Widger, W. R., Cramer, W. A., Herrman, R. G., & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 674-678.
Wilson, E., Forley, T. M., & Takamoto, J. Y. (1985) *J. Biol. Chem. 260*, 10288-10292.
Yang, F.-D., Yu, L., & Yu, C.-A. (1986) *Biophys. J. 49*, 96a.
Yu, C.-A., & Yu, L. (1980) *Biochemistry 19*, 5715-5720.
Yu, L., & Yu, C.-A. (1982) *Biochem. Biophys. Res. Commun. 108*, 1285-1292.
Yu, L., & Yu, C.-A. (1984) *Biochem. Biophys. Res. Commun. 123*, 1234-1239.
Yu, L., Yu, C.-A., & King, T. E. (1978) *J. Biol. Chem. 253*, 2657-2663.
Yu, L., Mei, O.-C., & Yu, C.-A. (1984) *J. Biol. Chem. 259*, 5752-5760.
Yu, L., Yang, F.-D., & Yu, C.-A. (1985) *J. Biol. Chem. 260*, 963-973.
Yu, L., Dong, J.-H. & Yu, C.-A. (1986a) *Biochim. Biophys. Acta 852*, 203-211.
Yu, L., Yang, F.-D., Yu, C.-A., Tsai, A.-L., & Palmer, G. (1986b) *Biochim. Biophys. Acta 848*, 305-311.

Membrane Reconstitution of High-Affinity α_2 Adrenergic Agonist Binding with Guanine Nucleotide Regulatory Proteins†

Marian H. Kim and Richard R. Neubig*
Departments of Pharmacology and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109
Received December 15, 1986; Revised Manuscript Received March 18, 1987

ABSTRACT: Regulation of adenylate cyclase by α_2 adrenergic receptors requires the inhibitory guanine nucleotide binding protein N_i. A role for this protein has also been suggested in the high-affinity binding of agonists to the α_2 receptor. We recently reported that alkaline treatment can selectively inactivate α_2 agonist binding and N_i in human platelet plasma membranes [Kim, M. H., & Neubig, R. R. (1985) *FEBS Lett. 192*, 321-325]. Binding of the full α_2 agonists epinephrine and 5-bromo-6-[*N*-(4,5-dihydroimidazol-2-yl)amino]quinoxaline (UK 14304) to these membranes was determined by competition and direct radioligand binding, respectively. The high-affinity GTP-sensitive binding of the agonists is lost after alkaline treatment. Binding of [^{3}H]UK 14304 was reconstituted by poly(ethylene glycol)-induced fusion of alkaline-treated platelet membranes with cell membranes containing N_i but no α_2 receptor or with lipid vesicles containing purified guanine nucleotide binding proteins (N-proteins) from bovine brain. The reconstituted binding was of high affinity (K_d = 0.4 ± 0.1 nM), accounted for a substantial fraction of the total α_2 receptors (B_{max} for [^{3}H]UK 14304 was 78 ± 23% of the B_{max} for [^{3}H]yohimbine), and was abolished in the presence of guanosine 5′-(β,γ-imidotriphosphate) (GppNHp). The brain-specific protein N_o (predominant guanine nucleotide regulatory protein from bovine brain) was also effective in reconstituting high-affinity α_2 agonist binding. The results presented here show that a guanine nucleotide regulatory protein of the N_o or N_i type is necessary for high-affinity α_2 agonist binding. These methods should also prove useful for future studies of receptor N-protein interactions.

Adenylate cyclase activity is stimulated by β-adrenergic receptors and is inhibited via α_2 adrenergic receptors [see Ross and Gilman (1980) and Limbird (1981) for review]. Stimulation of enzyme activity requires the guanine nucleotide regulatory protein N_s,[1] while inhibition of adenylate cyclase requires a distinct guanine nucleotide regulatory protein, N_i (Ross & Gilman, 1980; Kurose et al., 1983). Another GTP binding protein of unknown function, termed N_o, has recently been purified from bovine brain (Sternweis & Robishaw, 1984; Neer et al., 1984). The interactions of purified β-adrenergic receptors with N_s, N_i, and the catalytic subunit of adenylate cyclase have been examined after reconstitution into phospholipid vesicles (May et al., 1985; Cerione et al., 1983). In addition, Cerione and collaborators have recently reconstituted α_2-receptor-stimulated GTPase activity using purified N-proteins and partially purified α_2 receptors (Cerione et al., 1986).

In addition to the role of guanine nucleotide regulatory proteins in producing responses, there is evidence that the affinity of agonist binding to many receptors including α_2 (Hoffman et al., 1982; Michel et al., 1980) and β-adrenergic (Maguire et al., 1976; Blume, 1978), muscarinic (Jakobs et al., 1979), and opiate receptors (Kurose et al., 1983; Katada & Ui, 1982) is regulated by these proteins. Guanine nucleotides such as the nonhydrolyzable GTP analogue GppNHp selectively reduce the affinity of α_2-adrenergic agonist binding without reducing antagonist binding (Hoffman et al., 1982; Michel et al., 1980). Modification of the N_i protein by pertussis-toxin-catalyzed ADP-ribosylation in NG108-15 cells results in a loss of receptor-mediated inhibition of adenylate

† This work was supported by NSF Grant DCB-8409333 and by a grant-in-aid from the American Heart Association of Michigan. R.R.N. is a Hartford Foundation Fellow.
* Address correspondence to this author at the Department of Pharmacology, University of Michigan Medical School.

[1] Abbreviations: buffer A, 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.0, 2 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl; GppNHp, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); N-proteins, guanine nucleotide binding proteins; N_i, inhibitory guanine nucleotide regulatory protein; N_o, predominant guanine nucleotide regulatory protein from bovine brain; N_s, stimulatory guanine nucleotide regulatory protein; NAD, nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol); POB membranes, platelet membranes pretreated with phenoxybenzamine; SDS, sodium dodecyl sulfate; TED buffer, 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol (DTT); TME buffer, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA); UK 14304, 5-bromo-6-[*N*-(4,5-dihydroimidazol-2-yl)amino]quinoxaline.

0006-2960/87/0426-3664$01.50/0

cyclase activity and also reduces the binding affinity of α_2-adrenergic, opiate, and muscarinic agonists (Kurose et al., 1983). Similar effects of pertussis toxin to reduce α_2 agonist binding affinity have been reported in rat brain (Nomura et al., 1985) and adipocytes (Garcia-Sainz et al., 1984).

The cyc$^-$ variant of S49 lymphoma cells that lacks N_s has been very useful as a tool for studying the role of that protein in various biochemical systems (Ross et al., 1978). β-Adrenergic receptor mediated stimulation of adenylate cyclase and the GTP sensitivity of β-adrenergic agonist binding are absent in cyc$^-$ membranes. Since no such mutant cell was available for N_i, we utilized the approach of Citri and Schramm (1980), who found that treatment of turkey erythrocyte membranes with alkaline buffer inactivated N_s and adenylate cyclase catalytic activity while leaving β-adrenergic receptors intact. Similar results have been seen by Kassis et al. (1984) after alkaline treatment of HeLa cell membranes. This technique has also proved useful in studies of nicotinic acetylcholine receptors (Neubig et al., 1979). We recently demonstrated that alkaline treatment of human platelet membranes inactivates N_i as measured by pertussis-toxin-catalyzed [^{32}P]NAD labeling and markedly reduces high-affinity α_2 agonist binding (Kim & Neubig, 1985). Antagonist binding is minimally affected by this treatment. The alkaline-treated platelet membranes thus constitute a preparation containing a functional α_2 receptor but lacking a functional N_i.

In this paper, we describe the use of membrane–membrane fusion methods (Kassis et al., 1984; Schramm, 1979) to reconstitute high-affinity α_2 adrenergic agonist binding with purified N-proteins. This method is also shown to be applicable to reconstitution of agonist binding by use of a variety of natural membranes as the source of N-protein. The simplicity of the approach and the lack of a requirement for detergents suggest a significant generality for these methods in the study of receptor effector coupling. Some of these results have been reported previously in abstract form (Kim & Neubig, 1986).

Materials and Methods

Reagents. Poly(ethylene glycol) (M_r 8000), soybean phosphatidylcholine, cholesterol, and cholic acid were obtained from Sigma Chemical Co. (St. Louis, MO). The cholic acid was purified by the method of Ross and Schatz (1978) except that chromatography was performed on DEAE–Sephacel. GTPγS was from Boehringer Mannheim. Oxymetazoline hydrochloride was a gift from the Schering Corp. (Bloomfield, NJ). Phenoxybenzamine was a gift from Smith Kline & French and was precyclized for 15 min at 37 °C before use (Kunos et al., 1983). Purified pertussis toxin was a kind gift of Dr. L. Winderry, Michigan Department of Public Health (Lansing, MI). [^{3}H]Yohimbine (75–85 Ci/mmol), [^{3}H]UK 14 304 (84–88 Ci/mmol), and [^{35}S]GTPγS (1000–1200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Heptylamine–Sepharose was prepared as described (Shaltiel, 1974). All other chemicals were of reagent grade or better.

Preparation and Covalent Modification of Human Platelet Membranes. Human platelet concentrates purchased from the Detroit Chapter of the American Red Cross within 24 h of collection were used to prepare membranes as described by Neubig and Szamraj (1986). In brief, the turbid fraction from the interface of a 14.5%/34% discontinuous sucrose gradient was collected by centrifugation at 105000g, resuspended in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EGTA (TME buffer), quick frozen in a dry ice–ethanol bath, and stored at −70 °C until use.

Alkaline treatment of platelet membranes was performed as described by Kim and Neubig (1985). Briefly, purified platelet membranes in TME buffer were thawed and diluted 20-fold in 50 mM sodium phosphate, pH 11.75, and the pH was adjusted to 11.50 with 5 M NaOH. After 1 h on ice, the membranes were collected by centrifugation at 145000g for 30 min, resuspended in TME buffer at pH 7.6, quick frozen in a dry ice–ethanol bath, and stored at −70 °C for up to 2 weeks prior to use.

For inactivation of α_2 receptors, platelet membranes were incubated at a concentration of 4 mg of protein per milliliter in TME buffer containing 10 μM precyclized phenoxybenzamine for 30 min at 23 °C. The reaction was terminated by dilution with 10 volumes of ice-cold TME buffer containing 10 mg/mL bovine serum albumin, and membranes were centrifuged at 145000g for 30 min. The pellet was washed by resuspension in the same volume of buffer containing 10 mg/mL albumin, centrifuged again and resuspended in TME buffer without albumin, quick frozen, and stored at −70 °C for 1–2 weeks until use. The 10 μM phenoxybenzamine was chosen because that concentration inactivates greater than 95% of the [^{3}H]yohimbine binding. Phenoxybenzamine treatment did not affect pertussis toxin catalyzed [^{32}P]ADP ribosylation of the 41 000-dalton substrate in cholate extracts of these membranes (data not shown).

Preparation of Pertussis Toxin Treated C6 Glioma Cell Membranes. C6 glioma cells kindly provided by Dr. R. Simpson (Department of Pharmacology) were grown as monolayer cultures in Dulbecco's modified Eagle medium containing 10% fetal calf serum on plastic Petri dishes until confluency was achieved (4 or 5 days). In some experiments, cells were treated with 100 ng/mL purified pertussis toxin for 18 h prior to preparation of membranes. Cells were detached with Versene (5.5 mM dextrose, 5 mM KCl, 1 mM HEPES, pH 7.4, 137 mM NaCl, 0.5 mM EDTA), collected by centrifugation at 500g for 10 min, and washed twice in 10 mM Tris-HCl, pH 7.5, 0.5% NaCl, by centrifugation. Cells were then homogenized with 10 strokes in a Potter-Elvehjem homogenizer in buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT, and the membranes were collected by centrifugation at 20000g for 30 min. Membranes were washed once, resuspended in the same buffer, quick frozen, and stored at −70 °C for 1–2 weeks until use. Binding of 10 nM [^{3}H]yohimbine to C6 cell membranes was minimal (10 ± 7 fmol/mg of protein, n = 5).

Purification of N_o and N_i from bovine brain was performed by modifications of published methods (Sternweis & Robishaw, 1984; Neer et al., 1984; Huff et al., 1985). Differences from those procedures are outlined below. All procedures were performed at 0–4 °C. Bovine cerebral cortex (300 gm) collected from a local slaughterhouse and frozen in liquid N_2 was thawed briefly in 1.2 L of cold homogenization buffer (50 mM Tris, pH 8.0, 5% sucrose, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM DTT, 3 mM benzamidine hydrochloride, 1 mg/L soybean and lima bean trypsin inhibitors), homogenized in a Waring blender in five 10-s bursts, and rehomogenized with a Polytron homogenizer (30 s, setting 30). The homogenate was centrifuged at 20000g for 40 min, and pellets were washed once in 1.2 L of cold homogenization buffer by resuspension and centrifugation. After the pellets were resuspended in a minimal amount of homogenization buffer, the membrane paste was quick frozen and stored at −70 °C for up to a week. The membrane paste was thawed in TED buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT) including 3 mM benzamidine hydrochloride and 1 mg/L soybean and lima bean trypsin

inhibitors, rehomogenized, and centrifuged at 20000*g* for 40 min. Pellets were resuspended in 1 volume of TED, and an equal amount of TED containing 2.0% sodium cholate without activating ligands was added. The mixture was stirred on ice for 1 h and centrifuged at 143000*g* for 1 h. The soluble extract was collected and chromatographed on DEAE-Sephacel, Ultrogel AcA34, and heptylamine–Sepharose columns as described (Sternweis & Robishaw, 1984; Neer et al., 1984; Huff et al., 1985). Guanine nucleotide binding protein activity was monitored by measuring [^{35}S]GTPγS binding according to Sternweis and Robishaw (1984), and protein was determined by the method of Bradford (1976).

The peak of [^{35}S]GTPγS binding activity obtained from the heptylamine–Sepharose column contains polypeptides of M_r 41 000, 39 000, and 36 000 as detected by Coomassie Blue staining of 10% Laemmli gels as previously reported (Sternweis & Robishaw, 1984; Neer et al., 1984). The 39 000-dalton α subunit of N_o accounts for the bulk of the higher molecular weight component with a 41 000-dalton protein accounting for the remainder. There were approximately equal amounts of α and β subunit detectable by Coomassie Blue staining. Three different preparations of this combined N_o/N_i mixture were used for most of the reconstitution experiments described and all three contained roughly the same proportions of α_o, α_i, and β subunits. In all experiments with purified N-proteins, more than 95% of the protein detectable by Coomassie Blue staining was accounted for by the known subunits of N_o or N_i.

In some experiments N_o was further purified from the mixture by chromatography on DEAE-Sephacel in 0.5% Lubrol PX as described by Katada et al. (1986). Briefly, 18 mL ($\sim$4 mg of protein) of the N_o/N_i pool from the heptylamine–Sepharose column was diluted with 3 volumes of TED containing 0.67% Lubrol PX. The sample was loaded on a 1.5 × 20 cm DEAE-Sephacel column which had been preequilibrated with TED containing 0.5% Lubrol PX. The column was then washed with 50 mL of the equilibration buffer, and the N-proteins were eluted with a 160-mL gradient of the equilibration buffer containing 25–200 mM NaCl followed by an additional 50 mL of the high-salt buffer. Two peaks of protein eluted during the gradient. On sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and Coomassie Blue staining, the first peak contained only bands of 39 000 and 36 000 daltons (Figure 6, lane C). The second had nearly equal amounts of the 39 000- and 41 000-dalton bands in addition to some enrichment of the 36 000-dalton protein (see Figure 6, lane D). The first peak (which we will call N_o) was concentrated by filtration on Amicon YM 30 membranes and exchanged into TED containing 100 mM NaCl and 0.9% sodium cholate prior to incorporation into phospholipid vesicles.

Preparation of Phospholipid Vesicles Containing Nucleotide Regulatory Proteins. Twelve milligrams of soybean phosphatidylcholine and 1.2 mg of cholesterol in 1.12 mL of $CHCl_3$ were dried under N_2 and incubated with 4.6 mL of a 0.3% solution of sodium cholate in 25 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, and 100 mM NaCl (buffer A) at 23 °C for 5 min. The mixture was vortexed for 5 min and then sonicated at 4 °C under N_2 with a Branson microtip (setting 5.5) or in a sonicator bath (Bransonic 12) for 30–45 min until the mixture appeared translucent. Purified N-proteins (10 mg/mL) in TED buffer containing 100 mM NaCl and 0.9% sodium cholate were added to a final concentration of 0.3–2.9 mg of protein/mL. The cholate was removed by chromatography through a 50-mL Sephadex G50 column equilibrated in buffer A. Turbid fractions were pooled, quick frozen, and stored at −70 °C for up to 1 mo until use in reconstitution assays. The same concentrations of phospholipid, cholesterol, and detergent were used to prepare control phospholipid vesicles, except that no N-proteins were added to the lipid mixture.

Fusion of Receptor-Deficient Membranes or N-Protein-Containing Vesicles with pH 11.5 Treated Membranes. Phenoxybenzamine-treated platelet membranes and pH 11.5 treated platelet membranes were mixed and centrifuged at 145000*g* for 30 min. Equal amounts of protein from donor and acceptor membranes were used unless otherwise indicated. The mixed pellet was resuspended at 7 mg/mL in 50% (w/w) poly(ethylene glycol) (PEG) in buffer containing 5 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM EDTA, 135 mM NaCl, 5 mM KCl, and 20 mM Tris-HCl, pH 7.4, and incubated for 5 min at 25 °C (Kassis et al., 1984; Schramm, 1979). This mixture was diluted with 20 volumes of TME buffer and centrifuged at 145000*g* for 30 min at 4 °C. The pellet was washed once by resuspension in ice-cold TME buffer, recentrifuged, and resuspended in the same buffer for determination of ligand binding.

Fusion with phospholipid vesicles was performed exactly as described for fusion of phenoxybenzamine-treated membranes to pH 11.5 membranes, with the following exceptions. Lipid vesicles with or without N-protein were mixed with pH 11.5 treated membranes, diluted 10-fold in TME buffer, and centrifuged at 200000*g* for 2 h. The mixed pellet was resuspended in 50% PEG and incubated for 5 min at 25 °C. After a 20-fold dilution in TME buffer, the mixture was recentrifuged at 200000*g* for 30 min. The pellet was washed once as above and resuspended in TME buffer for determination of binding.

Equilibrium Binding of [^{3}H]UK 14 304 and [^{3}H]Yohimbine. Binding of the antagonist [^{3}H]yohimbine and the full α_2 agonist [^{3}H]UK 14 304 was measured at 23–25 °C as described by Neubig et al. (1985). Bound and free ligand were separated by vacuum filtration over Whatman GF/C filters or poly(ethylenimine)-treated GF/C filters (Neubig et al., 1985; Bruns et al., 1983). Nonspecific binding was defined by using 10^{-5} M yohimbine or 10^{-5} M oxymetazoline, respectively, as described (Neubig et al., 1985) and represented 8–17% of total binding for [^{3}H]yohimbine, 2–7% for [^{3}H]UK 14 304 binding to control membranes, and 40–46% for [^{3}H]UK 14 304 binding to alkaline-treated membranes prior to reconstitution. Data are presented as mean ± SD of triplicate determinations unless otherwise noted. Saturation binding experiments were analyzed by using the computer program SCAFIT (Munson & Rodbard, 1980). Hill coefficients and IC_{50}'s for competition curves were analyzed by linear least-squares fits of −log [(100 − % bound)/% bound] vs. −log [epinephrine] for points corresponding to the 10–90% specifically bound ligand. Correlation coefficients were 0.95–0.99 for all Hill plots calculated. Scintillation counting efficiency was 42–53%.

Miscellaneous. Protein was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard or, in the case of N-protein purification, the procedure of Bradford (1976) using lysozyme as a standard. SDS–polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (1970) using 10% acrylamide gels. Proteins were stained with Coomassie Brilliant Blue.

RESULTS

Agonist Binding to pH 11.5 Treated Platelet Membranes. Binding of the full α_2 agonist [^{3}H]UK 14 304 to control platelet membranes and pH 11.5 treated membranes is shown in Figure 1. As we showed previously for the partial α_2 agonist

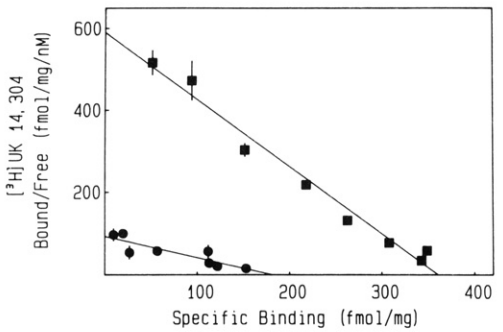

FIGURE 1: Binding of [^{3}H]UK 14 304 to control platelet membranes and pH 11.5 treated membranes. Binding of [^{3}H]UK 14 304 to control membranes (treated with 50 mM phosphate buffer, pH 7.6) and to membranes treated to pH 11.5 for 1 h and then returned to pH 7.6 was measured as described in Materials and Methods. Scatchard transformations of specific binding are shown for control (■) and pH 11.5 treated membranes (●). Nonspecific binding accounted for less than 15% of total binding for control membranes and 40% for pH 11.5 treated membranes at 10 nM [^{3}H]UK 14 304. These data are from a single experiment. Binding to pH 11.5 treated membranes has been similarly measured in three additional experiments (see text).

Table I: IC_{50} Values and Hill Coefficients for Epinephrine Competition with [^{3}H]Yohimbine Binding[a]

membranes	IC_{50} (μM)	Hill coeff	n
control membranes			
epinephrine alone	0.11 ± 0.10	0.46 ± 0.10	3
+GppNHp (10 μM)	0.86 ± 0.30°	0.76 ± 0.16$^+$	3
+NaCl (100 mM)	1.63 ± 0.75°	0.50 ± 0.17$^+$	3
pH 11.5 treated membranes			
epinephrine alone	1.6 ± 0.8	0.69 ± 0.15	5
+GppNHp (10 μM)	3.7 ± 2.2$^+$	0.65 ± 0.22$^+$	3
+NaCl (100 mM)	11.4 ± 0.4°	0.81 ± 0.18$^+$	4

[a] Binding of 5 nM [^{3}H]yohimbine was measured in the presence of 10–15 concentrations of (−)-epinephrine with the addition of GppNHp or NaCl as noted. Pseudo Hill plots of specific [^{3}H]yohimbine binding were analyzed as described in Materials and Methods. Values of the IC_{50} and Hill coefficient are shown as mean ± SD of the indicated number of determinations. Two-tailed paired t tests comparing results in the presence of NaCl or GppNHp with the results for the same membrane preparation without additions were performed; ° indicates significantly different results ($p < 0.02$), and + shows results that are not significantly different ($p > 0.10$).

[^{3}H]p-aminoclonidine, binding of the full agonist is markedly reduced by alkaline treatment. For [^{3}H]UK 14 304 concentrations from 0 to 2 nM a Scatchard plot of the binding is linear, since only the high-affinity-binding sites are occupied in this range (Figure 1). For alkaline-treated membranes we observed a K_d of 1.89 ± 0.34 nM and a B_{max} of 75 fmol/mg (range 15–172, $n = 4$) for [^{3}H]UK 14 304 compared to 0.88 ± 0.17 nM and 280 ± 20 fmol/mg previously reported for the high-affinity component of [^{3}H]UK 14 304 binding to control membranes (Neubig et al., 1985).

Binding of the full agonist (−)-epinephrine was determined indirectly by competition with the antagonist [^{3}H]yohimbine (Figure 2). Results of Hill analysis are shown in Table I. For control membranes, the concentration of epinephrine needed to inhibit 50% of [^{3}H]yohimbine binding (IC_{50}) was significantly increased by both GppNHp and sodium. For pH 11.5 treated membranes the IC_{50} was significantly greater than that seen for control membranes and was similar to that for control membranes in the presence of GppNHp. GppNHp does not affect the IC_{50} for epinephrine after pH 11.5 treatment of membranes. In contrast, the IC_{50} for epinephrine was significantly increased in the presence of 100 mM NaCl. These data indicate that while the effect of GppNHp on epinephrine binding is abolished for alkaline-treated membranes, the sensitivity to NaCl does not differ between the control and pH 11.5 treated membranes.

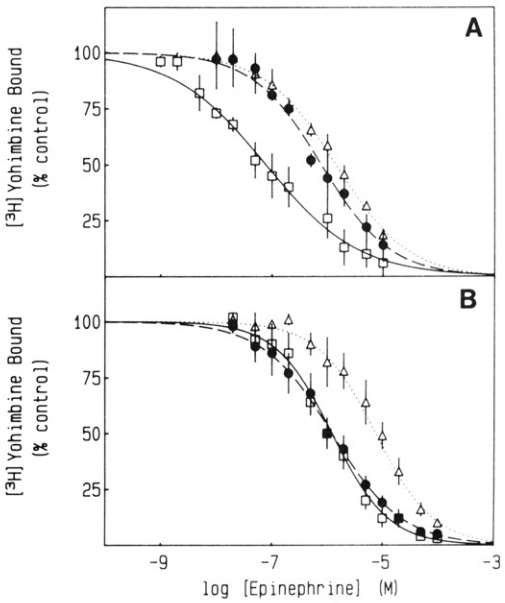

FIGURE 2: Epinephrine competition with [^{3}H]yohimbine binding to α_2 receptors on control and pH 11.5 treated platelet membranes. The binding of 5 nM [^{3}H]yohimbine to control (A) and pH 11.5 treated membranes (B) was measured in the presence of varying concentrations of (−)-epinephrine alone (□), (−)-epinephrine in the presence of 10 μM GppNHp (●), and (−)-epinephrine in the presence of 100 mM NaCl (△). Binding was allowed to equilibrate for 30–60 min at 23 °C and was measured in a volume of 500 μL as described under Materials and Methods. Samples contained 0.01% sodium ascorbate. These data are averages of three (A) or five (B) experiments, each performed in triplicate. The curves drawn are theoretical fits of the data to the Hill equation using the parameters indicated in Table I.

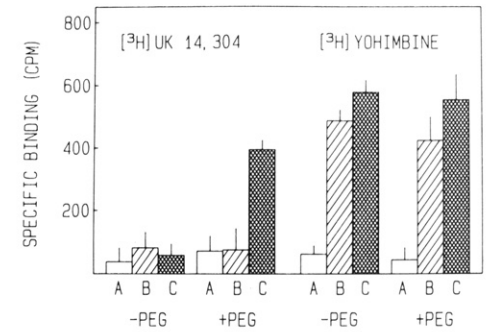

FIGURE 3: Agonist and antagonist binding to pH 11.5 treated membranes after fusion with phenoxybenzamine-treated platelet membranes. Equilibrium binding of 1 nM [^{3}H]UK 14 304 (left) and 10 nM [^{3}H]yohimbine (right) was measured in triplicate for phenoxybenzamine-treated membranes (bar A), pH 11.5 treated membranes (bar B), and a mixture of both (bar C), resuspended in the absence (−PEG) or the presence (+PEG) of 50% PEG. The samples for [^{3}H]UK 14 304 binding contained 0.065 mg of protein, while the samples for [^{3}H]yohimbine binding contained 0.032 mg of protein. Nonspecific binding was 50 cpm for [^{3}H]UK 14 304 and 200 cpm for [^{3}H]yohimbine measurements. This figure is representative of four experiments performed with similar results.

Reconstitution of High-Affinity [^{3}H]UK 14 304 Binding by Fusion with Receptor-Deficient Membranes. Poly(ethylene glycol)-induced fusion was used to prepare hybrid membranes from pH 11.5 treated platelet membranes and receptor-deficient donor membranes containing functional N-protein. The first source of membranes containing functional N_i protein was platelet membranes in which the α_2 receptor binding site was covalently inactivated by pretreatment with phenoxybenzamine (POB membranes). Figure 3 shows the specific binding of [^{3}H]UK 14 304 and [^{3}H]yohimbine to hybrid membranes

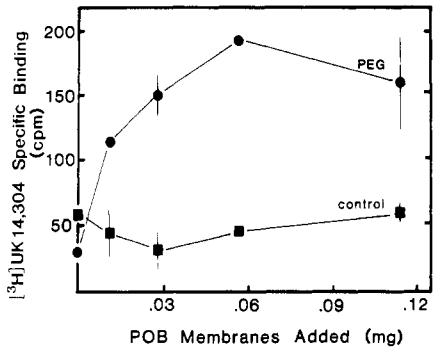

FIGURE 4: Dependence of agonist binding to pH 11.5 membranes on the amount of added phenoxybenzamine-treated membranes. Equilibrium binding of 1 nM [^{3}H]UK 14 304 was measured for a mixed membrane pellet of POB and pH 11.5 membranes treated without (■) or with (●) 50% PEG as described in Figure 3. The amount of pH 11.5 membrane added to the assay was constant at 0.058 mg of protein, and the amount of POB membrane is indicated on the abscissa. Nonspecific binding was 70 cpm for all samples. This experiment is representative of four experiments with comparable results.

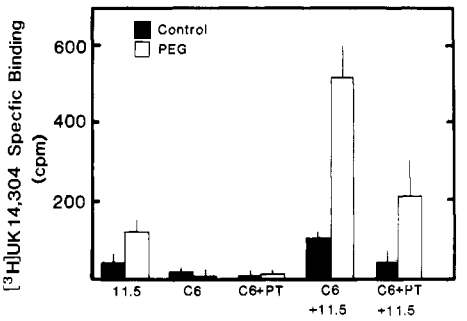

FIGURE 5: Effect of pertussis toxin on reconstitution of α_2 adrenergic agonist binding. Rat C6 glioma cells were treated with vehicle (C6) or 100 ng/mL of pertussis toxin for 18 h prior to membrane preparation (C6 + PT). pH 11.5 treated membranes were then mixed with C6 membranes in the absence (closed bars) and presence (open bars) of PEG, and specific binding of 1 nM [^{3}H]UK 14 304 was measured. There was 0.060 mg of protein from pH 11.5 and/or C6 membranes in these measurements. Nonspecific binding was 50–100 cpm. This experiment is one of three experiments performed with similar results.

prepared by PEG-induced fusion of POB membranes with pH 11.5 treated membranes. At the concentrations chosen, [^{3}H]UK 14 304 (1 nM) will occupy the majority of the high-affinity agonist binding sites, while 10 nM [^{3}H]yohimbine will occupy approximately two-thirds of the total α_2 receptor binding sites. [^{3}H]Yohimbine binding was nearly abolished (<8% of control) in POB membranes (bar A), as would be expected since the α_2 receptor binding site is covalently blocked. The pH 11.5 treated membranes (bar B) exhibited substantial [^{3}H]yohimbine binding. Binding to the mixture of the two types of membranes (bar C) was approximately equal to the sum of the two individual components. Treatment with PEG did not significantly alter the [^{3}H]yohimbine binding.

There was minimal high-affinity [^{3}H]UK 14 304 binding to either the POB membranes or the pH 11.5 treated membranes. Mixing and pelleting the two membranes did not enhance the binding. PEG treatment did not significantly alter binding to either POB or pH 11.5 membranes but PEG treatment of the combined pellet produced a (4.2 ± 0.6)-fold (mean ± SEM, n = 8) increase in [^{3}H]UK 14 304 binding.

Figure 4 depicts the effect of adding varying amounts of POB membranes on the reconstitution of [^{3}H]UK 14 304 binding to pH 11.5 treated membranes. Agonist binding was not increased when PEG was absent from the mixture (control), regardless of the amount of POB membrane added. Also, agonist binding was not enhanced by PEG in the absence of added POB membrane. In the presence of PEG, however, POB membranes produced a concentration-dependent reconstitution of high-affinity [^{3}H]UK 14 304 binding. Reconstitution increased up to a 1:1 ratio of protein from phenoxybenzamine and pH 11.5 treated membranes. Interestingly, in all four experiments, no additional reconstitution occurred if this ratio was exceeded. The increase in binding of [^{3}H]UK 14 304 to pH 11.5 treated membranes after fusion with POB membranes was abolished by the addition of 10 μM GppNHp to the binding assay (data not shown).

In order to provide additional evidence about the identity of the membrane component necessary for reconstitution, we studied the pertussis toxin sensitivity of this reconstitution. Since attempts to fully inactivate the N_i protein in platelet membranes with pertussis toxin were unsuccessful,[2] rat C6 glioma cells were used as an alternative source of functional N-protein. Intact C6 glioma cells were treated with pertussis toxin, and membranes prepared from the cells were then fused with pH 11.5 treated platelet membranes in the presence of PEG. Binding of 1 nM [^{3}H]UK 14 304 to the resulting hybrid membranes in which the C6 cells had been pretreated with or without pertussis toxin is shown in Figure 5. The alkaline-treated membranes bind [^{3}H]UK 14 304 poorly, and C6 membranes which do not contain α_2 receptors also do not exhibit binding of [^{3}H]UK 14 304 either with or without pertussis treatment. Fusion of C6 membranes to pH 11.5 treated membranes resulted in a 4.6 ± 0.6 fold (n = 3) increase in agonist binding to pH 11.5 treated membranes, using pH 11.5 membranes + PEG as a control ($p < 0.05$ unpaired t test).[3] Treatment of C6 cells with pertussis toxin consistently reduced the ability of those membranes to reconstitute high-affinity agonist binding to only 1.6 ± 0.3 fold (n = 3), a result not significantly different from the control value ($p > 0.2$).

Reconstitution of High-Affinity α_2 Adrenergic Agonist Binding by Fusion with Purified N-Proteins. Because the reconstitution was pertussis toxin sensitive, we determined the effect of purified pertussis toxin substrates on the reconstitution of high-affinity α_2 agonist binding. A mixture of the guanine nucleotide regulatory proteins N_i and N_o was purified from bovine brain (Figure 6, lanes A and B). These combined N_o/N_i preparations were inserted into phospholipid vesicles as described under Materials and Methods and the vesicles fused with pH 11.5 treated membranes in the presence of PEG. Binding of 1 nM [^{3}H]UK 14 304 and 10 nM [^{3}H]yohimbine was measured (Figure 7). The pH 11.5 treated membranes retain the ability to bind yohimbine but neither the phospholipid vesicles nor the N-protein containing vesicles bind the antagonist (Figure 7, right). The fusion of phospholipid vesicles and pH 11.5 treated membranes did not significantly affect yohimbine binding. A small decrease in [^{3}H]yohimbine binding was observed after fusion with N-protein-containing vesicles, but this was not seen consistently in all experiments.

The agonist binding properties of the fused membranes were quite different (Figure 7, left). Neither the pH 11.5 treated membranes nor the N-protein-containing vesicles alone exhibit

[2] M. Kim and R. Neubig, unpublished observations.

[3] The increase in [^{3}H]UK 14 304 binding induced by PEG alone was small and variable (compare Figures 3–5). It is possible that some residual N_i was present after alkaline treatment but sequestered away from the α_2 receptor. PEG may fuse vesicles containing α_2 receptor with those containing the residual N_i. In any case, this effect is very small compared to that seen when exogenous N-protein is included.

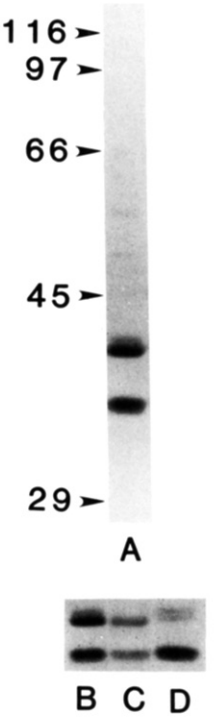

FIGURE 6: Purified guanine nucleotide binding proteins from bovine brain. Purified N-proteins were subjected to polyacrylamide slab gel electrophoresis on 10% Laemmli gels. Lanes B–D, N_o was resolved from a mixed N_o/N_i pool by DEAE chromatography as described under Materials and Methods and subjected to polyacrylamide slab gel electrophoresis. Proteins are stained with Coomassie Blue. Lane A, N_o/N_i mixture from heptylamine–Sepharose column. Lane B contains another preparation of mixed brain pertussis toxin substrates (N_o/N_i). Lane C shows the first peak from the second DEAE column (purified N_o) and lane D, the second peak from the DEAE column used in resolution of N_o from N_o/N_i. Material from lanes A and B and similar preparations containing the M_r 39 000 and 41 000 α subunits were used to prepare N-protein-containing vesicles for use in most of the reconstitution assays. Material from lane C was used in the experiment in Figure 8B.

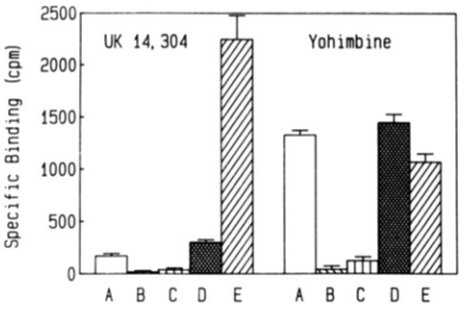

FIGURE 7: Agonist and antagonist binding to pH 11.5 treated membranes after fusion with phospholipid vesicles containing purified guanine nucleotide binding proteins. Equilibrium binding of 1 nM [^{3}H]UK 14 304 (left half of figure) and 10 nM [^{3}H]yohimbine (right half of figure) was measured in triplicate for (A) pH 11.5 membranes, (B) phospholipid vesicles, (C) phospholipid vesicles containing N_o/N_i, (D) phospholipid vesicles fused with pH 11.5 membranes, and (E) vesicles containing N_o/N_i fused with pH 11.5 membranes. All samples were treated with 50% PEG. Those for [^{3}H]UK 14 304 binding contained 0.144 mg of platelet membrane protein and 79 pmol of N_o/N_i. [^{3}H]Yohimbine binding was measured on samples containing half as much material. Nonspecific binding was 70–130 cpm for [^{3}H]UK 14 304 and 100–250 cpm for [^{3}H]yohimbine.

substantial high-affinity [^{3}H]UK 14 304 binding. The fusion of phospholipid vesicles with pH 11.5 treated membranes does not result in a significant enhancement of binding. However, when phospholipid vesicles containing N-protein are fused with pH 11.5 treated membranes, a 24-fold increase in [^{3}H]UK 14 304 binding is observed (range 15–50-fold). In contrast

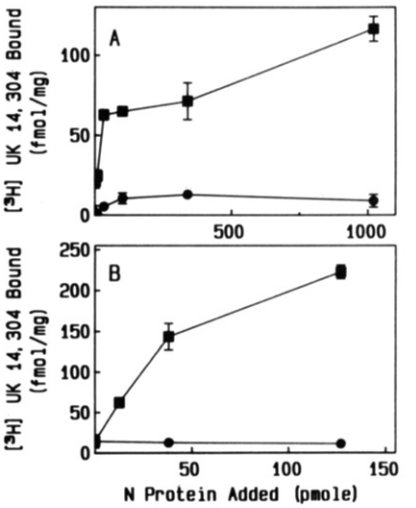

FIGURE 8: Specific binding of [^{3}H]UK 14 304 to pH 11.5 membranes fused with increasing amounts of N-protein-containing vesicles. (A) Equilibrium binding of 1 nM [^{3}H]UK 14 304 to pH 11.5 membranes fused with either vesicles containing N_o/N_i (■) or control phospholipid vesicles containing the equivalent amount of lipid (●) was measured. Fifty percent PEG was included in all samples. Agonist binding to pH 11.5 membranes alone is shown as the point at zero N-protein. Data are presented as fmol/mg of platelet membrane protein, and 100 fmol/mg corresponds to 650 cpm. The number of [^{3}H]yohimbine binding sites (estimated from 10 nM [^{3}H]yohimbine binding) was 441 fmol/mg. This experiment was performed three times with similar results. (B) Equilibrium binding of 1 nM [^{3}H]UK 14 304 was measured for pH 11.5 treated membranes fused to vesicles containing either purified N_o (■) or the equivalent amount of phospholipid alone (●). Fifty percent PEG was included in all samples. Binding of 1 nM [^{3}H]UK 14 304 to pH 11.5 membranes alone is shown as the point at zero N-protein. Data are presented as fmol/mg of protein in the pH 11.5 membranes, and 100 fmol/mg corresponds to 450 cpm. This experiment has been performed four times with three different preparations of N_o with similar results.

to the absolute dependence on PEG for the POB and C6 membrane reconstitutions, reconstitution with purified N_o/N_i in the absence of PEG results in approximately half as much high-affinity [^{3}H]UK 14 304 binding as seen for membranes reconstituted in the presence of PEG (data not shown).

The reconstitution of high-affinity agonist binding to pH 11.5 treated membranes was dependent on the amount of N-protein-containing vesicles added (Figure 8A). [^{3}H]UK 14 304 binding began to plateau at an amount of N-protein corresponding to 100 [^{35}S]GTPγS binding sites per [^{3}H]yohimbine binding site. The binding of [^{3}H]UK 14 304 then increased gradually up to the highest molar ratio tested (3000:1). The presence of phospholipid vesicles alone did not consistently or significantly increase high-affinity agonist binding. Even at the highest concentration of phospholipid vesicle used in the assay, the binding of 1 nM [^{3}H]UK 14 304 to the pH 11.5 membranes fused with phospholipid vesicles alone is less than 5% of that seen with N-protein-containing vesicles.

To determine whether N_o could reconstitute α_2 agonist binding or if the small amount of N_i present in the N_o/N_i pool was responsible for the reconstitution observed, we further purified N_o for reconstitution studies. Figure 6, lane C, shows a Coomassie Blue stained sodium dodecyl sulfate polyacrylamide gel of N_o prepared from the mixed N-proteins by chromatography on DEAE–Sephacel in 0.5% Lubrol PX (Katada et al., 1986). Reconstitution of high-affinity [^{3}H]UK 14 304 binding by this purified N_o is shown in Figure 8B. Of four different preparations of purified N_o obtained by using two different methods, three resulted in substantial reconstitution of [^{3}H]UK 14 304 binding. The average increase in

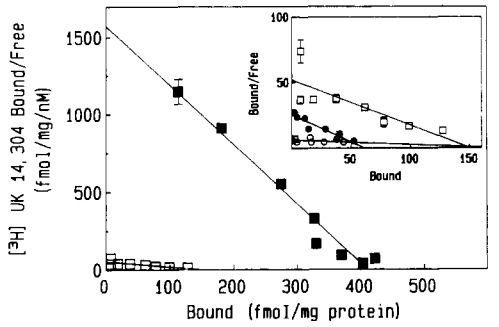

FIGURE 9: Scatchard plot of specific [^{3}H]UK 14 304 binding to reconstituted pH 11.5 treated membranes. Phospholipid vesicles containing N_o/N_i were fused with pH 11.5 membranes as described under Materials and Methods. Agonist binding to the reconstituted membranes in the absence (■) and the presence (□) of 10 μM GppNHp is shown in the main figure. The inset shows a Scatchard plot of the binding of [^{3}H]UK 14 304 to pH 11.5 membranes in the absence (●) or the presence (○) of 10 μM GppNHp. The binding to reconstituted membranes in the presence of GppNHp is included in the inset (□) for comparison. Each sample contained 0.023 mg of platelet membrane protein and 23 pmole of N_o/N_i. The units on the abscissa and the ordinate of the inset graph are the same as on the main graph. The B_{max} for [^{3}H]yohimbine as estimated from 10 nM yohimbine binding is 441 fmol/mg. Error bars depict the mean and SD of duplicate determinations.

binding was 13 ± 4 fold (mean SEM, n = 4).

Figure 9 shows a Scatchard plot of [^{3}H]UK 14 304 binding to pH 11.5 treated membranes and to pH 11.5 treated membranes fused with N-protein-containing vesicles. Treatment of platelet membranes with alkaline buffer results in a selective abolition of high-affinity [^{3}H]UK 14 304 binding sites (Figure 9 inset; see also Figure 1). For these pH 11.5 treated membranes alone, the K_d is 2.0 nM and B_{max} is 52 fmol/mg of protein. In the presence of GppNHp, the B_{max} is 17 fmol/mg and K_d is 5.5 nM. After the pH 11.5 treated membranes were fused with vesicles containing purified guanine nucleotide binding proteins (at a 1000-fold ratio of N-protein to α_2 receptor), there was an increase in both the affinity and the B_{max} for [^{3}H]UK 14 304 (Figure 9). For three such experiments, the K_d for reconstituted membranes was 0.34 nM (range 0.23–0.50), and the average B_{max} was 226 fmol/mg (range 67–405 fmol/mg). The B_{max} values for [^{3}H]UK 14 304 after reconstitution are comparable to the amount of [^{3}H]yohimbine binding, indicating a substantial degree of reconstitution (see summary in Table II).[4] In the presence of 10 μM GppNHp, the high-affinity binding of [^{3}H]UK 14 304 is abolished in the reconstituted membranes; K_d = 2.2 nM (range 1.2–3.2 nM) and B_{max} = 14 fmol/mg (range 9–19 fmol/mg).

DISCUSSION

In this paper we have further characterized the α_2 agonist binding properties of alkaline-treated platelet membranes (Kim & Neubig, 1985) and by use of reconstitution methods established that a guanine nucleotide regulatory protein is necessary for high-affinity α_2 adrenergic agonist binding. This is the first full report of the reconstitution of high-affinity α_2 adrenergic agonist binding by purified guanine nucleotide regulatory proteins. In addition, these results extend the utility of poly(ethylene glycol)-induced membrane–membrane fusion methods to studies of agonist binding using both native membranes and purified components as a source of N-protein.

As previously reported for the partial agonist [^{3}H]p-aminoclonidine (Kim & Neubig, 1985), binding of the full agonists UK 14 304 and epinephrine is markedly reduced after alkaline treatment of human platelet membranes. The decrease in B_{max} for [^{3}H]UK 14 304 binding is most likely due to loss of the high-affinity binding sites detectable under our assay conditions. We cannot accurately quantitate the low-affinity binding sites by the use of [^{3}H]UK 14 304 binding. The hypothesis that there is a conversion of agonist binding from high to low affinity is further supported by the data on epinephrine binding determined indirectly by competition studies. Epinephrine is still able to fully compete for [^{3}H]-yohimbine binding but with a significantly lower affinity. As predicted by our hypothesis that the effect of alkaline treatment is to inactivate the guanine nucleotide regulatory protein, this affinity is similar to that observed for native membranes in the presence of GppNHp. Also, there is no further decrease in epinephrine's affinity in alkaline-treated membranes upon addition of GppNHp. The alkaline-treated membranes still exhibit a significant increase in the IC_{50} for epinephrine in the presence of 100 mM NaCl. The Hill coefficients for the epinephrine competition curves were not significantly different from each other, regardless of the type of membrane or whether GppNHp or NaCl was included in the binding assay. It is not clear why the Hill coefficient for epinephrine binding in the presence of GppNHp is less than unity. Previous studies have shown that addition of GppNHp results in Hill coefficients of one in similar experiments (Michel et al., 1980). Concerning the effects of NaCl and GppNHp on agonist competition for [^{3}H]yohimbine binding to pH 11.5 treated membranes, similar results have been reported for heat-, NEM-, protease-, and digitonin-treated platelet membranes (Limbird & Speck, 1983). Our data are consistent with the conclusion drawn by those authors that the site of sodium's effect is on the α_2 receptor and not on N_i.

The exact biochemical lesion in pH 11.5 treated membranes cannot yet be definitively specified. As previously discussed (Kim & Neubig, 1985), the loss of pertussis substrate activity could result from effects on either the α or β subunits of N_i. The N-protein is not removed from the plasma membrane intact because neutralized supernatants from the pH 11.5 treatment show no pertussis toxin substrate activity.[2] Attempts to label N_i in the intact platelet membranes with pertussis toxin and [^{32}P]NAD followed by extraction of membranes with pH 11.5 buffer have yielded variable results which are difficult to interpret because of the very low efficiency of labeling (1% or less).[2] The results presented in this paper do definitively show that the loss of high-affinity α_2 agonist binding is due

Table II: Parameters of [^{3}H]UK 14 304 Binding to Reconstituted Platelet Membranes[a]

expt no.	B_{max} (fmol/mg) [^{3}H]UK 14 304 binding[a] control	B_{max} (fmol/mg) [^{3}H]UK 14 304 binding[a] reconstituted	B_{max} (fmol/mg) [^{3}H]yohimbine binding, reconstituted	B_{max}(UK 14 304)/ B_{max}(Yoh)
1	59[b] (2.3)	405 (0.23)	479[d]	0.85
2	37[c] (1.4)	211 (0.50)	219[d]	0.96
3	59[c] (2.0)	67 (0.32)	128[e]	0.52

[a] Binding of [^{3}H]UK 14 304 to control pH 11.5 treated membranes and to the same membranes after reconstitution with purified N-proteins (1000:1 molar excess) was measured for ligand concentrations from 0.1 to 2 nM. Scatchard transformations of specific binding as shown in Figure 9 were linear, and the B_{max} and K_d (nM) (in parentheses) were determined by analysis using SCAFIT. [^{3}H]Yohimbine binding to the reconstituted membranes was also determined to estimate the total recovery of α_2 receptor sites following reconstitution. [b] pH 11.5 treated membranes alone. [c] pH 11.5 treated membranes reconstituted with phospholipid vesicles and PEG. [d] Estimated from [^{3}H]yohimbine binding at 10 nM by assuming a K_d of 5 nM. [e] Determined from Scatchard analysis.

[4] Although experiment 3 showed no increase in B_{max} for [^{3}H]UK 14 304, the affinity increased approximately 6-fold as indicated by the decrease in the K_d.

to an effect on N_i rather than the α_2 receptor since nearly complete restoration of high-affinity [^{3}H]UK 14304 binding is produced by reconstitution with pure N-protein.

The pertussis toxin substrate in platelet membranes appears to consist solely of N_i as only a 41 000-dalton protein is labeled in platelet extracts (Neubig et al., 1985). The fact that N_i is the only pertussis substrate present in platelets and that our reconstitution is pertussis sensitive strongly suggest that N_i is the protein responsible for high-affinity α_2 adrenergic agonist binding in platelet membranes. Recent evidence indicates that rat C6 glioma cells contain both N_o and N_i, as well as N_s (Itoh et al., 1986). Because of the small amount of N_i present in our purified preparations of N-proteins, we were not able to obtain sufficient amounts of pure N_i to directly compare reconstitution by N_i and N_o. We have shown that there is not a significant difference between reconstitution by the N_o/N_i preparation and by the highly purified N_o. Because of the ability of many N-proteins to interact with receptors that may not be their primary activator in situ, reconstitution, such as reported here, can only elucidate the potential for and characteristics of interactions of receptors with N-proteins. Specifically, the ability of purified N_o to effectively reconstitute α_2 agonist binding clearly does not prove a role for this protein in platelets. As has been shown previously in studies of receptor-stimulated GTPase activity (Asano & Ogasawara, 1986; Kurose et al., 1986; Cerione et al., 1986), N_o does appear to interact well with receptors that inhibit adenylate cyclase.

The stoichiometry of N-proteins and α_2 receptors in our reconstitution studies is of interest. With purified N-proteins, a 100–300-fold molar excess of N-protein over receptor appears to be sufficient for nearly maximal reconstitution. This ratio is only marginally greater than the 70-fold excess of N-proteins over α_2 receptors found in the native platelet membrane (Neubig et al., 1985). Florio and Sternweis (1985) reported that maximal reconstitution of muscarinic agonist binding required a 1000-fold molar excess of N-proteins over muscarinic receptors. They attribute this requirement to the low efficiency of reconstitution and to the rather larger excess of N-protein preexisting in native brain membranes.

There have been other reports concerning the reconstitution of high-affinity agonist binding. The GTP sensitivity of agonist competition for muscarinic receptors was restored in a resolved receptor preparation by pure N-proteins (Florio & Sternweis, 1985). In that report, both N_o and N_i reconstituted agonist binding to muscarinic receptors. High-affinity muscarinic agonist binding has also been induced in membranes from embryonic chick hearts, which normally bind agonist with low affinity, by mixing the membranes with detergent extracts of adult chick hearts in which the muscarinic receptors had been inactivated (Galper, 1984). Addition of the nonreceptor component, presumably N_o or N_i, in the extract was reponsible for an enhancement of high-affinity muscarinic binding sites and a restoration of GppNHp sensitivity. Reconstitution of high-affinity agonist binding to $GABA_B$ receptors in NEM-treated and pertussis toxin treated brain membranes with purified GTP binding proteins has also been reported (Asano et al., 1985; Asano & Ogasawara, 1986). More recently, α_2 receptor stimulated GTPase activity was reconstituted in phospholipid vesicles containing partially purified α_2 receptor and N_o or N_i (Cerione et al., 1986). No data concerning the affinity of α_2 agonist binding were presented in that report. Also, there has been a preliminary report of reconstitution of clonidine binding to NG108-15 membranes (Ui et al., 1984).

The reconstitution method reported here is a novel approach to the technique of membrane reconstitution as opposed to solubilized reconstitution systems used by many other investigators. We have adapted this method, previously used to reconstitute β adrenergic receptor stimulated adenylate cyclase (Kassis et al., 1984) to α_2 adrenergic ligand binding. Because the platelet membrane treated with alkaline buffer is used as a source of "N_i minus" membrane, the receptor is never removed from its lipid environment. This may account for the excellent recovery of receptor activity and the high efficiency of reconstitution. With this method it should be possible to more clearly identify factors that regulate α_2 receptor–N-protein interactions. Two questions of interest are the affinity of α_2 receptor and N-proteins for each other in the presence and absence of agonist ligands and the possible existence of receptor heterogeneity to account for the low-affinity agonist binding (Neubig et al., 1985). The results presented here show that purified guanine nucleotide binding proteins from bovine brain are sufficient to reconstitute high-affinity α_2 adrenergic agonist binding. These procedures developed for reconstitution of high-affinity agonist binding should prove useful for further investigation of receptor–N-protein interactions.

ACKNOWLEDGMENTS

We thank Dr. Rita Huff from Upjohn Co., Kalamazoo, MI, for providing a preparation of purified N_o protein, Dr. Robert Simpson, Department of Pharmacology, for C6 glioma cells, Dr. Larry Winderry, Michigan Department of Public Health, for pertussis toxin, and Sheila Krawczyk for assistance with the epinephrine competition experiments. We also thank Dr. M. Gnegy for critically reviewing the manuscript and Linda Busha for preparing it.

Registry No. UK 14304, 59803-98-4; epinephrine, 51-43-4; poly(ethylene glycol), 25322-68-3.

REFERENCES

Asano, T., & Ogasawara, N. (1986) *Mol. Pharmacol. 29*, 244–249.

Asano, T., Katada, T., Gilman, A. G. & Ross, E. M. (1984) *J. Biol. Chem. 259*, 9351–9354.

Asano, T., Ui, M., & Ogasawara, N. (1985) *J. Biol. Chem. 260*, 12653–12658.

Blume, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1713–1717.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brandt, D. R., & Ross, E. M. (1986) *J. Biol. Chem. 261*, 1656–1664.

Bruns, R. F., Lawson-Wendling, K., & Pugsley, T. A. (1983) *Anal. Biochem. 132*, 74–81.

Cerione, R. A., Strulovici, B., Benovic, J. L., Lefkowitz, R. J., & Caron, M. G. (1983) *Nature (London) 306*, 562–566.

Cerione, R. A., Regan, J. W., Nakata, H., Codina, J., Benovic, J. L., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., Lefkowitz, R. J., & Caron, M. G. (1986) *J. Biol. Chem. 261*, 3901–3909.

Citri, Y., & Schramm, M. (1983) *Nature (London) 287*, 297–300.

Florio, V. A., & Sternweis, P. C. (1985) *J. Biol. Chem. 260*, 3477–3483.

Galper, J. B. (1984) *Circulation 70*, 69.

Garcia-Sainz, J. A., Boyer, J. L., Michel, T., Sawyer, D., Dohlman, H., & Lefkowitz, R. J. (1984) *FEBS Lett. 172*, 95–98.

Hoffman, B. B., Michel, T., Brenneman, T. B., & Lefkowitz, R. J. (1982) *Endocrinology 110*, 926–932.

Huff, R. M., Axton, J. M., & Neer, E. J. (1985) *J. Biol. Chem. 260*, 10864–10871.

Itoh, H., Kozasa, T., Shigekazu, N., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K., & Kaziro, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3776–3780.
Jakobs, K. H., Saur, W., & Schultz, G. (1979) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 310*, 113–119.
Kassis, S., Henneberry, R. C., & Fishman, P. H. (1984) *J. Biol. Chem. 259*, 4910–4916.
Katada, T., & Ui, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3129–3133.
Katada, T., Oinuma, M., & Ui, M. (1986) *J. Biol. Chem. 261*, 8182–8191.
Kim, M. H., & Neubig, R. R. (1985) *FEBS Lett. 192*, 321–325.
Kim, M. H., & Neubig, R. R. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 928.
Kunos, G., Kan, W. H., Greguski, R., & Venter, J. C. (1983) *J. Biol. Chem. 258*, 326–332.
Kurose, H., Katada, T., Amano, T., & Ui, M. (1983) *J. Biol. Chem. 258*, 4870–4875.
Kurose, H., Katada, T., Haga, T., Haga, K., Ichigama, A., & Ui, M. (1986) *J. Biol. Chem.* 261, 6423–6428.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Limbird, L. E. (1981) *Biochem. J. 195*, 1–13.
Limbird, L. E., & Speck, J. L. (1983) *J. Cyclic Nucleotide Protein Phosphorylation Res. 9*, 191–201.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maguire, M. E., VanArsdale, P. M., & Gilman, A. G. (1976) *Mol. Pharmacol. 12*, 335–339.
May, D. C., Ross, E. M., Gilman, A. G., & Smigel, M. D. (1985) *J. Biol. Chem. 260*, 15829–15833.
Michel, T., Hoffman, B. B., & Lefkowitz, R. J. (1980) *Nature (London) 288*, 709–711.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Neer, E. J., Lok, J. M., & Wolf, L. G. (1984) *J. Biol. Chem. 259*, 14222–14229.
Neubig, R. R., & Szamraj, O. (1986) *Biochem. Biophys. Acta 854*, 67–76.
Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
Neubig, R. R., Gantzos, R. D., & Brasier, R. S. (1985) *Mol. Pharmacol. 28*, 475–486.
Nomura, Y., Kitamura, Y., & Segawa, T. (1985) *J. Neurochem. 44*, 364–369.
Ross, E. M., & Schatz, G. (1978) *Methods Enzymol. 53*, 222–229.
Ross, E. M., & Gilman, A. G. (1980) *Annu. Rev. Biochem. 49*, 533–564.
Ross, E. M., Howlett, A. C., Ferguson, K. M., & Gilman, A. G. (1978) *J. Biol. Chem. 253*, 6401–6412.
Schramm, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1174–1178.
Shaltiel, S. (1974) *Methods Enzymol. 34*, 126–140.
Sternweis, P. C., & Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806–13813.
Ui, M., Katada, T., Murayama, T., & Kurose, H. (1984) in *Endocrinology* (Labrie, F., & Proulx, L., Eds.) pp 157–160, Elsevier, New York.

Radical Intermediates in the Oxidation of Octaethylheme to Octaethylverdoheme†

Noriyoshi Masuoka‡ and Harvey A. Itano*
Department of Pathology, University of California, San Diego, La Jolla, California 92093
Received July 23, 1986; Revised Manuscript Received January 8, 1987

ABSTRACT: Iron(III) oxyoctaethylporphyrin was isolated and purified as a dimer. The addition of tosylmethyl isocyanide to a solution of the dimer produced a monomer species, which was isolated and identified as bis(tosylmethyl isocyanide)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. The product of dissociation of the dimer by imidazole was bis(imidazole)iron(III) 5-oxyoctaethylporphyrin. The spectral properties of the product of dissociation of the dimer by pyridine and published data on bis(pyridine)oxymesoheme and bis(pyridine)oxyprotoheme were consistent with its identification as bis(pyridine)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. When this product was exposed to oxygen, a weak radical signal appeared in its electron spin resonance spectrum, which was attributed to the displacement of one of its pyridine ligands by O_2 to form (pyridine)(dioxygen)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. The pyridine oxygen radical converted spontaneously to octaethylverdohemochrome, which was purified and identified as bis(tosylmethyl isocyanide)iron(II) octaethylverdohemochrome hydroxide. The yield of verdohemochrome from iron oxyporphyrin was increased by the addition of phenylhydrazine or ascorbate. A scheme for the oxidation of iron(III) oxyporphyrin to iron(II) verdoheme by O_2 that proposes a mechanism for the expulsion of CO and the replacement of a methene bridge of the porphyrin ring by an oxa bridge is presented.

An intermediate product in the oxidation of pyridine protohemochrome to pyridine protoverdohemochrome by O_2 in the presence of ascorbic acid was characterized as an iron(III) hematin of an oxyporphyrin (Lemberg et al., 1937, 1938). Exposure of oxymesohemin IXβ dimethyl ester to O_2 resulted in mesoverdohemin dimethyl ester, an iron(III) compound (Jackson et al., 1968). Sano et al. (1981) reported that the electron spin resonance (ESR) spectrum of a mixture of iron oxymesoporphyrin isomers in alkaline pyridine solution at 77 K was characteristic of a high-spin iron(III) compound. When,

† This work was supported by National Institutes of Health Grant AM14982. FAB mass spectral determinations were performed at the Midwest Center for Mass Spectrometry, an NSF Regional Instrumentation Facility (Grant CHE-8211164).
‡ Present address: Department of Biochemistry, Okayama University Medical School, 2-5-1 Shikatacho, Okayama 700, Japan.

0006-2960/87/0426-3672$01.50/0