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Large-scale purification of α_2-adrenergic receptor-enriched membranes from human platelets. Persistent association of guanine nucleotides with nonpurified membranes

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A simple large-scale purification of α_2-adrenergic receptor-enriched membranes from human platelets is described. Binding of the antagonist [^{3}H]yohimbine is enriched 3–5-fold compared to a crude membrane fraction. Binding of low concentrations of the partial agonist ^{3}H-*p*-aminoclonidine is increased 15–20-fold due to a higher binding affinity for the purified membranes. A soluble inhibitor of ^{3}H-*p*-aminoclonidine binding to purified membranes is found even in thrice-washed crude platelet membranes. The guanine nucleotides GDP and GTP are found to account for this inhibitory activity. Forskolin-stimulated adenylate cyclase activity is also enriched in the purified membrane fraction. Adenylate cyclase activity is inhibited by α_2-agonist to a comparable extent in all membrane fractions. This membrane preparation should prove useful in studies of α_2-adrenergic receptor mechanisms.

Introduction

Catecholamines can produce either activation of adenylate cyclase via β-adrenergic receptors or inhibition via α_2-receptors [1,2]. Activation of the enzyme by β-adrenergic receptors has been extensively studied (see Ref. 3, for review). There are significant parallels between the mechanisms of receptor-mediated activation and inhibition. Both processes require guanine nucleotides [1] and appear to be mediated by guanine nucleotide binding proteins [3,4]. Gilman and collaborators have provided significant insight into the interactions of the stimulatory (N_s) and inhibitory (N_i) guanine nucleotide binding proteins with each other and with the catalytic subunit of adenylate cyclase [5].

In order to better understand the interactions of α_2-adrenergic receptors with the N_i protein it would be desirable to be able to accurately measure agonist and antagonist binding to this receptor. Few direct comparisons of agonist and antagonist binding to a single membrane preparation are available [6,7]. This is due in part to difficulties in measurements of agonist binding. Many investigators have used a washed crude

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Abbreviations: cAMP, adenosine 3′,5′-monophosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; I, purified membranes from interface of sucrose gradients; M, crude membranes; MW, washed crude membranes; N_i, inhibitory nucleotide binding protein; N_s, stimulatory nucleotide binding protein; *P*/*Y* ratio, specific binding of 3 nM ^{3}H-*p*-aminoclonidine/specific binding of 10 nM [^{3}H]yohimbine; SDS, sodium dodecyl sulfate; UK 14,304, 5-bromo-6-*N*(2-4,5-dihydroimidazolyl)quinoxaline.

membrane fraction from human platelets for α_2-receptor binding studies [6–9]. 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 [10] receptor system has facilitated biochemical and mechanistic studies of that receptor. Human platelets provide a ready source of α_2-adrenergic receptors [8.9,11] and many methods for purifying plasma membranes from human platelets have been reported [11–13]. One study of α_2-agonist binding to a partially purified plasma membrane preparation has been published [11].

In this report we describe a simple method to prepare large amounts of platelet membranes enriched in α_2-adrenergic receptors. The agonist and antagonist binding properties of these membranes are characterized. The increase in binding of low concentrations of agonists seen for the purified membranes in several-fold greater than that for antagonists. The persistence of guanine nucleotide inhibitors of agonist binding in washed crude membranes is demonstrated and may partially explain these observations. The purified membrane preparation described here should facilitate biochemical and mechanistic studies of the α_2-adrenergic receptor. A preliminary version of this report has been presented [14].

Materials and Methods

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

Radiochemicals. ^{3}H-*p*-Aminoclonidine (40.0–40.5 Ci/mmol), and [^{3}H]yohimbine (75.0–82.7 Ci/mmol) were obtained from New England Nuclear. [α-^{32}P]ATP and [^{3}H]cAMP were from Amersham. Radiochemical purity was checked by the TLC systems suggested by the manufacturers.

Drugs and chemicals. Oxymetazoline was a gift of Schering Corp. UK 14,304 was a gift of Pfizer Ltd. 1-Epinephrine tartrate, yohimbine, phenylmethylsulfonyl fluoride, and nucleotides were from Sigma. Forskolin was from Calbiochem-Behring. All other chemicals were reagent grade or better from standard suppliers.

Washing and freezing platelets. All steps prior to freezing the platelets were performed at room temperature using plastic tubes and pipettes. Platelet concentrates were diluted with an equal volume of 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), and centrifuged for 10 min at $200 \times g$ to remove contaminating erythrocytes. The supernatant was carefully decanted and centrifuged for 20 min at $1500 \times g$. The platelet pellets were resuspended in the same buffer by gentle trituration and pelleted again. The pellets were resuspended in 2 ml per unit of buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.2 M sucrose. These washed platelet pellets were quick frozen in a solid CO_2/ethanol bath and stored at -70°C for up to 2 months before use.

Membrane preparation. Frozen washed platelets (10–56 U per preparation) were supplemented with sufficient phenylmethylsulfonyl fluoride (0.1 M in ethanol) to produce a final concentration of 10^{-4} M. They were then thawed in a water bath at 15°C and immediately chilled on ice. 50 ml batches were sonicated twice with a Branson sonifier (micro tip, setting 6) for 10 s, chilling for 30–60 s between bursts in an ice-salt bath to prevent gel formation. The sonicate (15 ml per tube) was 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 $105\,000 \times g$ for 3 h in a Beckman Type 35 rotor. The clearly defined interface between 14.5 and 34% sucrose contained the purified membrane fraction (I membranes). This was diluted with two volumes of ice-cold distilled-deionized water, centrifuged at $105\,000 \times g$ for 60 min, and resuspended in a volume of one ml of buffer per unit of platelets. The buffer contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Tris-EGTA (pH 7.6). Aliquots were quick-frozen in a solid CO_2/ethanol bath and stored at -70°C for up to three months without loss of binding activity.

The large pellet (P) from the discontinuous gradient was resuspended in 2 ml per unit of the

same buffer. Aliquots of the total sonicate were pelleted at $43000 \times g$ for 20 min to prepare a crude membrane fraction (M). These membranes were washed thrice more by pelleting and resuspending in 1 ml/unit of buffer and called washed membranes (MW membranes). A washed membrane fraction similar to this is used by many investigators for binding studies. All samples were quick frozen and stored at −70°C.

[^{3}H]Yohimbine binding. Binding was measured in a final volume of 0.1 ml of buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA (pH 7.6) at room temperature (22–24°C). The reaction was initiated by addition of membranes and stopped after 30–60 min by dilution with 3 ml of buffer (50 mM Tris-HCl, 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 the dilution buffer (room temperature). Filters were dried for 15 min under a heat lamp and counted in 4 ml OCS (Amersham) at 32–42% efficiency. Nonspecific binding was determined in the presence of 10^{-5} M yohimbine and was less than 7% of total binding for I membranes and less than 10% for MW and P membranes [15].

^{3}H-p-Aminoclonidine binding. Binding was measured in a final volume of 1 ml of buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA (pH 7.6) at room temperature (22–24°C). Aliquots of membranes were diluted at least 20-fold in the same buffer and were warmed for 2 min in a 23°C bath before being added to the radioligand. The reaction was stopped at exactly 20 min by addition of 5 ml of buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.6) followed by immediate filtration over Whatman GF/C filters. The filters were washed twice with 10 ml of the dilution buffer and radioactivity measured as above. Nonspecific binding was determined in the presence of 10^{-5} M oxymetazoline as described [15].

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-*p*-Aminoclonidine binding curves were fit using a nonlinear least-squares procedure for one- and two-site models [16].

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^{-5} M; phosphocreatine, 5 mM; creatine phosphokinase, 50 U/ml; propranolol 10^{-5} M; and ATP, 0.2 mM (0.5 μCi [α-^{31}P]ATP per 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. [17]. Activity was linear to 20 min in the presence and absence of epinephrine. Assays were routinely conducted for 10–15 min at 30°C.

Measurement of catecholamine and nucleotide content. Frozen membrane fractions were thawed on ice. Protein was precipitated by addition of an equal volume of 0.8 M $HClO_4$ followed by centrifugation in an Eppendorf microfuge at 4°C. Supernatants were neutralized by addition of KOH and $KHCO_3$. Aliquots were assayed for catecholamine by the radioenzymatic method of Peuler and Johnson [18]. Nucleotides were quantitated by HPLC on a strong anion-exchange column [19].

Miscellaneaous. SDS-gel electrophoresis was performed by the method of Laemmli [20]. Lactate dehydrogenase was measured by the method of Wroblewski and La Due [21]. Protein was determined by the method of Lowry et al. [22] with bovine serum albumin as standard. Na^+, K^+-stimulated ATPase and acidic phosphodiesterase were measured as described [23,24]. All binding measurements are means of triplicate determinations unless otherwise indicated with values expressed as mean ± S.D.

Results

Platelet fractionation

A well defined membrane fraction (I) appears just below the interface between the 14.5% and 34% sucrose solutions. It is easily aspirated from the gradient without disturbing the firm pellet. This fraction contains approximately one eighth of the total membrane protein. It contains less than 1–2% of total platelet lactate dehydrogenase indicating minimal contamination by the cytosolic

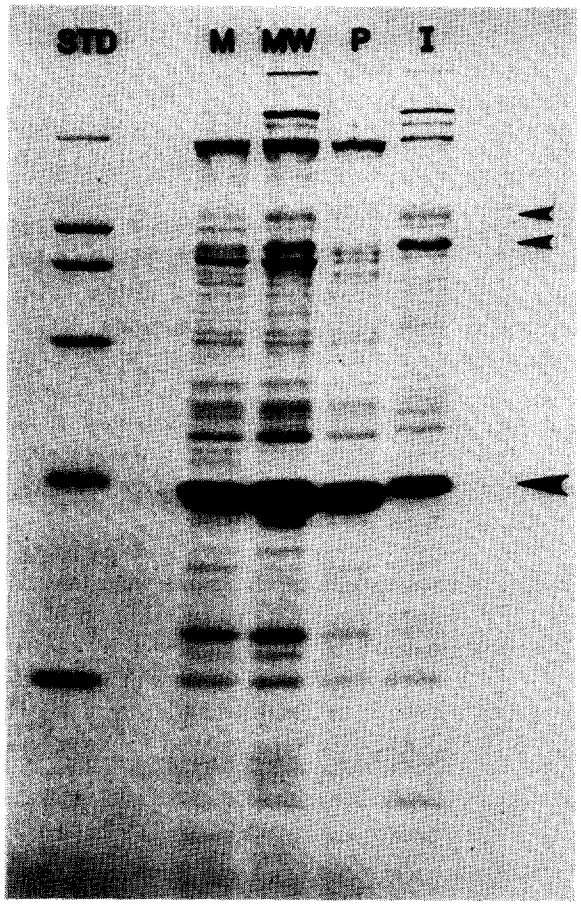

Fig. 1. SDS-polyacrylamide gel electrophoresis of different membrane fractions. Samples of each of the platelet membrane fractions containing 100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli [20]. 10% acrylamide gels were used and proteins stained with Coomassie blue. The light membranes (I) appear to have relatively more of the M_r 104000 and 129000 bands corresponding to the surface associated glycoproteins IIb and III (small arrows). The I membranes are depleted of the M_r 41800 protein reported to be actin (large arrow). Molecular weight standards from bottom to top (and their apparent M_r) are carbonic anhydrase (29000), ovalbumin (45000), bovine albumin (66000), phosphorylase *b* (97400), β-galactosidase (116000) and myosin (205000). I, sucrose interface membrane; M, crude membrane; MW, washed crude membrane; P, pellet membrane.

fraction. In contrast, the total membrane fraction (M), washed membrane fraction (MW) and pellet (P) from the sucrose gradient contained 9–31%, 3–6%, and 13–14%, respectively, of total lactate dehydrogenase, indicating more cytosolic contamination.

Attempts to measure Na^+ and K^+-stimulated or ouabain-inhibited ATPase activity as a plasma membrane marker were unsuccessful as has been reported by others [13]. Acidic phosphodiesterase, another possible plasma membrane marker, was found predominantly in the soluble fraction of human platelets making it a poor plasma membrane marker (unpublished data). SDS gel electrophoresis of I membranes shows less of a prominent M_r 41800 protein than is seen in M, MW and P membranes (Fig. 1). This protein band has been previously associated with actin. The I membranes are also relatively enriched in a pair of diffusely stained polypeptides of M_r 104000 and 129000, which are similar to the M_r of the surface associated glycoproteins IIB and III [25].

^{3}H-labeled α_2-adrenergic receptor ligand binding

Measurement of the binding of the antagonist, [^{3}H]yohimbine, and the agonist, ^{3}H-*p*-aminoclonidine, to the various membrane fractions was done for twelve different preparations of the purified membranes. Single ligand concentrations were used; 10 nM [^{3}H]yohimbine which will saturate most (more than two-thirds) of the binding sites and 3 nM ^{3}H-*p*-aminoclonidine which should occupy predominantly the high-affinity agonist binding sites [7,15]. Results shown in Table I indicate a 3.7-fold purification of [^{3}H]yohimbine binding sites in the purified plasma membranes (I) versus the crude membrane (M) fraction. Part of this purification is probably due to removal of contaminating or loosely bound cytoplasmic proteins as there is a 1.5-fold purification from washing the membranes alone (MW). The remaining 2.5-fold purification may thus be attributed to removal of membrane-containing organelles not containing the α_2-adrenergic receptor. In order to determine whether the increase in [^{3}H]yohimbine binding was due to a change in the dissociation constant or to an increase in the number of binding sites per milligram protein, binding isotherms were measured (0.5–30 nM [^{3}H]yohimbine). A comparison of Scatchard plots of [^{3}H]yohimbine binding to the total washed membrane fraction (MW) and the interface membranes (I) revealed more binding sites in the latter, 253 ± 80 vs. 530 ± 46 fmol/mg protein ($n = 4$ and 8, respectively, data not shown). There was no difference in the dissociation con-

stants for the two membrane fractions, 5.8 ± 0.8 nM and 5.3 ± 1.2 nM, respectively. Another consistent finding was that the total amount of receptor recovered in the combined I and P fractions was approx. 50% more than that in M or MW fractions (Table I).

The 17-fold increase in binding of 3 nM ^{3}H-*p*-aminoclonidine upon comparison of results for M and I membranes is even more striking than that seen for [^{3}H]yohimbine (Table I). A quantitative measure of the discrepancy between agonist and antagonist binding is the ratio of ^{3}H-*p*-aminoclonidine bound at 3 nM divided by [^{3}H]yohimbine bound at 10 nM times 100 (P/Y ratio). The P/Y ratio is indicative of the fraction of α_2-receptors binding ^{3}H-*p*-aminoclonidine with high affinity. It is lowest in M and P membranes, 5.2 and 5.5%, respectively. It is highest in I membranes at 27.1% while MW membranes are intermediate at 10.8%.

Complete binding curves were constructed for ^{3}H-*p*-aminoclonidine and [^{3}H]yohimbine binding to I and MW membranes to explore the origin of this discrepancy. Fig. 2 (panels A & B) shows that Scatchard plots of [^{3}H]yohimbine binding are linear, indicating a single class of binding sites. For this particular preparation there was only a 1.5-fold greater number of [^{3}H]yohimbine binding sites in I vs. MW membranes. ^{3}H-*p*-Aminoclonidine binding to I membranes is characterized by a nonlinear Scatchard plot. Fitting these data with a nonlinear least-squares method gives estimates of 30 fmol/mg high-affinity sites with a K_d of 0.21 nM and 327 fmol/mg low-affinity sites with a K_d of 4.8 nM. In contrast, the MW membranes had no high-affinity ^{3}H-*p*-aminoclonidine binding. With the small amount of binding to MW, parameter estimates are only approximate, but a K_d of 41 nM and B_{max} of 420 fmol/mg were obtained. Thus, the striking increase in binding of low concentrations of the partial agonist ^{3}H-*p*-aminoclonidine upon purification of the membranes is due more to the increase in binding affinity than to an increase in the number of binding sites. Notably, the K_d for binding to MW membranes is the same as that seen for I membranes in the presence of p[NH]ppG [7,15]. A more detailed characterization of the ligand binding properties of these purified membranes is reported elsewhere [15].

Origin of reduced agonist binding to crude washed membranes

Many mechanisms could account for the reduced affinity of agonist binding in the crude washed membranes (MW fraction): different receptors for agonist and antagonist, inactivation of agonist binding by proteases, degradation of labelled ligand, presence of inhibitors of agonist binding (e.g. endogenous catecholamines, GTP, or sodium ion). The following experiments were done to evaluate these mechanisms.

TABLE I

[^{3}H]YOHIMBINE AND ^{3}H-*p*-AMINOCLONIDINE BINDING TO DIFFERENT MEMBRANE FRACTIONS

Specific [^{3}H]yohimbine (10 nM) and ^{3}H-*p*-aminoclonidine (3 nM) binding to M, MW, P and I membrane fractions from 12 different preparations was measured as described in Materials and Methods. The total unwashed membrane pellet (M) was used to determine yields and purification factors for [^{3}H]yohimbine binding. The percent of [^{3}H]yohimbine binding sites also occupied by 3 nM ^{3}H-*p*-aminoclonidine is shown as P/Y. This is a semiquantitative measure of the amount of high-affinity ^{3}H-*p*-aminoclonidine binding (see text). Yields of ^{3}H-*p*-aminoclonidine binding sites are not presented because its bindings is not linear with membrane concentration (Fig. 3). The amount of membrane used in each assay was typically 0.4–0.8 mg/ml for M, 0.3–0.7 mg/ml for MW, 0.3–0.6 mg/ml for P and 0.1–0.3 mg/ml for I.

Membrane fraction	[^{3}H]Yohimbine			^{3}H-*p*-Aminoclonidine	
	Spec. act. (fmol/mg)	Yield (%)	Purification factor	Spec. act. (fmol/mg)	P/Y (%)
M	131 ± 19	100	1.0	6.9 ± 1.8	5.2 ± 0.9
MW	160 ± 17	105 ± 25	1.5 ± 0.4	17.1 ± 3.2	10.8 ± 1.5
P	136 ± 22	99 ± 15	1.0 ± 0.1	7.0 ± 1.2	5.5 ± 1.0
I	457 ± 47	52 ± 13	3.7 ± 0.5	116.0 ± 12	27.1 ± 1.6

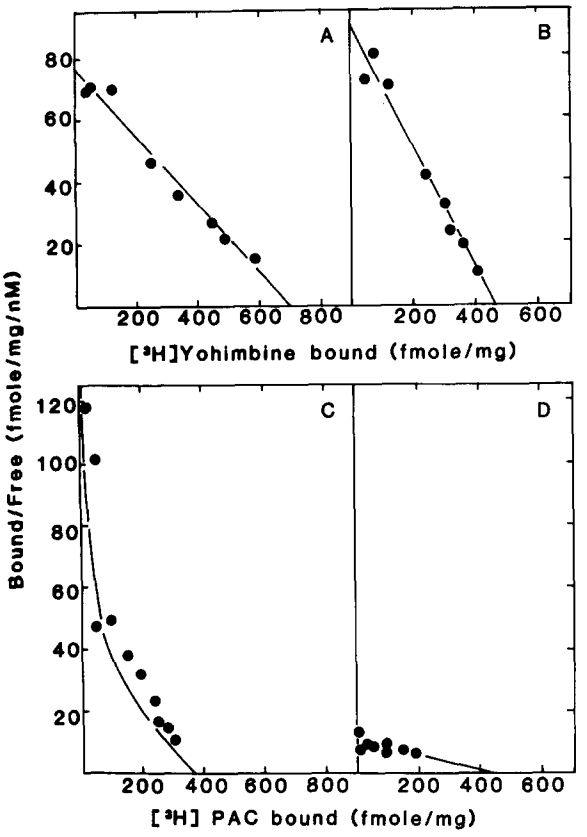

Fig. 2. Scatchard plots of [^{3}H]yohimbine and ^{3}H-*p*-aminoclonidine ([^{3}H]PAC) binding to the I and MW membrane fractions. (A and B) Binding of [^{3}H]yohimbine (1–30 nM) to I (panel A) and MW (panel B) membranes was measured as described in Materials and Methods. Linear least-squares fits of the Scatchard transformations yielded for I membranes a K_d of 9.2 nM and B_{max} of 699 fmol/mg protein and for MW membranes a K_d of 5.1 nM and B_{max} of 459 fmol/mg protein. As discussed in the text there was no significant difference in K_d for yohimbine binding to I and MW membranes while the B_{max} was significantly higher for the former. (C and D) Binding of ^{3}H-*p*-aminoclonidine (0.2–30 nM) to I (panel C) and MW (panel D) membranes was measured as described in Materials and Methods. The solid lines are nonlinear least-squares fits of the data. A two-site model best fit the binding to I while a one-site model adequately fit MW (see text). The same scales are used for ordinate and abscissa for all graphs. I, sucrose interface membrane; MW, washed crude membrane.

First, linearity of ligand binding with increasing protein concentrations was studied. For the purified membranes (I fraction), binding of both agonist (3 nM ^{3}H-*p*-aminoclonidine) and antagonist (10 nM [^{3}H]yohimbine) were linear to 0.4–0.5 mg protein/ml (data not shown). In contrast, MW membranes show essentially no increase in agonist binding as the membrane concentration is doubled from 0.2 to 0.4 mg protein/ml (squares, Fig. 3B). [^{3}H]Yohimbine binding to MW membranes increased linearly with the amount of protein despite the 10-fold higher protein concentration (2–4 mg/ml) in the antagonist binding assay. This observation suggested that an inhibitor or inactivation of receptor or radioligand was the

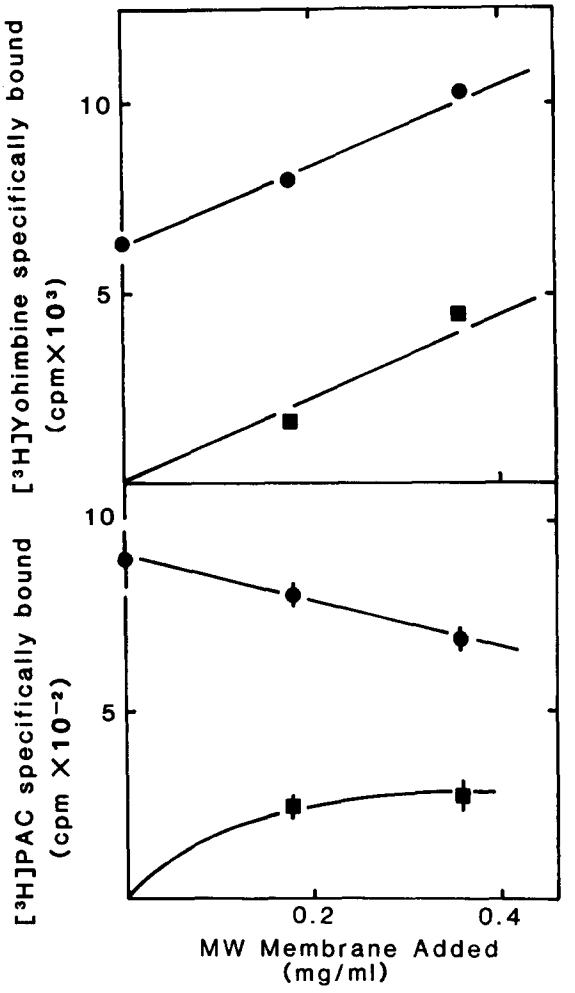

Fig. 3. Effect of mixing I and MW membranes on binding of [^{3}H]yohimbine and ^{3}H-*p* – aminoclonidine. Binding of [^{3}H]yohimbine (10 nM, top) and ^{3}H-*p*-aminoclonidine (3 nM, bottom) to the indicated amount of MW membranes was performed in the presence (circles) and absence (squares) of 0.224 mg of I membranes. [^{3}H]Yohimbine binding was additive and linear with respect to protein concentrations. Agonist binding to I membranes was reduced in the presence of MW membranes suggesting that an inhibitor is present in the latter. I, sucrose interface membrane; MW, washed crude membrane.

mechanism. Thin-layer chromatography of ^{3}H-*p*-aminoclonidine incubated with MW membranes for 30 min at 25°C showed no evidence of degradation of ligand (data not shown). Mixing MW and I membranes revealed an actual decrease in ^{3}H-*p*-aminoclonidine binding when MW membranes were added circles (Fig. 3B). [^{3}H]Yohimbine binding increased in an additive manner as expected. These observations strongly suggested the presence of a selective inhibitor of agonist binding present in the thrice-washed platelet membranes.

Epinephrine and norepinephrine concentrations in the membrane fractions were M 2.5 and 16.4 nM; MW 1.0 and 2.6 nM; P 2.0 and 17.3 nM and I 2.2 and 6.1 nM, respectively. These samples were always diluted at least 20-fold prior to measurement of ^{3}H-*p*-aminoclonidine binding so there should be no effect from endogenous catecholamines. The concentrations of epinephrine and norepinephrine present in the MW membranes even undiluted, are insufficient to account for the inhibitory activity seen.

The unwashed membranes, M, and even the thrice washed crude membranes, MW, contain appreciable contents of the guanine nucleotides GTP and GDP (Table II). The concentrations achieved in binding assays with M and MW membranes are greater than the IC_{50} for inhibition of ^{3}H-*p*-aminoclonidine binding (0.6 and 0.9 mM, respectively, data not shown). The adenine nucleotide content is not sufficient to inhibit agonist binding ($IC_{50} > 100$ μM for ATP and ADP, data not shown). The concentrations of GTP and GDP in the I membranes are markedly lower, but GDP may produce a small amount of inhibition of agonist binding when high membrane protein concentrations are used.

Two additional washes of MW membranes do not increase ^{3}H-*p*-aminoclonidine binding or reduce the inhibition of ^{3}H-*p*-aminoclonidine binding to I membranes (data not shown). If MW membranes are pelleted and resuspended after a freeze-thaw step, the binding of ^{3}H-*p*-aminoclonidine increases and the inhibitory activity decreases (Table III). This suggests that the soluble inhibitor of agonist binding (presumably the guanine nucleotides) can be partially removed by washing after a freeze-thaw step.

Distribution of forskolin-stimulated adenylate cyclase in membrane fractions

Adenylate cyclase activity in the different membrane fractions was measured in the presence of 10^{-5} M forskolin (Table IV). The relative enrichment in adenylate cyclase activity closely parallels the enrichment in [^{3}H]yohimbine binding sites with the greatest activity in the I membrane fraction. Epinephrine-induced inhibition of adenylate cyclase was present in all fractions. The percentage of inhibition was greatest in the crude M fraction but because of experimental variability, the dif-

TABLE II

AGONIST BINDING AND NUCLEOTIDE CONTENT OF MEMBRANE FRACTIONS

Membrane fractions from two sucrose gradient purifications were analyzed for [^{3}H]yohimbine and ^{3}H-*p*-aminoclonidine binding. The percentage of sites occupied by the agonist with high affinity was estimated by the *P/Y* ratio (see Table I and text). Nucleotide content of perchloric acid extracts of the membrane fractions were determined as described in Materials and Methods. Results are expressed as μmol/100 pmol [^{3}H]yohimbine binding sites because agonist binding measurements are typically performed at a receptor concentration of 50–100 pM. Thus the values presented reflect the concentration of nucleotide in a typical binding assay in units of μM. Values in parentheses indicate the range of duplicate determinations. I, sucrose interface membrane; M, crude membrane; MW, washed crude membrane; P, pellet membrane.

Fraction	*P/Y* (%)	μmol/100 pmol [^{3}H]yohimbine binding			
		GTP	GDP	ATP	ADP
I	32 (29, 34)	0.07 (0.06, 0.07)	0.5 (0.4, 0.6)	0.4 (0.7, 0.1)	1.2 (1.7, 0.9)
M	4 (4, 4)	1.9 (1.4, 2.3)	5.4 (5.2, 5.5)	11.0 (11.0, 11.0)	26.7 (29.4, 24.0)
MW	11 (6, 16)	0.9 (0.5, 1.4)	2.5 (2.4, 2.5)	4.3 (3.7, 4.8)	14.0 (12.9, 15.1)
P	6 (5, 6)	2.1 (1.4, 2.7)	5.7 (5.5, 5.8)	11.7 (11.3, 12.0)	30.7 (31.7, 29.7)

TABLE III

EFFECT OF WASHED CRUDE MEMBRANE SUPERNATANT ON ^{3}H-*p*-AMINOCLONIDINE BINDING

Aliquots of I and MW membranes were thawed and kept at 4°C. Half of the MW membranes were centrifuged for 30 min at 140000 × *g*. The supernatant (MW supernatant) was collected and the pellet (MW pellet) resuspended in the same volume of buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.6). Specific binding of 3 nM ^{3}H-*p*-aminoclonidine to the indicated samples is shown. The expected binding is calculated from that seen for I alone, MW alone or MW supernatant. Values are means of triplicate determinations that differed by less than 10%. I, sucrose interface membrane; MW, washed crude membrane.

Sample	Specifically bound ^{3}H-*p*-aminoclonidine (cpm)		
	Observed	Expected	% of expected
I alone	640	640	100
MW alone	212	212	100
I + MW	459	852	54
MW supernatant	0	0	–
I + MW supernatant	262	640	41
MW pellet	315	212	149
I + MW pellet	680	955	70

ference in percent inhibition among fractions was not statistically significant. It has been previously reported that adenylate cyclase inhibition is greatest in cude membrane preparations [11].

TABLE IV

ADENYLATE CYCLASE ACTIVITY IN MEMBRANE FRACTIONS

Adenylate cyclase activity was measured in the presence of 10^{-5} M forskolin at 30°C for 10–15 min as described in Materials and Methods. Results are mean ± S.D. of three experiments each performed in triplicate. Results for relative enrichment were calculated for each membrane preparation and the results averaged to give the value reported. I, sucrose interface membrane; M, crude membrane; MW, washed crude membrane; P, pellet membrane.

Membrane fraction	Forskolin-stimulated adenylate cyclase (pmol/min per mg protein)	Relative enrichment	Inhibition by epinephrine (%)
M	253 ± 74	1.0	39 ± 25
MW	468 ± 127	2.0 ± 0.9	35 ± 15
P	383 ± 93	1.4 ± 0.2	37 ± 2
I	823 ± 74	3.5 ± 1.1	23 ± 12

Basal adenylate cyclase activity in I membranes was 30–90 pmol of cAMP produced/min per mg protein ($n = 7$). Epinephrine (10^{-4} M) inhibited adenylate cyclase by 26–38% ($n = 5$). The pharmacologic specificity of this response was studied. Adenylate cyclase activity was 30 ± 2, 19 ± 1, 19 ± 2, and 28 ± 2 pmol/min per mg protein in the presence of no additions, 10^{-4} M UK 14,304, 10^{-4} M epinephrine and 10^{-4} M epinephrine plus 10^{-5} M yohimbine, respectively. The similar inhibition by epinephrine and the selective α_2-agonist UK 14,304 and the reversal by the α_2-antagonist yohimbine confirm the α_2-adrenergic nature of adenylate cyclase inhibition in human platelet membranes [7,11,26].

Discussion

We have described a simple large scale preparation of α_2-adrenergic receptor-enriched membranes from human platelets. The 3.7-fold enrichment of [^{3}H]yohimbine binding in the purified membranes (I) is consistent with the expected plasma membrane location of α_2-adrenergic receptors. This enrichment reduces the nonspecific binding of [^{3}H]yohimbine and allows higher receptor concentrations to be used in binding assays without overloading the glass fiber filters with protein. The preparation is highly reproducible and stable when frozen in aliquots. It provides a means to study α_2-adrenergic agonist and antagonist binding on the same membrane sample under a wide variety of conditions [15].

Preliminary studies using the method of Barber and Jamieson [12] yielded similar results. However, we have found sonication to result in less cytosotic contamination as determined by measurements of lactate dehydrogenase than the glycerol lysis method (data not shown). Our method differs from that reported by Mooney et al. [11] in the use of sonication rather than N_2 decompression for platelet disruption. This method is simpler and easier to scale up to large amounts of material (> 60 U). Also we avoid the use of colchicine which has been shown to affect the guanine nucleotide binding protein of the adenylate cyclase system [27].

Our method could be used as an initial step in the complete purification of α_2-receptor. Struct-

ural integrity of the receptor may be better preserved by this method because of the absence of detergents. The 30–50% yield of α_2-receptors is a limiting factor, but attempts to improve this with a second sonication are in progress. Currently, a preparation starting with 40–60 units of platelets yields 50–200 pmol of α_2-receptors in the enriched membranes. The α_2-adrenergic receptor has also been partially purified from detergent extracts of crude human platelet membranes [28]. Recently, the α_2-adrenergic receptor from a rat adrenocarcinoma cell line has been purified to apparent homogeneity [29]. The structural integrity of receptors purified in this manner remains to be determined.

The striking finding concerning this purified membrane preparation is the markedly greater agonist binding than is seen with the crude, washed membrane fraction (MW). This is due both to the enrichment in receptor binding sites, as reflected by [^{3}H]yohimbine binding, and more importantly to a marked increase in the affinity of ^{3}H-*p*-aminoclonidine binding. The low affinity of ^{3}H-*p*-aminoclonidine binding to MW membranes has been demonstrated to be due in part to a soluble inhibitor of agonist binding. Endogenous catecholamines do not seem to explain these results as very low concentrations are present in the assay. Concentrations are lowest in the MW fraction while ^{3}H-*p*-aminoclonidine binding is clearly greatest in the I fraction. The persistent association of GDP and GTP with the crude washed membranes appears to explain the selective loss of agonist binding. These data reiterate the difficulty in eliminating guanine nucleotides from membrane preparations and confirm the utility of sucrose gradient methods for preparation of membranes relatively free of guanine nucleotides [30].

The enrichment of adenylate cyclase activity closely parallels that of [^{3}H]yohimbine binding. This is expected for two activities presumed to be in the same subcellular location (i.e. plasma membrane). The lack of enhancement in epinephrine-induced inhibition of adenylate cyclase is likely due to the fact that even in the crude membrane fractions, the α_2-adrenergic receptor and adenylate cyclase catalytic subunit are in the same membrane vesicles. Thus, the coupling between α_2-receptor and adenylate cyclase is not substantially changed by removal of non-plasma membrane components. The reduced percentage inhibition of forskolin-stimulated adenylate cyclase in the purified membranes ($23 \pm 12\%$ vs. $35 \pm 15\%$) may be due to the longer time required for preparation of this membrane fraction. In more extensive studies of epinephrine-induced inhibition of basal adenylate cyclase, however, a larger percentage inhibition was seen ($31 \pm 5\%$).

The partially purified platelet plasma membrane preparation described in this paper may represent a useful step in purification of the α_2-adrenergic receptor. It also provides a reproducible system for studies of the mechanisms of α_2-adrenergic agonist binding and responses in the membrane environment.

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