliminated by 100 μM GppNHp (Fig. 4, *top*). [^{3}H]Yohombine binding is minimally affected by the guanine nucleotide analog (Fig. 4, *bottom*). There was a 1.3-fold decrease in the K_d for [^{3}H]yohimbine in this experiment, but in two other experiments there was no change in the K_d or the number of [^{3}H]yohimbine-binding sites. Binding of the [^{3}H]PAC in the presence of GppNHp is best fit by a K_d of 41 nM with 355 fmol/mg of binding sites. Thus, GppNHp reduces the affinity of both the component with K_d of 0.6 nM and that with K_d of 8 nM.

In order to directly compare the stoichiometries of binding of all three ligands, saturation binding curves for the antagonist, [^{3}H]yohimbine, the full agonist, [^{3}H]UK 14,304, and the partial agonist, [^{3}H]PAC, were measured using the same membrane preparation (Fig. 5). Binding of the agonists [^{3}H]UK 14,304 and [^{3}H]PAC never exceeds that of [^{3}H]yohimbine. The total binding of [^{3}H]PAC detected (i.e., both high and low affinity) corresponds roughly to the amount of high affinity binding of [^{3}H]UK 14,304. The additional lower affinity binding of [^{3}H]UK 14,304 does not bring the total amount of [^{3}H]UK 14,304 binding to that seen for [^{3}H]yohimbine. This is expected in light of the very low affinity (K_d = 170 nM) of UK 14,304 observed for ~25% of the [^{3}H]yohimbine sites in competition studies (see below).

Inhibition of [^{3}H]yohimbine binding by nonradioactive ligands was studied in the presence and absence of 10 μM GppNHp. The IC_{50} values are shown in Table 1. As reported by numerous investigators (7, 11, 13), the guanine nucleotide analog reduces the apparent affinity of agonists in inhibiting the binding of the ^{3}H-labeled antagonist. These effects were small but reproducible. Because even the purified membranes contain detectable amounts of guanine nucleotides,[3] detailed inhibition curves in the absence of guanine nucleotide were repeated using a more dilute membrane suspension in a larger final volume (Fig. 6).[4] The IC_{50} values for agonists and partial agonists were reduced in the larger assay volume (epinephrine, 40 versus 280 nM; UK 14,304, 9 versus 40 nM; PAC, 20 versus 42 nM), whereas that for yohimbine was slightly increased (11 versus 5.6 nM). Quantitative analysis of detailed inhibition curves was done for the latter experiments using LIGAND. The results of this analysis are shown in Table 2. The antagonist, yohim-

[4] At this dilution, the GTP concentration is less than 0.03 μM and GDP was less than 0.2 μM in the final assay mixture.

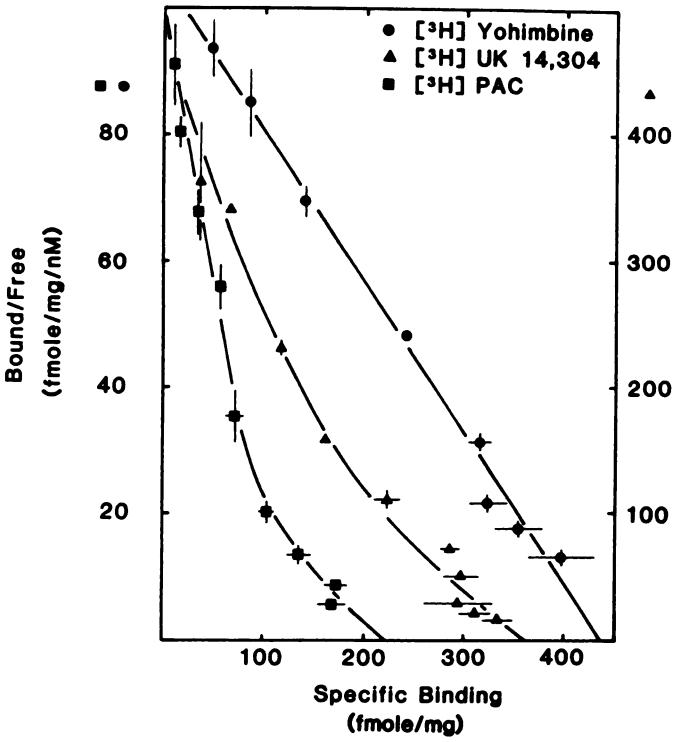

FIG. 5. *Scatchard plot of specific binding of [³H]yohimbine, [³H]UK 14,304, and [³H]PAC to the same membrane preparation*

Binding of [³H]yohimbine (●), [³H]UK 14,304 (▲), and [³H]PAC (■) to aliquots from the same membrane preparation was determined as in Figs. 2–4. Scatchard plots are shown with the same *abscissa* for all three ligands. *Solid lines* are arbitrary curves through the data. This experiment has been repeated on three different membrane preparations with identical results (see the text and Table 2 for a summary of nonlinear least squares fits of the data).

bine, was best fit by a single-site model with a K_i of 4.9 nM, which is in good agreement with the directly determined K_d of 6.2 nM. Inhibition of [³H]yohimbine binding by UK 14,304 and epinephrine was adequately fit by two-site models (Table 2). The high affinity K_i for UK 14,304 is in reasonably good agreement with the directly determined high affinity K_d, and the low affinity K_i, 169 nM, is high enough that in the direct binding experiments one would expect to only partially saturate those sites.

Inhibition of [³H]yohimbine binding by PAC was best fit by a three-site model, although the significance was marginal ($p = 0.07$). The parameters obtained for the two high affinity sites were in reasonable agreement with those obtained from the direct binding measurements. As in the case of UK 14,304, the low affinity PAC binding would not be detected in the direct measurements. This is consistent with the observation that [³H]PAC never occupies more than 60% of the number of binding sites that [³H]yohimbine does.

Inhibition of adenylate cyclase in purified membranes by α_2 agonists. Basal adenylate cyclase activity in the purified plasma membrane preparation was 63 ± 11 pmol/min/mg. Inhibition by 0.1 mM epinephrine was 31.6 ± 2.4% ($n = 6$), similar to that seen by Insel *et al.* (25). UK 14,304 (0.1 mM) produced 30.6 ± 0.8% inhibition of adenylate cyclase which was 99 ± 11% ($n = 3$) of the inhibition produced by 0.1 mM epinephrine in the same experiments (Fig. 7). In contrast, PAC produced only 11.6 ± 2.4% inhibition which was 46 ± 10% of the inhibition produced by epinephrine ($n = 3$). These data show that UK 14,304 is a full agonist for inhibition of adenylate cyclase in human platelet membranes and confirm that the partial agonist nature of PAC (13, 25).

The concentrations of agonists producing half-maximal responses are 3 μM for UK 14,304, 20 μM for (−)-epinephrine, and 3 μM for PAC. These concentrations of agonists are one to two orders of magnitude greater than even the low affinity component of ligand binding. It

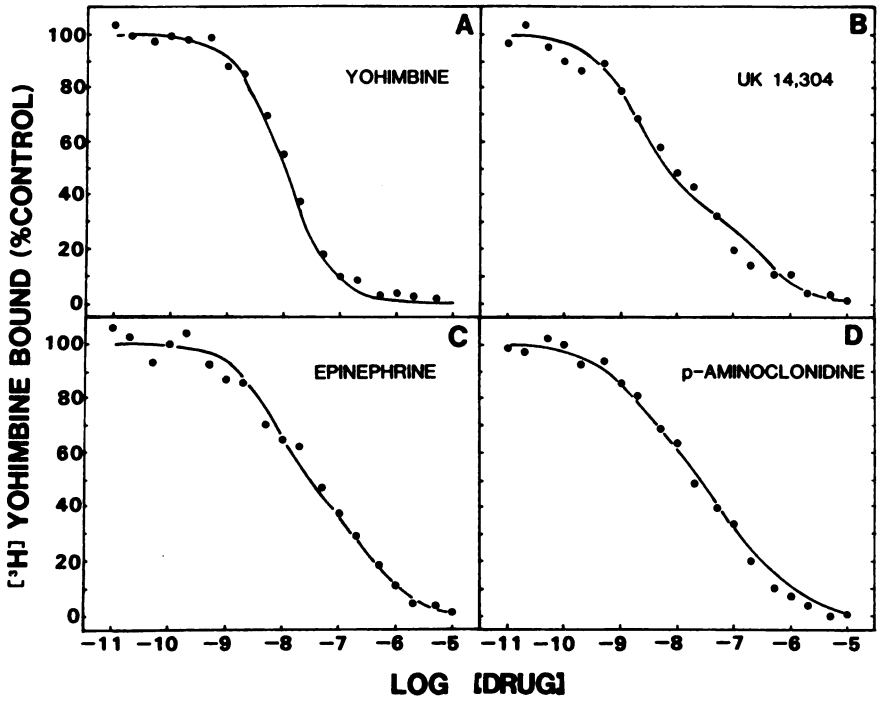

FIG. 6. *Inhibition of [³H]yohimbine binding to platelet membranes by α_2-adrenergic ligands*

Equilibrium binding of 5 nM [³H]yohimbine was measured in the presence of various concentrations of (A) yohimbine, (B) UK 14,304, (C) (−)-epinephrine, or (D) PAC. The final volume was 0.5 ml and incubation was at 23° for 60–90 min for UK 14,304 and 30–60 min for the other ligands. Data points are the means of two experiments each performed in triplicate. *Solid lines* are the theoretical values for a one-site model for (A) yohimbine ($K_I = 4.9$ nM), a two-site model for (B) UK 14,304 ($K_{I1} = 1$ nM, 65% of sites, $K_{I2} = 150$ nM, 35%) and (C) (−)-epinephrine ($K_{I1} = 4$ nM, 60%, $K_{I2} = 200$ nM, 40%), and a three-site model (D) for PAC ($K_{I1} = 0.65$ nM, 32%, $K_{I2} = 20$ nM, 48%, and $K_{I3} = 380$ nM, 20%). The K_D used for [³H]yohimbine binding in the theoretical calculations was 5 nM.

TABLE 2

Summary of analysis of agonist and antagonist binding data

Results obtained from nonlinear least squares analysis of direct radioligand binding (D) and competition for binding of [^{3}H]yohimbine (C) are summarized. Direct binding experiments were analyzed individually and parameters were averaged (arithmetic mean ± SE). Competition experiments were analyzed as a combined data set with the scaling factor varied as an independent parameter (27). Results are shown for both two- and three-site models even though a two-site fit is adequate for the full agonists.

		B_{max}			K_d			No.[a]	p[b]
		Type 1	Type 2	Type 3	Type 1	Type 2	Type 3		
		fmol/mg			*nM*				
Yohimbine	D		507 ± 53			6.2 ± 1.4		8	
		% of yohimbine sites							
	C		100%			4.9		2	(>0.5)
PAC	D	15 ± 6	39 ± 6	ND[c]	0.61 ± 0.18	7.9 ± 1.4	ND	5	
Two site	C	65		35	24		186	2	(<0.001)
Three site	C	32	48	20	0.65	20	380	2	(0.07)
UK 14,304	D	65 ± 6		ND	0.88 ± 0.17		ND	6	
Two site	C	73		27	2.6		169	2	(<0.001)
Three site	C	26	54	18	0.3	7.6	338	2	(>0.40)
Epinephrine									
Two site	C	64		36	7.9		247	2	(<0.001)
Three site	C	16	54	30	0.29	15.0	256	2	(>0.25)

[a] Number of experiments of each type.

[b] Estimate of the probability that the model indicated results in a statistically better fit than the model with one fewer site.

[c] ND, not determined.

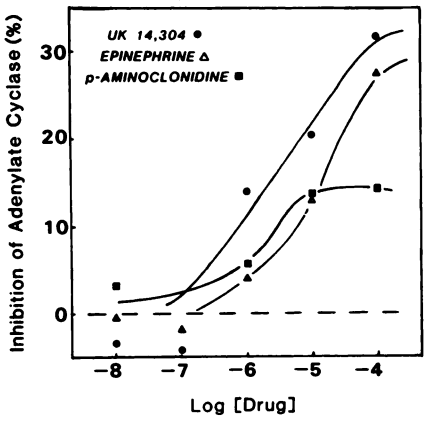

FIG. 7. *Inhibition of adenylate cyclase in purified membranes by α_2-agonists*

Adenylate cyclase activity was measured for 10 min at 30° in the presence of the indicated concentrations of UK 14,304 (●), (−)-epinephrine (△), and PAC (■) as described in Materials and Methods. Basal activity was 88 ± 8 pmol/min/mg and data are expressed as the per cent inhibition of basal activity. Tubes containing epinephrine also contained 0.01% sodium ascrobate, which did not affect activity. Data points are means of triplicate determinations from a single experiment which has been done three times with identical results.

should be remembered that the adenylate cyclase assays are conducted at a higher temperature (30°) and in the presence of both GTP and sodium ion. The latter have additive effects in reducing the affinity of agonist binding (7).

Quantitation of N_i protein in purified platelet membranes. Pertussis toxin-catalyzed ADP ribosylation of membrane N_i proteins was measured in cholate extracts as described in Materials and Methods. Only a single polypeptide of M_r 40,700 (N_i) was labeled in platelet membranes (Fig. 8, *lane B*). In contrast, two labeled bands can be seen following pertussis toxin-catalyzed [^{32}P]ADP ribosylation of cholate extracts from bovine brain synaptic plasma membranes (Fig. 8, *lane A*). As previously reported (26, 27), the faster migrating band is more intense and presumably corresponds to the substrate of M_r 39,000 predominant in brain (also called N_o). The band labeled in the platelet membranes appears to comigrate with the slower migrating band from brain which was reported to have an M_r of 41,000, in good agreement with our results. Increasing the exposure time during autoradiography did not reveal any evidence for a faster migrating "shoulder" on the labeled peak for platelet membranes. This suggests that human platelet membranes contain only N_i, the 41,000-dalton substrate for pertussis.[5]

Preliminary experiments showed that 2% sodium cholate yielded optimal extraction and labeling of the N_i protein so that this concentration was used rather than the 1% employed by Bokoch *et al.* (4). The [^{32}P]NAD labeling was saturated with respect to toxin concentration and time, and incorporation of [^{32}P]NAD into the M_r 40,700 protein was linear with the amount of cholate extract used in the assay (data not shown). Five determinations of the amount of N_i protein in four different preparations of purified membranes yielded an average of 25.8 (range 9.2–47.2) pmol/mg of protein. The average ratio of N_i protein to the amount of α_2-receptor (determined from [^{3}H]yohimbine binding curves) was 63 (range 23–112). The amount of protein used to calculate pico-

[5] Since we can detect two substrates in brain membranes which contain approximately 90% N_o and 10% N_i, it is likely that if N_o is present in platelet membranes, it accounts for less than 10% of the pertussis toxin substrate activity.

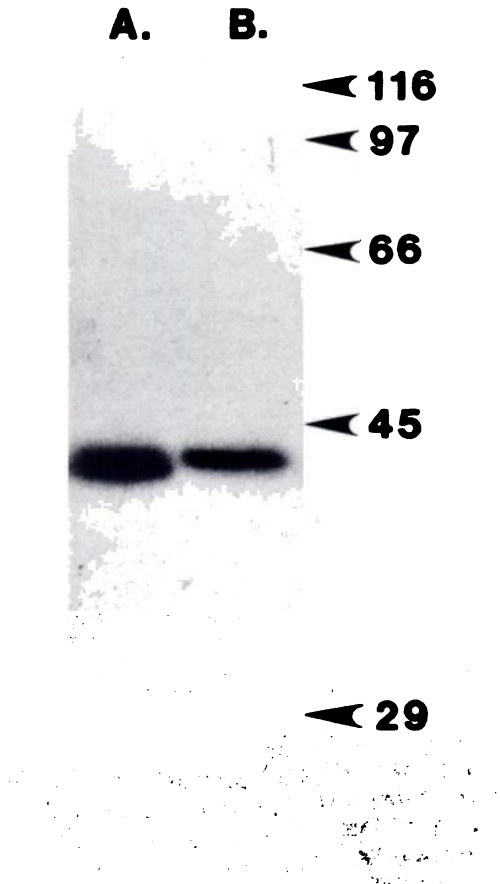

FIG. 8. *[^{32}]ADP ribosylation of detergent extracts from human platelet and bovine brain*

Cholate extracts of (A) bovine brain synaptic plasma membranes and (B) purified human platelet plasma membranes were labeled by incubation with [^{32}P]NAD (2 μM) and IAP (12.5 μg/ml) as described in Materials and Methods. Following reduction with 0.18 mM dithiothreitol and alkylation with 2.5 mM *N*-ethylmaleimide (35), samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (21) on 10% gels. Gels were dried and autoradiography was performed as described in Materials and Methods. *Numbered arrows* indicate the position and value of molecular weight of marker proteins.

moles per milligram of N_i was the protein in the original membrane. Consequently, any errors such as incomplete extraction or incomplete labeling of N_i would result in an underestimation of the amount of N_i. These values are thus a *lower* limit of the amount of N_i present. Calculations from the data of Katada *et al.* (5) yield a similar value of 17 pmol of N_i/mg of protein in an unpurified platelet membrane preparation. Clearly there is a marked excess of N_i protein over the amount of α_2-receptor in platelet membranes.

DISCUSSION

We report here a quantitative characterization of the binding of α_2-adrenergic agonists and antagonists using a newly reported plasma membrane preparation from human platelets.[3] This preparation is especially useful in studies of α_2-agonist binding because of its minimal contamination with endogenous guanine nucleotides.

Direct measurements of the binding of the α_2-adrenergic full agonist [^{3}H]UK 14,304 to human platelet membranes have not, to our knowledge, been previously reported. The binding is of high affinity, is reversible and exhibits α_2-adrenergic pharmacologic specificity. The nonlinear Scatchard plots and nonexponential association and dissociation kinetics suggest that UK 14,304 does not bind to a single class of homogeneous α_2-receptors. This has previously been reported for the binding of other radiolabeled agonists (11) and partial agonists (10–12, 21) to human platelet membranes. Loftus *et al.* (15) have described the binding of [^{3}H]UK 14,304 to rat cerebral cortex membranes. They reported a single high affinity binding site (K_d = 1.4 nM) but referred to preliminary data indicating an additional low affinity component in the equilibrium binding. A preliminary report of [^{3}H]UK 14,304 binding to membranes from a human colonic adenocarcinoma cell line indicates two affinities (28).

A number of previous studies have included partial characterizations of agonist and antagonist binding. Mooney *et al.* (13) reported two apparent affinities for binding of the partial agonist [^{3}H]PAC to a similar partially purified membrane fraction from human platelets. They did not present parallel information on antagonist binding to allow a comparison of agonist and antagonist binding stoichiometries. Additional direct comparisons of agonist and antagonist binding have used crude platelet membranes (10–12). Shattil *et al.* (12) found a single class of [^{3}H]clonidine-binding sites with a K_d of 25 nM and a B_{max} of 64 fmol/mg of protein (18% of the number of [^{3}H]dihydroergocryptine sites). These authors subsequently reported a B_{max} for [^{3}H]yohimbine that is only two-thirds of that for [^{3}H]dihydroergocryptine (30); consequently, [^{3}H]clonidine would occupy 24% as many sites as [^{3}H]yohimbine. Garcia-Sevilla *et al.* (10) reported two affinities for [^{3}H]clonidine binding, 5 nM and 19 nM. These corresponded to sites present in amounts of 19% and 41% of the [^{3}H]yohimbine binding. In a comprehensive study, U'Prichard *et al.* (11) found biphasic Scatchard plots for both [^{3}H]PAC (K_d = 0.7 nM and 9 nM) and [^{3}H]epinephrine (K_d = 2 nM and 40 nM). The total numbers of sites observed were 180 fmol/mg for [^{3}H]yohimbine, 130 fmol/mg for [^{3}H]epinephrine, and 109 fmol/mg for [^{3}H]PAC. Except for the 3-fold increase in the density of binding sites, our results with [^{3}H]yohimbine and [^{3}H]PAC are quite similar to those of U'Prichard *et al.* (11).

The high affinity component of [^{3}H]UK 14,304 binding reported here corresponds to 65 ± 6% of the total number of [^{3}H]yohimbine-binding sites measured in the same membrane preparations. In contrast, [^{3}H]PAC binds to only 15 ± 6% of the total number of [^{3}H]yohimbine sites with high affinity (K_d = 0.6 nM) and to an additional 39 ± 6% with an intermediate affinity (K_d = 7.9 nM). Thus, the sum of the number of high affinity and intermediate affinity [^{3}H]PAC-binding sites is roughly comparable to the total number of high affinity [^{3}H]UK 14,304-binding sites and is significantly less than the number of [^{3}H]yohimbine-binding sites. The studies of inhibition of [^{3}H]yohimbine binding by UK 14,304 and PAC reveal the origin of the discrepancy between the number of agonist-

and antagonist-binding sites. An additional low affinity binding component (K_i = 150–400 nM) is seen for both UK 14,304 and PAC, corresponding to 20–35% of the total yohimbine-binding sites. The competition binding studies also show dissociation constants for the high affinity binding sites which are in reasonably good agreement with those determined by direct binding methods. The combination of both direct and indirect measures of PAC binding, however, provides the most compelling argument for the presence of *three* apparent affinities for the partial agonist.

Difficulties in reliably estimating ligand binding stoichiometries have been pointed out for the nicotinic acetylcholine receptor (29). The radioligand-specific radioactivities in these studies have not been independently determined as described in Ref. 29 because of the difficulties associated with analysis of the competition assay in the presence of nonlinear Scatchard plots. The binding stoichiometry studies have, however, been reproducible over five batches of [^{3}H]yohimbine and four batches of [^{3}H]PAC and [^{3}H]UK 14,304. Also, similar results are seen in indirect studies of PAC and UK 14,304 binding determined by inhibition of [^{3}H]yohimbine binding.

The agonist binding data in this paper have been analyzed in terms of multiple independent binding sites for purposes of comparison to literature values. It has been pointed out (9, 31) that the ternary complex model can effectively simulate a two-site mass action model and that the parameters obtained do not relate to any physically identifiable entities. It is useful to examine some predictions of the ternary complex model to see if it can account for our data. DeLean *et al.* (9) noted that ligand binding depended on the amount of coupling protein present. They obtained the best fit between the model and their experimental data by assuming equal amounts of β-adrenergic receptor and coupling protein. The nonhyperbolic binding curves in this model depend on depletion of the coupling protein. Thus, a large excess of N protein over the amount of receptor would result in hyperbolic binding curves. We have performed theoretical calculations of the amount of ligand binding for the ternary complex model (see Appendix). The effect of varying the N protein (N) from 0.01 to 30 times the amount of receptor (R) was studied and a wide range of affinity constants was tested. For N equal to 3 times R there was only minimal deviation from a linear Scatchard plot. For N equal to 10 times R or greater there was no detectable deviation. When N was less than R, strikingly biphasic Scatchard plots generally resulted. Wreggett and DeLean (31) recently described some predictions of the ternary complex model as it relates to the D_2-dopamine receptor in bovine pituitary. They similarly concluded that a stoichiometrically limiting amount of coupling protein (X in their nomenclature) must be present to account for their experimental data.

Actual measurements of the amount of N_i (40,700-M_r pertussis toxin substrate) ranged from 23 to 112 times the amount of α_2-receptor in the membranes. There does not appear to be a detectable amount of the 39,000-M_r pertussis toxin substrate (N_o) in human platelet membranes. In light of the theoretical calculations just discussed, it appears that a *simple* ternary complex model in which R and N_i are *homogeneous entities free to diffuse in the membranes* cannot account for the biphasic agonist binding curves.

Studies of receptor-mediated release of guanine nucleotides from human platelet (32, 33) and hamster adipocyte membranes (34) give an estimate of the amount of N_i protein capable of a functional interaction with α_2-receptors. Motulsky and Insel (33) suggest that α_2-adrenergic and ADP receptors interact with distinct pools of N_i. In contrast, Murayama and Ui (34) show in hamster adipocytes that α_2-receptors and prostaglandin E_1 receptors can both interact with a common pool of N_i. In all of these studies, the amount of guanine nucleotide released per α_2-receptor was in the range of 0.6–3. This is significantly less than the amount of N_i determined from pertussis labeling (a 20- to 100-fold molar excess). Because of the necessity to "preload" the N_i protein with radiolabeled guanine nucleotide analog and the short time (3–5 min) of the nucleotide release assay, receptor-specific guanine nucleotide release will be an underestimate of the number of N_i proteins with which a receptor could interact during an hour-long binding reaction. Alternatively, the N_i protein may be heterogeneous in that not all of the N_i detected by pertussis labeling is functional in coupling to the α_2-adrenergic receptor.

The three apparent affinities for PAC are even more difficult to reconcile with the simple ternary complex model. Indeed, a partial agonist which has less difference in its affinities for the free and N_i-complexed receptor should actually show more linear rather than less linear Scatchard plots (compare Fig. 9, *top* and *bottom*). In none of our simulations were three apparent affinities observed. U'Prichard and colleagues (11) have previously pointed out the inadequacy of the ternary complex model to account for [^{3}H]PAC binding in rat cortex. They have also suggested that the "super high" affinity binding of [^{3}H]PAC may represent a desensitized receptor due to storage of platelets prior to preparation of membranes. Against this hypothesis are the data of Garcia-Sevilla *et al.* (10). Using fresh platelets, they found two apparent high affinities for clonidine which only accounted for 60% of the number of [^{3}H]yohimbine-binding sites (10).

Binding of muscarinic agonists to brain membranes (35) and cardiac sarcolemma (36) also requires a three-site model. Mattera *et al.* (36) propose a modification of the ternary complex model to include receptors coupled and not coupled to N_i. This hypothesis is consistent with the arguments advanced here, that either receptor heterogeneity or compartmentation of N_i must be present. A simple mass-action complex formation between R and N_i should not result in "uncoupled" receptors.

The data presented here are compatible with an alternative hypothesis of heterogeneous receptors. As discussed by U'Prichard *et al.* (11), binding of the partial agonist, PAC, is best fit by a model including three classes of binding sites (SH, superhigh; H, high; and L, low, in their nomenclature). According to this model, UK 14,304 would bind to both SH and H sites with identical affinity. The nonexponential binding kinetics,

even at concentrations of [^{3}H]UK 14,304 which should only occupy its high affinity binding site, suggests that the sites distinguished by [^{3}H]PAC at equilibrium may be distinguishable by [^{3}H]UK 14,304 in kinetic studies. Receptor heterogeneity could represent two or three completely distinct receptors or posttranslational modifications (e.g., phosphorylation glycosylation or proteolysis) of a single receptor. Also, interaction of a single receptor with a heterogeneous N_i protein may be compatible with the data.

Although the simple ternary complex model cannot *quantitatively* account for α_2-adrenergic agonist and partial agonist binding, it is likely that N_i does affect the affinity of α_2-receptors for agonists. The effects of guanine nucleotides (Ref. 11, Fig. 4) and IAP (3) to eliminate high affinity agonist binding suggest a role of N_i. Also, inactivation of N_i by alkaline treatment of platelet membranes selectively abolishes agonist binding (37). Consequently, a model incorporating both receptor heterogeneity and ternary complex formation, similar to the one discussed for the muscarinic receptor (36), is likely to best account for the observations reported here. Identification of the structural determinants of the heterogeneity of agonist binding may reveal important clues about activation, desensitization, or regulation of α_2-adrenergic receptors.

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APPENDIX: EFFECT OF N PROTEIN CONCENTRATION ON LIGAND BINDING IN A TERNARY COMPLEX MODEL

A "mobile receptor" model was used in a quantitative analysis of β-adrenergic agonist binding (9). DeLean *et al.* (9) used the term "ternary complex" to characterize the model. It has since been invoked to explain the nonlinear Scatchard plots of agonist binding to a wide variety of receptors including α_2-adrenergic receptors in platelets and D_2-dopamine receptors in pituitary (31). A recent quantitative analysis concerns the D_2-dopamine receptor (31).

DeLean *et al.* (9) specifically avoided making conclusions about the identity of the third component in their model. More recently it has been widely speculated that the nucleotide-binding proteins, N_s (for receptors that stimulate adenylate cyclase) and N_i (for inhibitory receptors) are the third component. The ability to quantitate these proteins in membrane preparations by toxin-catalyzed ADP ribosylation makes a theoretical characterization of the effect of the concentration of the third component on ligand binding of significant interest. As previously reported (9, 31), there is a marked effect of the concentration of N on the shape of ligand binding curves.

The model used here is the complete ternary complex model. The nomenclature used here reflects the hypothesis that a guanine nucleotide-binding protein is the third component but is otherwise identical to that of DeLean *et al.* (9, 31). N_o and R_o refer to total N protein and receptor, respectively.[6] *AR* and *ARN* refer to ligand-occupied receptor and the ternary complex. *N* and *R* refer to free, or uncomplexed, nucleotide-binding protein and receptor, respectively.

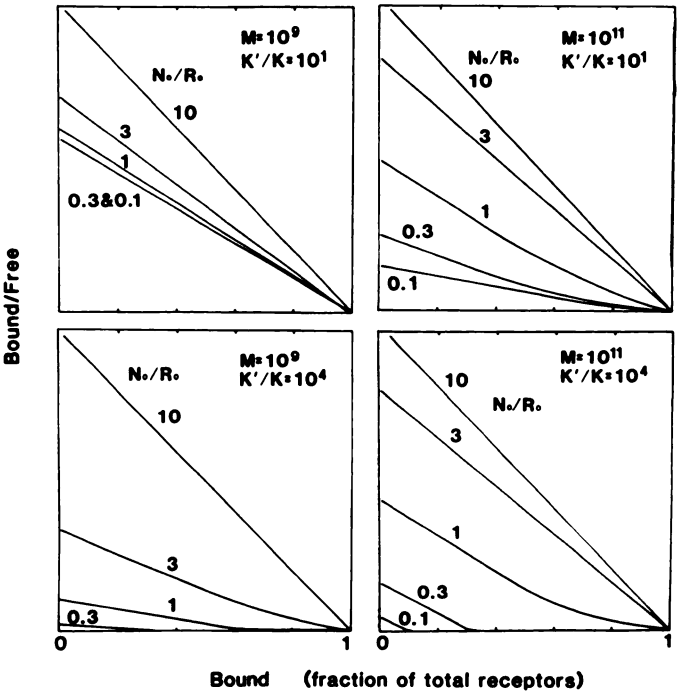

FIG. 9. *Effect of N protein concentration on predictions of a ternary complex model of ligand binding*

Predicted ligand binding for the cyclic ternary complex model (9) was calculated as described in the Appendix. The total receptor concentration used, R_o, was 10^{-11} M. K, the association constant for ligand binding to the free receptor, was fixed at 10^7 M^{-1}. The other parameters were varied: N_o, from 0.01 to 30 times R_o, K'/K from 10^1 to 10^5, and M, from 10^9 to 10^{13}. Scatchard transformations of the results were performed to assess deviation of the predicted binding from linearity and to allow comparison to the experimental data. Results from four parameter sets were chosen to illustrate the range of results obtained. The extent of coupling of R and N in the absence of ligand varies from 1% for $M = 10^9$ to 99% for $M = 10^{13}$. The values of the bound/free ratio corresponding to the maximum shown on the graph are 1.0×10^{-4} (*upper left*), 8.9×10^{-1} (*lower left*), 9.0×10^{-4} (*upper right*), and 8.8×10^{-1} (*lower right*).

The association constants are defined as follows:

$$K = AR/A \cdot R \quad \text{(A1)}$$

$$K' = ARN/A \cdot RN \quad \text{(A2)}$$

$$M = RN/R \cdot N \quad \text{(A3)}$$

$$L = ARN/AR \cdot N \quad \text{(A4)}$$

[6] In this context, N_o refers to the total amount of the inhibitory nucleotide-binding protein, N_i, as distinguished from the free, N, or receptor-bound, RN, protein. It has no relation to the recently described nucleotide-binding protein from brain which has also been called G_o (26).

Only three constants are independent because of thermodynamic constraints (i.e., $K \cdot L = K' \cdot M$). Substitution of these equations into the mass conservation equations

$$N_o = N + RN + ARN \quad \text{(A5)}$$

$$R_o = R + AR + RN + ARN \quad \text{(A6)}$$

and elimination of N yields a quadratic equation for the concentration of noncomplexed receptor, R:

$$a \cdot R^2 + b \cdot R + c = 0 \quad \text{(A7)}$$

where

$$a = (1 + K \cdot A)(M + M \cdot K' \cdot A) \quad \text{(A8)}$$

$$b = 1 + K \cdot A + M(N_o - R_o) + M \cdot K' \cdot A \cdot (N_o - R_o) \quad \text{(A9)}$$

$$c = -R_o . \quad \text{(A10)}$$

The free receptor concentration, R, can be calculated from Eqs. A7–A10, and all other species can be calculated from the value of R.

A computer program was written in BASIC for an IBM personal computer to calculate the amount of ligand A bound (i.e., $AR + ARN$) for a range of concentrations of A with a variety of values of the parameters R_o, N_o, K, K', and M. R_o and K were kept constant, whereas N_o/R_o was varied from 0.01 to 30, M was varied from 10^9 to 10^{13} M^{-1}, and K'/K was varied from 10^1 to 10^5. M determines the extent of association of R and N in the absence of agonist. Since $R_o = 10^{-11}$, most of N is complexed with R for $M = 10^{13}$ and very little is for $M = 10^9$. Also, K'/K, the relative affinities of agonist for RN and R, determines the extent to which agonist will induce formation of the ARN ternary complex.

Fig. 9 shows Scatchard transformations of representative theoretical predictions for agonist binding. It is striking to note that with excess N (i.e., $N_o/R_o > 1$), the Scatchard plots are nearly linear. Specifically, for $N_o/R_o > 10$, all of the binding is present in a single component for all parameter sets tested. Thus, the simple ternary complex model does not seem to explain either the [^{3}H]PAC or [^{3}H]UK 14,304 binding given the measured excess of N_i over R of 23- to 112-fold. Even using more conservative estimates of a 3-fold excess of N_i over α_2-adrenergic receptor (32, 34) results in linear Scatchard plots.

REFERENCES

1. Limbird, L. E. Activation and attenuation of adenylate cyclase. *Biochem. J.* **195:**1–13 (1981).
2. Ross, E., and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49:**533–564 (1980).
3. Kurose, H., T. Katada, T. Amano, and M. Ui. Specific uncoupling by islet-activating protein of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **258:**4870–4875 (1983).
4. Bokoch, G. M., T. Katada, J. K. Northup, 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).
5. Katada, T., J. K. Northup, G. M. Bokoch, M. Ui, and A. G. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase: Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J. Biol. Chem.* **259:**3578–3585 (1984).
6. Maguire, M. E., P. M. VanArsdale, and A. G. Gilman. An agonist-specific effect of guanine nucleotides on binding to the beta adrenergic receptor. *Mol. Pharmacol.* **12:**335–339 (1976).
7. Michel, T., B. S. Hoffman, and R. J. Lefkowitz. Differential regulation of α_2 adrenergic receptor by Na^+ and guanine nucleotides. *Nature* **288:**709–711 (1980).
8. Limbird, L. E., D. M. Gill, and R. J. Lefkowitz. Agonist-promoted coupling of the β-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **77:**775–779 (1980).
9. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255:**7108–7117 (1980).
10. Garcia-Sevilla, J. A., P. A. Hollingsworth, and C. B. Smith. α_2-Adrenoreceptors on human platelets: selective labelling by [^{3}H]clonidine and [^{3}H]yohimbine and competitive inhibition by antidepressant drugs. *Eur. J. Pharmacol.* **74:**329–341 (1981).
11. U'Prichard, D. C., J. C. Mitrius, D. J. Kahn, and B. D. Perry. The α_2-adrenergic receptor: multiple affinity states and regulation of a receptor inversely coupled to adenylate cyclase, in *Molecular Pharmacology of Neurotransmitter Receptors* (T. Sagawa, H. I. Yamamura, and K. Kuriyama, eds.) Raven Press, New York, 53–72 (1983).
12. Shattil, S. J., M. McDonough, J. Turnbull, and P. A. Insel. Characterization of alpha-adrenergic receptors in human platelets using [^{3}H]clonidine. *Mol. Pharmacol.* **19:**179–183 (1982).
13. Mooney, J. J., W. C. Horne, R. I. Handin, J. J. Schildkraut, and R. W. Alexander. Sodium inhibits both adenylate cyclase and high-affinity ^{3}H-labeled *p*-aminoclonidine binding to alpha$_2$-adrenergic receptors in purified human platelet membranes. *Mol. Pharmacol.* **21:**600–608 (1982).
14. Deleted in proof.
15. Loftus, D. J., J. M. Stolk, and D. C. U'Prichard. Binding of the imidazoline UK 14,304, a putative full α_2-adrenoceptor agonist, to rat cerebral cortex membranes. *Life Sci.* **35:**61–69 (1984).
16. Ueda, T., P. Greengard, K. Berzins, R. S. Cohen, F. Blomberg, D. J. Grab, and P. Siekewitz. Subcellular distribution in cerebral cortex of two proteins phosphorylated by a cAMP-dependent protein kinase. *J. Cell Biol.* **83:**308–319 (1979).
17. Boyd, N. D., and J. B. Cohen. Kinetics of binding of [^{3}H]acetylcholine and [^{3}H]carbamylcholine to *Torpedo* postsynaptic membranes: slow conformational changes of the cholinergic receptor. *Biochemistry* **19:**5344–5353 (1980).
18. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107:**220–239 (1980).
19. Faden, V. B., and D. Rodbard. EXPFIT, Exponential Data Processing. BTIC Computer Code Collection, Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville.
20. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58:**541–548 (1974).
21. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685.
22. 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).
23. Clare, K. A., M. C. Scrutton, and N. T. Thompson. Effects of α_2-adrenoceptor agonists and of related compounds on aggregation of, and on adenylate cyclase activity in, human platelets. *Br. J. Pharmacol.* **82:**467–476 (1984).
24. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethyleneimine-treated filters. *Anal. Biochem.* **132:**74–81 (1983).
25. Insel, P. A., D. Stengel, N. Ferry, and J. Hanoune. Regulation of adenylate cyclase of human platelet membranes by forskolin. *J. Biol. Chem.* **257:**7485–7490 (1982).
26. Sternweis, P. C., and J. D. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* **259:**13806–13813 (1984).
27. Neer, E. J., J. M. Lok, and L. G. Wolf. Purification and properties of the inhibitory guanine nucleotide binding regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* **259:**14222–14229 (1984).
28. Turner, J. T., and D. B. Bylund. α_2 Adrenergic receptors and inhibition of VIP stimulated adenylate cyclase in HT-29 cells. *Fed. Proc.* **44:**509 (1985).
29. Neubig, R. R., and J. B. Cohen. Equilibrium binding of [^{3}H]tubocurarine and [^{3}H]acetylcholine by *Torpedo* postsynaptic membranes: stoichiometry and ligand interactions. *Biochemistry* **24:**5464–5475 (1979).
30. Motulsky, H. J., and P. A. Insel. [^{3}H]Dihydroergocryptine binding to α adrenergic receptors of human platelets: a reassessment using the selective ligands [^{3}H]prazosin, [^{3}H]yohimbine, and [^{3}H]rauwolscine. *Biochem. Pharmacol.* **31:**2591–2597 (1982).
31. Wreggett, K. A., and A. DeLean. The ternary complex model: its properties

and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland. *Mol. Pharmacol.* **26**:214–227 (1984).
32. Michel, T., and T. J. Lefkowitz. Hormonal inhibition of adenylate cyclase. *J. Biol. Chem.* **257**:13557–13563 (1982).
33. Motulsky, H. J., and P. A. Insel. ADP- and epinephrine-elicited release of [^{3}H]guanylylimido diphosphate from platelet membranes. Implications for receptor-N_i stoichiometry. *FEBS Lett.* **164**:13–16 (1983).
34. Murayama, T., and N. Ui. [^{3}H]GDP release from rat and hamster adipocyte membranes independently linked to receptors involved in activation or inhibition of adenylate cyclase. *J. Biol. Chem.* **259**:761–769 (1984).
35. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **24**:723–736 (1978).
36. Mattera, R., B. J. R. Pitts, M. L. Entman, and L. Birnbaumer. Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system. *J. Biol. Chem.* **260**:7410–7421 (1985).
37. Kim, M. H., and R. R. Neubig. Parallel inactivation of α_2 adrenergic agonist binding and N_i by alkaline treatment. Fed. Proc. **44**:509 (1985).