The Mammalian β_2-Adrenergic Receptor: Reconstitution of Functional Interactions between Pure Receptor and Pure Stimulatory Nucleotide Binding Protein of the Adenylate Cyclase System†

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ABSTRACT: Pure β-adrenergic receptors (β-AR) isolated from guinea pig lung and pure guanine nucleotide binding regulatory protein (N_s) of adenylate cyclase isolated from human erythrocytes have been inserted into phospholipid vesicles, resulting in the functional coupling of these two components. The reconstitution of receptor and N_s interactions results in the establishment of a guanine nucleotide sensitive state of the receptor that binds agonists with high affinity. Competition curves of isoproterenol for labeled antagonist binding to vesicles containing both β-AR and N_s are biphasic and reveal two affinity states, one of high ($\sim$2 nM) and the other of low affinity ($\sim$300 nM). In the presence of guanine nucleotides, the competition curves become monophasic and are shifted to a single low-affinity state for the agonist similar to the situation observed in membrane preparations. In addition, the interactions of the receptor and N_s lead to the induction of a GTPase activity in N_s. The GTPase activity can be stimulated by β-adrenergic agonists such as isoproterenol (2–5-fold) and is completely blocked by antagonists such as alprenolol in a stereoselective manner. The established hormone responsive activity retains the β_2-adrenergic specificity conferred by the pure receptor, and similar extents of stimulation (up to 4-fold) are observed with pure receptor from frog erythrocytes, indicating a similar efficiency of coupling between receptors from different species and N_s. These experiments demonstrate that no components, other than the pure β-AR and N_s, are necessary to elicit these activities and now open the way for attempting to elucidate the molecular mechanisms by which transduction of receptor occupancy into N_s activation leads to the stimulation of the rate of cAMP formation.

The mechanism of stimulation of adenylate cyclase by hormones has been the subject of intense investigative efforts for the past 10 years. Probably the most thoroughly studied system is the β-adrenergic receptor–adenylate cyclase complex, which consists of at least three distinct components, these being (1) the β-adrenergic receptor (β-AR), which contains the binding site for catecholamine agonists, (2) the nucleotide regulatory protein (N_s), which binds guanine nucleotides and mediates regulation of adenylate cyclase activity by hormone–receptor complexes, and (3) the effector adenylate cyclase itself. To date, two of these three components have been purified to apparent homogeneity. The β-adrenergic receptor binding proteins have been purified from frog erythrocytes (Shorr et al., 1982a) and turkey erythrocytes (Shorr et al., 1982b), as well as from the lungs of several mammalian species (Benovic et al., 1984; Homcy et al., 1982). The purified receptors from amphibian and mammalian species are single polypeptides that both bind ligands with appropriate specificity and are capable of conveying adrenergic responsiveness to cells that contain N_s and adenylate cyclase but no endogenous receptors (Cerione et al., 1983a). Pure preparations of N_s from rabbit liver (Northup et al., 1980; Sternweis et al., 1981) and turkey (Hanski et al., 1981) and human erythrocytes (Codina et al., 1984; Hanski & Gilman, 1982) are oligomeric proteins that upon reconstitution into cyc^- mutants of S49 lymphoma cells, which lack a functional stimulatory guanine nucleotide binding regulatory component, restore the hormone stimulation of adenylate cyclase activity. At the present time, the catalytic moiety, adenylate cyclase, has been resolved from N_s (Ross, 1981; Strittmatter & Neer, 1980) but has not been purified to a significant degree.

On the basis of studies in intact membranes (De Lean et al., 1980), it has been suggested that the interactions of the receptor with N_s result in an increase in the affinity of the receptor for agonists. The binding of guanine nucleotides to the ternary agonist–receptor–N_s complex appears to result in the dissociation of a modified N_s–GTP (N_s^*–GTP) complex and a return of the receptor to the lower affinity state for agonists. The actual stimulation of the catalytic moiety of adenylate cyclase is thought to reflect a direct interaction between the enzyme and N_s^*–GTP with the activation cycle being terminated by the hydrolysis of GTP to GDP and the return of N_s to its initial conformation (Pfeuffer, 1979; Cassel & Selinger, 1976). Since pure N_s appeared to lack GTPase activity (Northup et al., 1982), it has been commonly assumed that the GTPase activity is elicited by the interaction of N_s^*–GTP with the catalytic component of adenylate cyclase. However, recently, it has been reported that the interaction of partially purified turkey erythrocyte β-AR and purified rabbit liver N_s in phospholipid vesicles results in a hormone-responsive GTPase activity (Brandt et al., 1983).

While the above-mentioned studies have provided clues to the mechanisms of hormone activation of adenylate cyclase, the functional insertion of the individual pure components of a receptor-coupled adenylate cyclase system into a defined lipid milieu is prerequisite to understanding the molecular mechanism of such a system. Especially relevant are the mechanistic implications of the direct interactions of the pure β-AR and pure N_s proteins. Are these two proteins sufficient to induce high affinity agonist binding, and GTPase activity, or are other unidentified components necessary to mimic binding and catalytic effects observed in membranes and crude receptor–N_s systems? We describe here the first successful reconstitution of functional interactions between pure β-AR

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and N_s proteins and demonstrate that the interactions of these two proteins can induce both a high-affinity agonist binding state in the receptor and a GTPase activity. These studies also provide further insight into the nature of hormone regulation of β-AR and N_s interactions.

Materials and Methods

Materials. Soybean phosphatidylcholine (PC), bovine serum albumin (fraction V), poly(ethylene glycol), and (−)-isoproterenol were obtained from Sigma Chemical Co. (St. Louis, MO). Octyl β-D-glucopyranoside (octyl glucoside) was obtained from Calbiochem-Behring (San Diego, CA), alprenolol was a gift from Hassle Pharmaceuticals (Molndal, Sweden), and digitonin was from Gallard Schlesinger (Carle Place, NY). [^{125}I]Iodocyanopindolol ([^{125}I]CYP) and [^{3}H]-dihydroalprenolol were from New England Nuclear Corp. (Boston, MA). Extracti-gel D was from Pierce Chemical Co. (Rockford, IL). Southern grass frogs (*Rana pipiens*) were obtained from Nasco (Fort Atkinson, WI) and guinea pig lungs were from Pel-Freeze Biologicals (Rogers, AR).

Preparation of β-AR. Purified frog erythrocyte membranes and guinea pig lung membranes were prepared as described (Caron & Lefkowitz, 1976; Benovic et al., 1984). The β-adrenergic receptors were solubilized from purified erythrocyte and lung plasma membranes with digitonin and purified ~500–2000-fold by affinity chromatography on Sepharose–alprenolol gel as described previously (Shorr et al., 1982a; Benovic et al., 1984). Partially purified receptor preparations were concentrated in an Amicon concentration cell with a YM-30 membrane to ~50 pmol/mL as assayed by [^{3}H]dihydroalprenolol binding (Caron & Lefkowitz, 1976). These preparations were then purified to apparent homogeneity by high-pressure liquid chromatography (HPLC) chromatography (two successive passes for preparations from frog erythrocytes [as described by Shorr et al. (1982)] or one pass for preparations from guinea pig lung [as described by Benovic et al. (1984)]). Final concentrations of the pure β-adrenergic receptor fractions ranged from 5 to 25 pmol/mL while specific activities ranged from 12 000 to 18 000 pmol/mg of protein (Shorr et al., 1982a; Benovic et al., 1984). Iodinated frog erythrocyte receptor was prepared by pooling the peak of receptor binding activity after an initial HPLC chromatography, iodinating the pooled material by the chloramine T method (Shorr et al., 1982a), desalting on Sephadex G-50 columns to remove unreacted Na^{125}I, and finally purifying the iodinated receptor by a second HPLC chromatography as described above. A similar procedure was performed with guinea pig lung receptor except that the receptor binding activity, after chromatography on the Sepharose–alprenolol gel, was pooled, concentrated, iodinated, and then subjected to an HPLC chromatography.

Preparation of the Stimulatory Nucleotide Regulatory Protein (N_s). N_s was purified in quantities from 500 to 1000 μg, starting from ca. 50 g of washed erythrocyte membrane protein, by subjecting a cholate extract to a modification (Codina et al., 1984) of the method of Sternweis et al. (1981) in which NaF/Mg^{2+} buffers were avoided and replaced with 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM β-mercaptoethanol, and 30% ethylene glycol in 20 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (NaHepes), pH 8.0 (buffer A). As reported in detail elsewhere (Hildebrandt et al., 1984), the modified procedure, although longer and more tedious, yields a N_s protein that is heterotrimeric instead of heterodimeric. The preparations used in this paper were from step 8A (Codina et al., 1984) and were stored at −70 °C in buffer A containing in addition 0.1—5% Lubrol PX and 150 mM NaCl.

Insertion of β-AR and N_s into Phospholipid Vesicles. The insertion of pure β-adrenergic receptor (β-AR) and pure nucleotide regulatory protein (N_s) into phospholipid vesicles was performed as follows: β-AR (0.1–4 pmol in 300 μL of 100 mM tris(hydroxymethyl)aminomethane (Tris)–SO_4 and 0.1% digitonin, pH 7.4) and N_s (4–60 pmol in 20–40 μL of 10 mM Hepes, 1 mM EDTA, 20 mM β-mercaptoethanol, 30% ethylene glycol, 150 mM NaCl, 150 μg/mL bovine serum albumin, and 0.1–5% Lubrol PX) were incubated with BSA (2 mg/mL), sonicated soybean PC (1.5 mM), octyl glucoside (0.85%), alprenolol (0.4–4 μM), 20–30 mM NaCl, and 2–3 mM Tris-HCl (pH 7.4) in a final volume of 0.5 mL for 30 min on ice. In all cases, the pH of the Tris buffers was adjusted at room temperature. Prior to use in reconstitution experiments, the PC was sonicated 5–15 min in 100 mM NaCl and 10 mM Tris-HCl, pH 7.4, with a bath-type sonicator (Laboratory Supply, Hicksville, NY). The detergent concentrations were then reduced by chromatography on an Extracti-gel column (1 mL of gel), which was pretreated with 100 mM NaCl and 10 mM Tris-HCl (pH 7.4) buffer containing 2 mg/mL bovine serum albumin and then equilibrated with the same buffer without albumin. Elution of the protein–lipid vesicle fraction was performed in 100 mM NaCl and 10 mM Tris-HCl (pH 7.4). The eluates (2 mL) from the Extracti-gel columns were incubated with poly(ethylene glycol) (6–8000) (final concentration ~12% w/v) for 10 min at room temperature, then diluted 5–10-fold with 100 mM NaCl and 10 mM Tris-HCl (pH 7.4), and centrifuged at 250000*g*–300000*g* for 1.5–2.5 h at 4 °C. The resultant protein–lipid pellets were resuspended in 75 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, and 1.5 mM EDTA in a final volume of 0.4–0.9 mL and assayed for receptor binding, by elution through Sephadex G-50 columns as described earlier (Cerione et al., 1983b) and GTP hydrolytic activity as described below. Concentrations of β-AR throughout the text are expressed in terms of [^{125}I]iodocyanopindolol binding activity while concentrations of N_s are expressed in terms of total protein as determined by the method of Lowry et al. (1951). The recovery of N_s after reconstitution was assessed by measuring the binding of [^{35}S]GTPγS in the presence of excess $MgCl_2$ (50 mM) (Northup et al., 1982). Concentrations of lipids are expressed in terms of lipid phosphate and were determined by the method of Ames & Dubin (1960) with dimyristoylphosphatidylcholine (Sigma) as a standard.

(−)-Isoproterenol Competition for [^{125}I]Iodocyanopindolol Binding to β-AR Inserted into Phospholipid Vesicles. Solutions of phospholipid vesicles (1–1.5 mL) containing β-AR and N_s were assayed for binding with [^{125}I]iodocyanopindolol (10–50 pM) in the presence and absence of isoproterenol. All binding assays were performed in 75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, and 1.5 mM EDTA for 30 min at 25 °C. Free and bound antagonist were separated by elution through Sephadex G-50 columns preequilibrated in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% digitonin or in 75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA, and 0.1% digitonin. In some cases, binding also was performed in the presence of 10^{-4} M guanylyl 5′-(imidophosphate). Competition binding data were analyzed by a nonlinear least-squares curve-fitting procedure based on a model for complex ligand–receptor systems according to the law of mass action (Kent et al., 1980; De Lean et al., 1980).

GTP Hydrolytic Activity (GTPase). GTPase activity was assayed by incubating 15–20 μL of vesicles (alone) or vesicles

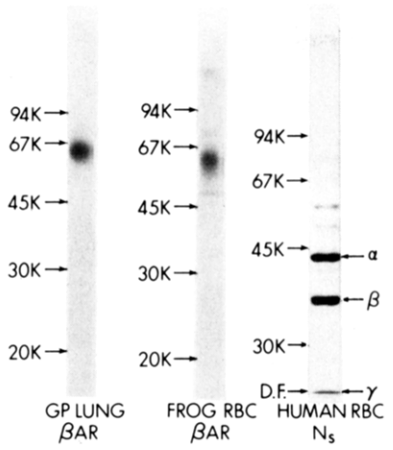


FIGURE 1: SDS–PAGE patterns of β-AR and N_s preparations. The left lane shows a SDS–PAGE pattern of the iodinated purified guinea pig (GP) lung receptor used in these experiments. The middle lane shows a SDS–PAGE pattern of the iodinated purified frog erythrocyte (RBC) receptor preparation. Iodination of these receptor preparations was performed as described under Materials and Methods. Aliquots of the receptor samples were run on 8% SDS–PAGE with the dried gel being exposed for 24 h to Kodak XAR-5 film and developed manually. The arrows to the left of these two lanes show the relative mobility of known molecular weight standards (phosphorylase *b*, M_r 94000; albumin, M_r 67000; ovalbumin, M_r 45000; carbonic anhydrase, M_r 30000; soybean trypsin inhibitor, M_r 20000). The right lane shows a SDS–PAGE pattern of the Coomassie blue stained human erythrocyte (RBC) N_s preparation for samples run on 10% SDS–PAGE.

containing β-AR, N_s, or β-AR + N_s in a total volume of 0.1 mL (30 °C) containing 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, 0.2% BSA, 0.5 mM ascorbic acid, 1 mM adenylyl 5′-(imidodiphosphate), and 100–200 nM [γ-^{32}P]GTP (~40000 cpm/pmol). GTPase was stopped by the addition of 10 μL of cold 50% trichloroacetic acid (TCA) with immediate chilling on ice. The mixture was centrifuged for 20–30 min at 2500 rpm and then 90 μL of the supernatant was removed and assayed for ^{32}P-labeled inorganic phosphate (P_i). The determination of [^{32}P]P_i was performed by adding the supernatant from the TCA precipitation to 4 mL of 1.25% (w/v) ammonium molybdate in 1.2 M HCl containing 50 μM potassium phosphate (Avron, 1960). A total of 5 mL of 2-methyl-2-propanol–benzene (1:1) was immediately added to this solution; the mixture was vortexed for 20 s, and 2 mL was counted from the top layer (total volume 5 mL).

Results

Purification of β-AR and N_s and Insertion of These Components into Phospholipid Vesicles. The β-adrenergic receptors from frog erythrocyte and guinea pig lung plasma membranes were purified by a combination of an affinity chromatography step using Sepharose–alprenolol gel coupled with size-exclusion high-performance liquid chromatography (Shorr et al., 1982a; Benovic et al., 1984). The N_s was purified as indicated under Materials and Methods from cholate extracts of human erythrocyte membranes by a sequence of chromatographic steps that avoided the use of activating ligands as stabilizing agents (Codina et al., 1984). Figure 1 shows an autoradiogram of a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of each of the purified iodinated receptor preparations used in the experiments described below as well as a Coomassie blue staining pattern of a SDS–PAGE of a N_s preparation. The guinea pig lung β-AR was purified to apparent homogeneity (M_r 64000) while the frog erythrocyte β-AR preparation used in these studies was ~90% pure (M_r 58000) as determined by densitometric scanning of autoradiograms of SDS–PAGE patterns of ^{125}I-labeled β-AR. The N_s preparations used in these experiments (as shown in Figure 1, right lane) contained predominantly two major Coomassie blue stainable bands at M_r 42000 (α subunit) and 35000 (β subunit) and two minor contaminants (<2% of the total protein by densitometric scanning) that varied from preparation to preparation and that in the particular preparation shown in Figure 1 were of M_r 57000 and 50000. As will be reported in detail elsewhere (Codina et al., 1984; Hildebrandt et al., 1984), these preparations also contain a peptide of M_r ca. 5000 (γ subunit), which under the particular conditions of electrophoresis used in Figure 1 runs with the dye front (DF). The presence of a third smaller subunit suggests a heterotrimeric structure for N_s similar to the structure for the nucleotide regulatory protein transducin of the rhodopsin system (O'Brian, 1982). These preparations of N_s are free of endogenous guanine nucleotide and, on treatment with Gpp(NH)p and 50–100 mM Mg^{2+} at 32 °C, undergo subunit dissociation as assessed by a change in the hydrodynamic behavior of the M_r 42000 subunit.

Methods for the insertion of solubilized and purified N_s into cell membranes or phospholipid vesicles have been well described and basically can be performed by a simple dilution of the N_s–detergent solutions into the membrane milieu (Pedersen & Ross, 1982; Ross et al., 1978). Conversely, methods for the insertion of purified β-adrenergic receptors into lipid vesicles have only recently become available (Cerione et al., 1983a,b; Brandt et al., 1983). Since the goal of this study was to examine the interactions of pure β-AR and N_s, it was essential that the levels of detergents present in these preparations following solubilization and purification of both components be effectively reduced; otherwise, the coupling between β-AR and N_s would be inhibited. Therefore, a coinsertion protocol was used in these studies. Preparations of pure guinea pig lung β-AR and human erythrocyte N_s could be coinserted into phosphatidylcholine vesicles by incubating these components together with octyl glucoside and then reducing the detergent concentration by chromatography on Extracti-gel, a detergent-adsorbing matrix. The efficiency of insertion of the β-AR preparations ranged from 10 to 20%, as assessed by [^{125}I]iodocyanopindolol binding activity, whereas the efficiency of insertion of N_s ranged from 40 to 85%, as estimated by [^{35}S]GTPγS binding activity with essentially the procedure as described by Northup et al. (1982).

Agonist Binding to Phospholipid Vesicles Containing β-AR and N_s. Binding studies performed on plasma membrane preparations from frog erythrocytes, turkey erythrocytes, and various mammalian species yield agonist competition curves, by use of antagonist radioligands, that are shallow (De Lean et al., 1980; Dickinson et al., 1981), indicating two classes of binding sites. These curves are shifted to a lower agonist potency in the presence of guanine nucleotides and become steeper, reflecting a transition toward a single class of agonist binding sites. Similar results are obtained when agonist competition of antagonist binding is examined in phospholipid vesicles containing pure guinea pig lung β-AR and human erythrocyte N_s. As shown in Figure 2, the binding profile for isoproterenol competition of [^{125}I]CYP binding is shallow and biphasic. In the presence of guanine nucleotides, the curve is steepened and shifted to the right, reflecting a reduction in the proportion of receptors in an agonist high-affinity state with a concomitant increase in the fraction of receptors displaying low-affinity agonist binding. For the data shown in Figure 2, the percent of high- (K_d ~ 2 nM) and low-affinity (K_d ~ 300 nM) binding sites in vesicles containing β-AR and

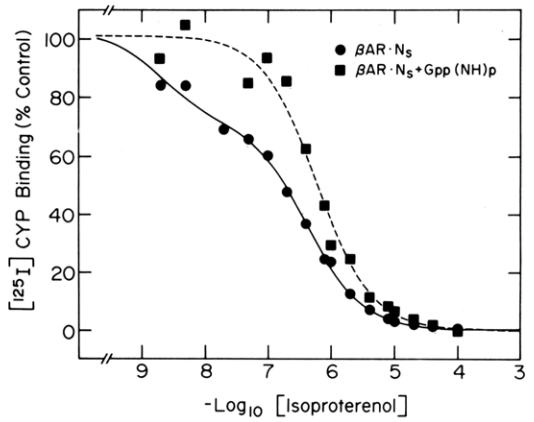


FIGURE 2: (−)-Isoproterenol competition for [^{125}I]iodocyanopindolol ([^{125}I]CYP) binding to phospholipid vesicles containing β-AR and N_s. Guinea pig β-AR (6.7 pmol) and human erythrocyte N_s (6.0 pmol) were incubated with phosphatidylcholine in reconstitution incubations as described (Materials and Methods), and then the isolated phospholipid vesicles were assayed in the absence (●) and presence (■) of guanylyl 5′-(imidophosphate) for binding to the β-AR (Materials and Methods). Each point represents the mean of duplicate determinations, and the data shown are representative of two experiments.

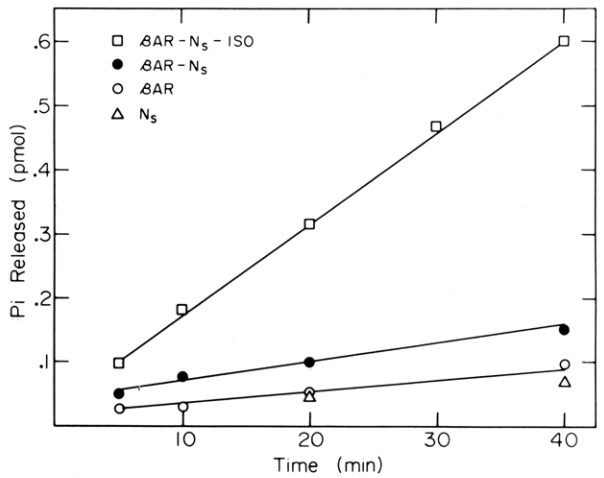


FIGURE 3: Time course of GTPase activity for phospholipid vesicles containing β-AR and N_s. Guinea pig lung β-AR (3.6 pmol) and human erythrocyte N_s (10.1 pmol) were added to reconstitution incubations either together or separately. GTPase assays were performed on vesicles in 75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, and 1.5 mM EDTA containing guinea pig β-AR alone (○), N_s alone (△), and guinea pig β-AR and N_s in the presence (□) and absence (●) of 10^{-6} M (−)-isoproterenol at 30 °C as described (Materials and Methods). The data shown are representative of two to three experiments, and each point is the average from triplicate determinations.

N_s is 30 and 70%, respectively, compared to essentially 100% low-affinity agonist binding sites in the presence of guanine nucleotides.

When receptor is inserted into vesicles in the absence of N_s, a steep competition curve is obtained, consistent with only a single class of low-affinity agonist binding sites (data not shown). This situation is similar to results obtained when binding is performed on solubilized and purified receptor preparations where the receptor is uncoupled from the nucleotide binding protein (Shorr et al., 1982a,b; Benovic et al., 1984). The data presented here (Figure 2) are thus consistent with an agonist-promoted association of the pure β-AR and N_s components in the phospholipid vesicles to form a high-affinity guanine nucleotide sensitive ternary complex (HRN) (De Lean et al., 1980).

GTPase Activity in Phospholipid Vesicles Containing β-AR and N_s. Figure 3 shows the GTPase activity for phospholipid

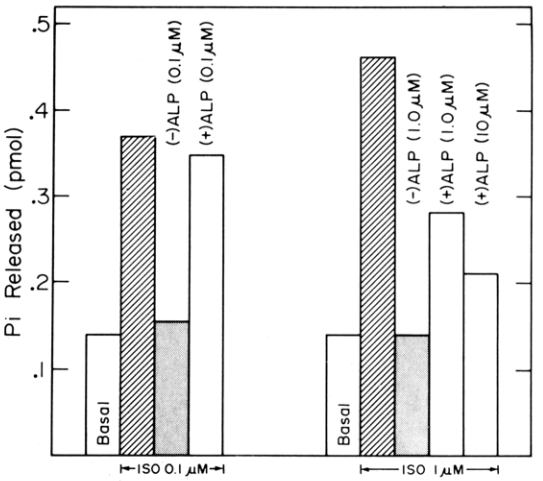


FIGURE 4: Effects of β-adrenergic agonists and antagonists on the GTPase activity in phospholipid vesicles containing β-AR and N_s. Guinea pig lung β-AR (4.8 pmol) and human erythrocyte N_s (10.1 pmol) were added to reconstitution incubations, and GTPase assays were performed on the isolated vesicles for 40 min at 30 °C. The concentrations of (−)-isoproterenol, (−)-alprenolol, and (+)-alprenolol included in the different assay incubations are as indicated. Basal represents the activity for reconstituted β-AR and N_s incubated in the absence of added drugs. Each bar graph represents the average of triplicate determinations.

vesicles containing N_s alone, guinea pig lung β-AR alone, and β-AR and N_s in the presence and absence of the β-agonist isoproterenol. Upon the insertion of β-AR and N_s in the same phospholipid vesicles, a marked stimulation of the GTPase activity is observed with isoproterenol. As shown (Figure 3), the hormone-stimulated GTPase activity is linear over the time period examined (3–40 min). Phospholipid vesicles containing β-AR (alone), or N_s (alone), consistently show little or no net production of inorganic phosphate compared to assays performed with vesicles containing no protein. Moreover, incubations of vesicles alone, or vesicles containing only β-AR, with isoproterenol also result in no change in the production of inorganic phosphate (data not shown). However, a basal GTPase activity for vesicles containing both β-AR and N_s is always observed with the extent of this activity being dependent on the amounts of β-AR and N_s (see below).

Characteristics of the GTPase Activity. Figure 4 shows that the isoproterenol stimulation of the GTPase activity can be completely abolished by the β-antagonist alprenolol, and similar results are obtained with propranolol (data not shown). The ability of the antagonists to reverse this stimulation displays the expected stereoselectivity for catecholamine receptors with the (−) isomers being more potent than the respective (+) isomers. In addition, the antagonist blockade was concentration dependent. As shown in Figure 5, the hormone-stimulated GTPase activity shows appropriate pharmacology such that agonists stimulate the activity with a typical β_2-adrenergic specificity (iso > epi > norepi).

The data presented in Table I indicate that the hormone responsiveness of the GTPase activity depends on the amounts of β-AR and N_s in the vesicles. As pointed out earlier, the interactions of these components (in the absence of hormone) result in a basal GTPase activity that becomes significant at increasing levels of β-AR and N_s (entry 1). Under such conditions, the GTPase activity is linear up to 40 min (data not shown); however, little or no isoproterenol-induced stimulation of GTPase activity is observed. The lack of agonist stimulation of GTPase activity, for the cases where there are high levels of N_s in the vesicles (Table I, entry 1), is not due to an increased amount of GTPase activity for N_s alone. While

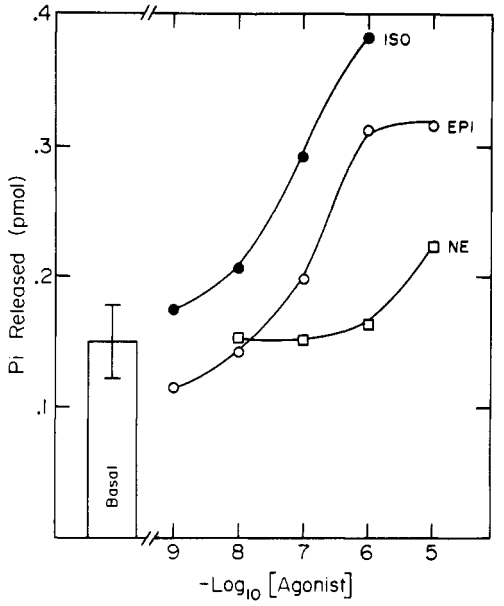


FIGURE 5: Dose–response curves for stimulation of GTPase activity in phospholipid vesicles containing β-AR and N_s by (−)-isoproterenol (●), (−)-epinephrine (○), and (−)-norepinephrine (□). Guinea pig lung β-AR (4.8 pmol) and human erythrocyte N_s (10.1 pmol) were added to reconstitution incubations, and GTPase assays were performed on the isolated vesicles for 40 min at 30 °C. Each point represents the average of triplicate determinations. The bar graph shows the mean GTPase activity for vesicles containing β-AR and N_s in the absence of agonists and for similar vesicles in the presence of both agonists and 10^{-6} M (−)-alprenolol.

Table I: Comparison of GTPase Activities in Phospholipid Vesicles Containing Varying Amounts of β-AR and N_s[a]

sample	P_i formed (fmol/min) with β-AR–N_s	P_i formed (fmol/min) with β-AR–N_s + Iso	x-fold stimulation by isoproterenol	n
(1) GP β-AR (0.56 pmol) + N_s (36 pmol)	19	21	1.1	2
(2) GP β-AR (0.15 pmol) + N_s (43 pmol)	9.2 ± 0.7	16.9 ± 0.9	1.9 ± 0.4	4
(3) GR β-AR (0.59 pmol) + N_s (6 pmol)	3.5 ± 0.3	11.7 ± 3.0	3.6 ± 0.4	3

[a] The quantities of β-AR and N_s in parentheses represent the amounts of these components in the vesicle pellets after centrifugation for 1.5–2.5 h at 250000*g*–300000*g* (see Materials and Methods). The amounts of β-AR were determined by [^{125}I]CYP binding as described under Materials and Methods; the amounts of N_s were determined by assuming an average efficiency of reconstitution (determined by [^{35}S]-GTPγS binding; Northup et al., 1982) of 69%—the mean of four typical experiments. The values listed for P_i formation and x-fold stimulation by isoproterenol are the means from the results of different experiments; in each experiment, triplicate determinations of GTPase activity were performed for every condition tested. The values for x-fold stimulation by isoproterenol are the means of the stimulations observed in the individual experiments rather than the ratios of the means of P_i released under each condition. GTPase assays were performed at 30 °C for 40 min. The isoproterenol concentrations ranged from 10^{-6} to 10^{-4} M; 10^{-6} M isoproterenol usually gives close to maximal stimulation. GP, guinea pig lung; Iso, isoproterenol; *n*, number of experiments.

it was reported that N_s, at high concentrations of Mg^{2+} (50 mM), is capable of GTPase activity in the absence of receptor and hormone (Brandt et al., 1983), this latter activity, for all entries in Table I (where [$MgCl_2$] = 10–12 mM), is low (<2.5 fmol of P_i generated/min). Reducing the amount of either component, i.e., β-AR (entry 2) or N_s (entry 3), in the vesicles,

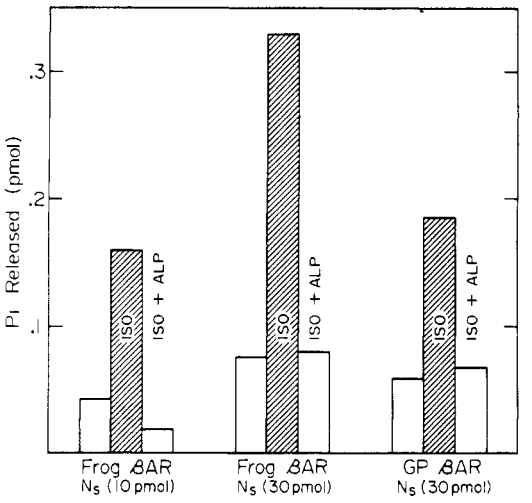


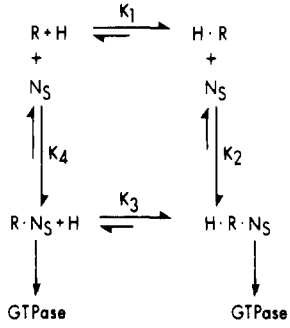
FIGURE 6: GTPase activity in phospholipid vesicles containing β-AR and N_s. Guinea pig lung β-AR (1.9 pmol) or frog erythrocyte β-AR (1.7 pmol) was added to reconstitution incubations with human erythrocyte N_s in the indicated amounts, and the isolated vesicles were assayed for GTPase activity for 40 min at 30 °C. [(−)-Isoproterenol] = 10^{-6} M; [(−)-alprenolol] = 10^{-6} M. Each bar graph represents the average of triplicate determinations, and the data shown are representative of two experiments.

lowers the basal GTPase activity and results in a significant increase in the agonist stimulation of GTPase activity. Recent experiments indicate that still higher "fold" stimulation by isoproterenol (5–10-fold, data not shown) can be obtained by further reducing the amount of β-AR in the vesicles to ~50 fmol, for conditions where the amount of N_s is higher (as in entries 1 and 2), or by reducing the ratio of N_s to β-AR in the vesicles to ~5 (when β-AR ≥ 500 fmol). As will be discussed further below, these results are compatible with a scheme where β-AR and N_s interact in the absence of hormone and suggest that hormone binding is translated into an increased interaction between these components.

Also of interest in this system is the effect of receptor occupancy by an antagonist. As alluded to earlier, the antagonist alprenolol can block the isoproterenol-stimulated GTPase activity elicited by the interaction of β-AR and N_s. However, under conditions where the basal GTPase activity is considerable, occupation of the receptor by the antagonist alprenolol itself effects a significant reduction in this activity in a stereoselective fashion. At 10^{-8} M (−)-alprenolol, the GTPase activity of the β-AR–N_s complexes is reduced by about 25% compared to essentially no effect by 10^{-8} M (+)-alprenolol (data not shown). The maximum stereoselective inhibition afforded by the antagonist alprenolol appears to level off around 35%.

Insertion of Purified β_2-AR of Frog Erythrocyte and N_s. To determine whether the interaction of N_s with β-AR could be demonstrated by using receptor from another source, the insertion of N_s together with receptor isolated from frog erythrocytes into phospholipid vesicles was performed. The incubation of vesicles containing highly purified frog β-AR and human N_s with isoproterenol also results in a significant stimulation of GTPase activity (3.7–4.4-fold, see Figure 6). Hormone stimulation again is completely blocked by antagonist, and the extent of stimulation again is comparable to that observed with similar concentrations of pure guinea pig lung β-AR (Figure 6). The hormone stimulation is also comparable to that obtained with equivalent amounts of affinity-purified frog erythrocyte β-AR (stimulation ranging from 3- to 8-fold, data not shown), supporting earlier reports suggesting that the ability of β-AR to promote stimulation of adenylate cyclase

Scheme I



activity is maintained throughout receptor purification (Cerione et al., 1983a,b).

Discussion

This paper describes the successful reconstitution of functional interactions between two pure components (β-AR and N_s) of a hormone-responsive adenylate cyclase system. An important outcome of these studies is that the interactions of just these two components after their insertion into phospholipid vesicles result in both the appearance of a high-affinity state of β-AR for agonists and the induction of GTP hydrolytic activity in N_s. Agonist (isoproterenol) competition curves for [^{125}I]CYP binding to vesicles containing β-AR and N_s are complex and indicative of two classes of binding sites, just as is the case in intact membranes (De Lean et al., 1980). The curves can be shifted to the right (lower potency) and steepened in the presence of guanine nucleotides. When isoproterenol competition curves are performed in vesicles containing β-AR alone, the curves are monophasic and already shifted to the right, which further highlights the importance of the N_s component for high-affinity agonist binding. This situation is similar to the results obtained when comparing isoproterenol competition curves performed in membrane preparations with those performed in solubilized or purified β-AR preparations where agonist high-affinity binding is absent due to the complete uncoupling of the receptor from N_s (Shorr et al., 1982a,b; Benovic et al., 1984).

The induction of GTPase activity in N_s following its interaction with β-AR provides a sensitive monitor for β-AR–N_s coupling since the background hydrolytic activity (due to N_s alone) under the conditions of these experiments is virtually zero. The GTPase activity can be stimulated by hormone in a dose-dependent manner; the extent of this stimulation is dependent on the potency of the agonist and is blocked in a stereoselective manner by antagonists. Similar extents of stimulation are observed with either guinea pig lung or frog erythrocyte β-AR, suggesting a conservation of the domains at which N_s interacts with these receptors.

A second important outcome of these studies is the observation that when large amounts of β-AR and N_s are inserted into phospholipid vesicles, a significant GTPase activity is observed even in the absence of hormone. These results indicate that β-AR and N_s are able to interact in the absence of agonist occupancy of the receptor, as summarized in Scheme I. Here, receptor is represented by R, N_s is the stimulatory guanine nucleotide regulatory component, H is a hormone (agonist), and K_1–K_4 are the equilibrium constants for the various interactions. Since high concentrations of Mg^{2+} (50 mM) also induce GTPase activity in N_s in the absence of receptor (Brandt et al., 1983), it seems possible that the interaction between β-AR and N_s causes a significant increase in the affinity of N_s for Mg^{2+}, resulting in a receptor-dependent GTPase activity at the lower Mg^{2+} concentrations used in these studies. Binding experiments indicate that K_3 is greater than K_1 since hormone binds more tightly to R·N_s complexes than to R alone. This, in turn, requires that $K_2 > K_4$; i.e., N_s binds more tightly to HR complexes than to R alone. Thus, the fact that hormone stimulation of GTPase activity is best observed when the activity induced by R·N_s formation (in the absence of hormone) is low indicates that hormone stimulation is a reflection of the increased ability of N_s to interact with hormone–receptor complexes (H·R) as compared to unoccupied receptor (R). The observation that alprenolol causes a small but significant stereoselective reduction in the GTPase activity obtained in the absence of hormone (i.e., in the basal activity) suggests that antagonist binding to the receptor may somewhat hinder its ability to interact with N_s.

The time course for the GTPase activity of reconstituted β-AR–N_s preparations indicates that this catalytic turnover is quite slow. The fact that the GTPase experiments are difficult to perform at saturating GTP (Brandt et al., 1983) prevents an accurate determination of turnover number. Under the conditions of our experiments, we observe turnover numbers ranging from 0.3 to 2 min^{-1} when expressed per mole of N_s—as determined by [^{35}S]GTPγS binding (Northup et al., 1982). The lower values are obtained under conditions where [N_s] $\gg$ [β-AR] and are likely a reflection of a lower percentage of the total N_s molecules being coupled to β-AR. The slow rate of hydrolysis is not unexpected since this hydrolytic pathway is thought to be intimately associated with the deactivation of adenylate cyclase. Therefore, by necessity, it must be slow in relation to the initial activation of the enzyme, which is felt to occur subsequent to the N_s*–GTP–adenylate cyclase interaction.

It should be stressed that the rate of isoproterenol-stimulated GTPase activity observed in our experiments with pure receptor and N_s compares favorably with values recently reported in membranes and in reconstituted systems utilizing much less pure receptor preparations (Brandt et al., 1983). On the other hand, the extent of the GTP-mediated shifts in agonist binding observed in our experiments is quite comparable to those demonstrated in membrane preparations and much more dramatic than that recently reported in reconstitution experiments of crude receptor and nucleotide binding protein preparations by Kelleher et al. (1983). These findings reemphasize the functional integrity of the pure components (R and N_s) and the fact that their purification is not associated with any impairment of their intrinsic biological activities. Moreover, they demonstrate that indeed the sole components required for the transduction of receptor occupancy into regulation of N_s activity are the purified β-AR and N_s proteins.

The demonstration of a functional reconstitution of the interactions between two pure components of the adenylate cyclase system will now permit investigations of important questions concerning the basic mechanism of adenylate cyclase activity, which previously could not be unambiguously addressed in less pure systems. For example, what functional domains (peptides) and groups (amino acids) on the β-AR and N_s are involved in their interaction? This can be probed by limited proteolysis and chemical modification studies. Is hormone stimulation of GTPase (and adenylate cyclase) principally a reflection of a facilitation of β-AR–N_s complex formation, or are other steps affected by hormone binding such as the dissociation of N_s into its component peptides? A detailed study of the subunit association states of N_s and the interaction of these subunits with β-AR in the phospholipid environment, as probed, for example, in cross-linking exper-

iments, should provide answers to these questions.

Acknowledgments

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Registry No. GTPase, 9059-32-9; adenylate cyclase, 9012-42-4; (−)-isoproterenol, 51-31-0; guanylyl 5′-(imidodiphosphate), 34273-04-6; (−)-epinephrine, 51-43-4; (−)-norepinephrine, 51-41-2.

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