Selectivity of the β-Adrenergic Receptor among G$_s$, G$_i$'s, and G$_o$: Assay Using Recombinant α Subunits in Reconstituted Phospholipid Vesicles[†]

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ABSTRACT: The selective regulation of G$_s$ (long and short forms), G$_i$'s (1, 2, and 3), and G$_o$ by the β-adrenergic receptor was assessed quantitatively after coreconstitution of purified receptor, purified G-protein βγ subunits, and individual recombinant G-protein α subunits that were expressed in and purified from *Escherichia coli*. Receptor and βγ subunits were incorporated into phospholipid vesicles, and the α subunits bound to the vesicles stoichiometrically with respect to βγ. Efficient regulation of α subunit by receptor required the presence of βγ. Regulation of G proteins was measured according to the stimulation of the initial rate of GTPγS binding, steady-state GTPase activity, and equilibrium GDP/GDP exchange. The assays yielded qualitatively similar results. GDP/GDP exchange was a first-order reaction for each subunit. The rate constant increased linearly with the concentration of agonist-liganded receptor, and the dependence of the rate constant on receptor concentration was a reproducible measurement of the efficiency with which receptor regulated each G protein. Reconstituted α$_s$ (long or short form) was stimulated by receptor to approximately the extent described previously for natural G$_s$. Both α$_{i,1}$ and α$_{i,3}$ were regulated with 25–33% of that efficiency. Stimulation of α$_o$ and α$_{i,2}$ was weak, and stimulation of α$_o$ was barely detectable over its high basal exchange rate. Reduction of the receptor with dithiothreitol increased the exchange rates for all G proteins but did not alter the relative selectivity of the receptor.

G TP-binding regulatory proteins (G proteins) convey information from cell-surface receptors to effector proteins that synthesize or release cytoplasmic second messengers. Selective interactions between receptors and G proteins and between G proteins and effectors are therefore central to reliable signal transduction. Receptors were first thought to be highly selective for individual G proteins. Prototypes for such selectivity are the β-adrenergic and glucagon receptors, which stimulate adenylyl cyclase via G$_s$.[1] However, it is now apparent that a signal can be routed from one receptor to multiple G proteins or from one G protein to several effectors [see Ross (1989) for a review]. The M1-muscarinic cholinergic receptor can stimulate phospholipase C activity through both pertussis toxin sensitive and pertussis toxin insensitive G proteins (Ashkenazi et al., 1989). Receptors for bradykinin and neuropeptide Y regulate a Ca^{2+} channel through a complex combination of G$_o$ and several G$_i$'s (Ewald et al., 1989). The β-adrenergic receptor, which usually regulates G$_s$, can also interact with G$_i$ (Abramson et al., 1985, 1987; Asano et al., 1984) and, perhaps, other G proteins (Barber et al., 1989).

G proteins are heterotrimers. The α subunit is activated by binding GTP and, when activated, regulates the activity of effector proteins. The α subunits are a family of homologous proteins that display strong conservation of amino acid sequence, particularly around the GTP-binding site [see Gilman (1987) and Bourne et al. (1991) for reviews]. A unique α subunit defines each G protein and is assumed to determine selectivity for both receptors and effectors. Receptors promote the activation of G proteins by accelerating the binding of GTP to the α subunit, which is otherwise a slow

process. It is thus likely that the receptor-binding domains on α subunits are similar and conserved but that each is adequately distinctive to provide the appropriate level of selectivity among receptors.

It is a major challenge to understand the physiological selectivity of a receptor among a cell's complement of G proteins. A receptor's selectivity can be defined according to its activation of effector proteins: channels, adenylyl cyclase, or phospholipase C. Such definition depends on knowing which G proteins are present in the cell, which can couple to each effector protein, and the efficiency with which the effector is stimulated. More direct assays of a receptor's ability to regulate a specific G protein, such as guanine nucleotide binding or GTPase activity, are not usually feasible in a native membrane because it is difficult or impossible to assign the binding or hydrolytic reaction to any one G protein. Measuring the regulation of purified G proteins by receptors in reconstituted systems provides an alternative test of selectivity (Asano et al., 1984; Cerione et al., 1986; Senogles et al., 1987, 1990). Assays are straightforward and can be both quantitative and reproducible. Selectivity determined in vitro gives information about which G proteins are potential targets of a receptor rather than which G protein conveys a signal in a specific cell. Such information can be combined with information on the cell's spectrum of G proteins and receptors to suggest likely signal transduction pathways.

Purifying individual G proteins to study selectivity in vitro is difficult because these homologous proteins tend to cofractionate. Even if the preparation is "homogeneous" with respect to the G-protein α subunit, the identity of βγ subunits among different preparations cannot be assumed. Recently,

[1] Abbreviations: GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); DTT, dithiothreitol; INE, isoproterenol; G and α, each followed by a subscript, denote a specific GTP-binding regulatory protein or its distinctive α subunit.

the two forms of α_s, the three α_i's, and α_o have been produced in *Escherichia coli* (Graziano et al., 1989; Linder et al., 1990). Preparations of each are not contaminated by other α subunits and can be recombined with a uniform, although heterogeneous, preparations of $\beta\gamma$ subunits to form a trimeric G protein (Parker et al., 1991; Kurose et al., 1991). To address the biochemical basis of receptor–G-protein selectivity, we reconstituted the β-adrenergic receptor in phospholipid vesicles with different recombinant α subunits and $\beta\gamma$ subunits. This system has allowed us to establish a quantitative assay of receptor–G-protein regulation and determine the receptors' selectivity among the various α subunits.

Experimental Procedures

Materials. (−)-Propranolol, (−)-alprenolol, (±)-cyanopindolol, and Lubrol 12A9 were gifts from Ayerst Laboratories, Hassle Pharmaceuticals, Dr. G. Engel of Sandoz Pharmaceuticals, and ICI, Ltd. GDP and GTP were from Sigma. GTPγS from Boehringer Mannheim was purified as described by Asano et al. (1984). Pertussis toxin was purchased from List Biologicals. [^{35}S]GTPγS, Na^{125}I, [^{32}P]NAD, [^{3}H]dihydroalprenolol, and [γ-^{32}P]GTP were from Dupont/NEN. [α-^{32}P]GTP was from Dupont/NEN or Amersham. 1-Palmitoyl-2-oleoylphosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were from Avanti Polar Lipids. Dimyristoylphosphatidylcholine and phosphatidylglycerol prepared from egg lecithin were from Sigma. The sources of other reagents have been listed previously (Asano et al., 1984; Brandt & Ross, 1986).

Protein Purification. β-Adrenergic receptor was purified from turkey erythrocytes either exactly as described by Brandt and Ross (1986) or using a modification of that protocol in which membranes were prepared according to Cooper et al. (1989) and receptors were solubilized using a mixture of digitonin and deoxycholate (10:1). Soluble receptor was assayed using [^{3}H]dihydroalprenolol (Fleming & Ross, 1980).

Recombinant bovine G$_s$ α subunits (long and short forms) were purified from *E. coli* according to the procedure of Graziano et al. (1989). Recombinant α subunits of rat G$_{i,1}$, G$_{i,2}$, G$_{i,3}$, and G$_o$ were purified according to the procedure of Linder et al. (1990). A preparation of a 41-kDa α subunit, presumably $\alpha_{i,1}$ or $\alpha_{i,3}$, was purified from bovine brain according to Mumby et al. (1988) and was a gift from Dr. I.-H. Pang of this department. Bovine α_o, purified according to Sternweis and Robishaw (1984), was a gift from Dr. P. J. Casey, also of this department.

Bovine brain $\beta\gamma$ subunits, partially purified by the method of Casey et al. (1989) through heptylamine–Sepharose chromatography, were a gift of Dr. P. J. Casey. To remove contaminating α subunits, $\beta\gamma$-containing fractions were further chromatographed on FPLC Mono Q (Casey et al., 1989) (preparation 1) or on hydroxyapatite (preparation 2). Hydroxyapatite chromatography was performed in 20 mM NaHepes (pH 8.0), 50 mM NaCl, 1% cholate; $\beta\gamma$ subunits were eluted with a 0–80 mM linear gradient of KP$_i$ in the same buffer. The major peak of $\beta\gamma$ from either preparation contained less than 1% contaminating α subunit according to the binding of [^{35}S]GTPγS. The $\beta\gamma$ subunits were assayed according to their ability to support the ADP-ribosylation of α_o by pertussis toxin (Casey et al., 1989). The presence of α_s does not interfere with this determination (not shown).

Reconstitution. β-Adrenergic receptor and G-protein subunits were reconstituted into phospholipid vesicles using modifications of the method of Brandt and Ross (1986). 1-Palmitoyl-2-oleoylphosphatidylcholine and phosphatidylglycerol (2:1, w/w) were dispersed by sonication in a solution of 0.3% deoxycholate and 0.06% cholate in 20 mM NaHepes (pH 8.0), 2 mM MgCl$_2$, 1 mM EDTA, and 100 mM NaCl at a final total concentration of 1 mg/mL. Receptor and G-protein subunits were added to the lipid dispersion to give a final lipid concentration of 0.5 mg/mL in an initial reconstitution mixture of 50-μL total volume. The mixture was then chromatographed to remove detergent on Ultrogel AcA34 (3 × 150 mm) in 20 mM NaHepes (pH 8.0), 1 mM EDTA, and 100 mM NaCl with either 2 or 3 mM MgCl$_2$. Ultrogel AcA 34 was used for gel filtration because its larger pore size allows the separation of reconstituted vesicles from unreconstituted proteins, detergent micelles, and small molecules (nucleotides, detergents). Vesicles eluted at the void volume of the column in a total volume of about 250 μL, and unreconstituted proteins eluted later with the detergent. Typically, only 5–10% of the β-adrenergic receptor in the original mixture was recovered in the vesicles. This yield of receptor is considerably lower than that previously reported (Brandt & Ross, 1986) and appears to reflect the change from Sephadex G-50 to UltroGel AcA34 as the matrix used for removal of detergent. The recovery of G-protein subunits is discussed under Results. Between 15 and 50% (usually ~30%) of reconstituted α subunit was sensitive to regulation by receptor in the vesicles. Less than quantitative coupling has been a constant observation. It probably reflects incorrect orientation of receptor and/or G protein in the vesicles and the distribution of small numbers of receptors among vesicles.

In other experiments (not shown), similar results were obtained using a phospholipid mixture of phosphatidylethanolamine and phosphatidylserine (3:2 w/w). The addition of 10% cholesteryl hemisuccinate to either phospholipid mixture was also without significant effect.

In some experiments, [α-^{32}P]GTP was bound to α subunits prior to reconstitution by incubation in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, and 10 mM MgCl$_2$ with a 5–10-fold molar excess of [α-^{32}P]GTP (5–200 cpm/fmol). Incubations were 10 min at room temperature for α_s, 30 min at room temperature for α_o, and 90 min at 30 °C for the α_i's. Incubations were terminated by chilling to 0 °C and addition of EDTA to 1 mM in excess over total Mg^{2+}. The [α-^{32}P]-GDP-liganded α subunits were then reconstituted with receptor and G$_{\beta\gamma}$ as described above. The amount of GDP bound to α was determined by diluting an aliquot of vesicles to 50 μL with the reconstitution buffer, adding 100 μL of buffer containing 20 mM NaHepes (pH 8.0), 100 mM NaCl, 0.1% Lubrol 12A9, 1 mM GTP, 0.1% 2-mercaptoethanol, 0.1 mM (−)-propranolol, 30 μM AlCl$_3$, 10 mM MgCl$_2$, and 10 NaF at 0 °C, and filtering the sample on Schleicher & Schuell BA85 nitrocellulose filters exactly as described for the assay of GTPγS binding [see below and Asano et al. (1984)]. To confirm the identity of the ^{32}P-labeled nucleotide as GDP, reconstituted vesicles were denatured by incubation in 70% methanol, and an aliquot of the supernatant was subjected to thin-layer chromatography on poly(ethylenimine)–cellulose in 0.75 M Tris–OH/0.45 M HCl/0.4 M LiCl (Bochner & Ames, 1982). All radioactivity migrated with the GDP standard.

The specific activity of [α-^{32}P]GDP-liganded G$_\alpha$ was determined by assays of bound ^{32}P and total GTPγS-binding activity (see below). Comparison of the specific activities of ^{32}P-labeled nucleotide before and after loading the G$_\alpha$'s indicated that 60–90% of the total G$_\alpha$ was labeled during the loading protocol. Reconstituted, [α-^{32}P]GDP-liganded α subunits retained over 90% of their ligand after 2 days at 0 °C.

Assays of Reconstituted Vesicles. Reconstituted receptor was assayed by the binding of the β-adrenergic antagonist [^{125}I]iodocyanopindolol using filtration on glass fiber filters to remove unbound ligand as previously described (Fleming & Ross, 1980). Because detergent-solubilized receptor flows through these filters, unreconstituted receptor was not detected by this assay.

The total amount of α subunit was assayed according to the binding of [^{35}S]GTPγS in the presence of Lubrol 12A9 and 30 mM MgCl$_2$ exactly as described by Asano et al. (1984), except that the concentration of GTPγS in the assay was 2 μM and the incubation at 30 °C was for 1 h. Experiments using unlabeled GTPγS confirmed that all of the [^{32}P]GDP that had been bound to G$_\alpha$ was released during this assay.

Receptor-stimulated [^{35}S]GTPγS binding (Asano et al., 1984; Brandt & Ross, 1986) and receptor-stimulated GTPase activity (Brandt et al., 1983) were assayed essentially as described previously. The assay buffer contained 20 mM Na-Hepes (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1 mM ascorbate, 200 nM nucleotide, and either 1.1 mM MgCl$_2$ for $\alpha_{i,2}$ and α_o or 3 mM MgCl$_2$ for all other α subunits.

Reconstituted vesicles that contained [α-^{32}P]GDP-liganded α subunits were assayed for receptor-stimulated GDP release under conditions similar to those used for the assays of receptor-stimulated GTPγS binding and GTPase activity, except that the buffer contained 3 mM MgCl$_2$ and the unlabeled guanine nucleotide shown in the figure legends (usually GDP). GDP release assays were quenched at 0 °C in the buffer described for the GDP-binding assay. Bound GDP was stable after quenching for at least 3 h. Unless otherwise noted, all assays of receptor–α subunit interaction were performed at 30 °C.

To measure initial rates of GDP release at different concentrations of receptor (see Table IV), vesicles that contained [^{32}P]GDP-liganded G protein and varying concentrations of β-adrenergic receptor were incubated in the presence of 1 μM GDP and either 0.1 μM (–)-propranolol (basal) or 10 μM (–)-isoproterenol. Bound [α-^{32}]GDP was assayed over 30–60 s, as appropriate to the rates. Data from duplicate four point time courses were fit to a first-order dissociation model (see text, eq 2 and 3):

$$\ln \{[\text{GDP bound}]/[\text{GDP bound at } t = 0]\} = -k_{\text{obs}}t$$

The values of k_{obs} increased linearly with increasing concentrations of receptor. The slopes of plots of k_{obs} vs receptor concentration (dk_{obs}/d[R]) were determined by linear least-squares fits.

Data Analysis. The data shown are generally means of duplicate determinations. First-order rate constants were derived using a weighted nonlinear curve-fitting program (KINETIC, Elsevier-BIOSOFT), and linear regressions were performed using an unweighted least-squares algorithm.

RESULTS

Role of G-Protein $\beta\gamma$ Subunits in the Reconstitution of Receptor with Recombinant α Subunits. G-Protein $\beta\gamma$ subunits are required for the association of isolated α subunits with lipid bilayers (Sternweis et al., 1986). This requirement also holds for recombinant α subunits expressed in *E. coli*. In the absence of $\beta\gamma$, the association of $\alpha_{s,\text{long}}$ with the vesicles was negligible. Essentially all of the α_s was recovered within the included volume of the column, while reconstituted vesicles were found in the void volume (not shown). The total recovery of α_s activity was also low, probably because $\beta\gamma$ subunits stabilize α subunits against denaturation (Northup et al., 1983). When $\beta\gamma$ subunits were included in the mixture to be
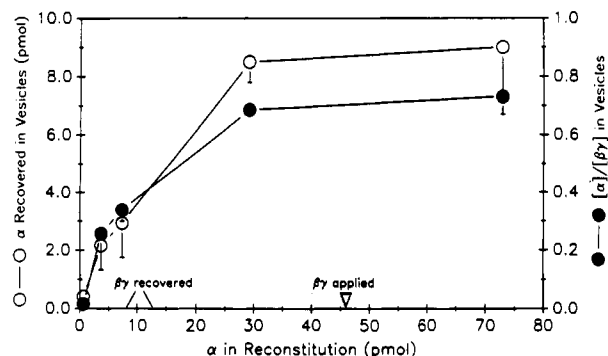

FIGURE 1: Reconstitution of increasing amounts of α_s with a fixed amount of $\beta\gamma$ and phospholipid. Increasing amounts of $\alpha_{s,\text{long}}$ were mixed with $\beta\gamma$ subunits (46 pmol of preparation 1) and phospholipid and chromatographed on AcA34. Vesicles in the void volume were pooled, and both α and $\beta\gamma$ subunits were assayed. The recovery of α_s in each batch of vesicles is expressed both as the total amount (O) and as the molar ratio of α to $\beta\gamma$ (●). Error bars show the range of values in two reconstitutions.

Table I: Recovery of α Subunits after Reconstitution[a]

	$\beta\gamma$ 1	$\beta\gamma$ 2
$\alpha_{s,\text{long}}$	0.73 ± 0.045 (8)	0.53 ± 0.054 (3)
$\alpha_{s,\text{short}}$	0.37 ± 0.071 (7)	0.96 ± 0.127 (3)
$\alpha_{i,1}$	0.52 ± 0.078 (7)	0.44 ± 0.077 (3)
$\alpha_{i,2}$	0.48 ± 0.072 (7)	0.28 ± 0.049 (3)
$\alpha_{i,3}$	0.61 ± 0.090 (7)	0.35 ± 0.068 (3)
α_o	0.17 ± 0.067 (8)	0.39 ± 0.075 (3)

[a] Individual α subunits were reconstituted with β-adrenergic receptor (4–8 pmol) and one of two preparations of $\beta\gamma$ subunits. Recoveries are expressed as molar ratios of α to $\beta\gamma$ in the vesicles. For $\beta\gamma$ preparation 1, reconstitution mixtures contained 23 pmol of $\beta\gamma$ and 10–20 pmol of each α subunit. Under these conditions, the recovery of $\alpha_{s,\text{long}}$ saturated at ~0.8 mol of α/mol of $\beta\gamma$. For preparation 2, 50 pmol of $\beta\gamma$ and 12.5 pmol of the indicated α were reconstituted. Data shown are means ± standard error for the indicated number of reconstitutions.

reconstituted (Figure 1), $\alpha_{s,\text{long}}$, $\beta\gamma$, receptor, and phospholipid all eluted primarily in the void volume, indicating that the proteins were associated with phospholipid vesicles. The total recovery of α_s activity also increased. When increasing amounts of $\alpha_{s,\text{long}}$ were reconstituted with a fixed amount of $\beta\gamma$ (Figure 1), the recovery of α reached a maximum at about 0.8 mol of α/mol of $\beta\gamma$, which suggests that a maximum of one molecule of α subunit was bound per $\beta\gamma$ dimer. The slight deviation from stoichiometric binding shown here and observed in other experiments may result from the relatively low affinity for $\beta\gamma$ that is displayed by recombinant α subunits expressed in *E. coli* (Linder et al., 1990, 1991) or it may represent a consistent overestimation of the concentration of $\beta\gamma$ subunits.

Association of the other α subunits with vesicles also reached a plateau at <1 mol/mol of $\beta\gamma$ (Table I). The lower average recovery of the α_i's and α_o relative to the two species of α_s is qualitatively predicted by the observation of Sternweis and Robishaw (1984) that the α_i's and α_o have lower affinities for $\beta\gamma$ than does α_s. Two different preparations of $\beta\gamma$ subunits were used for these experiments. The recovery of $\alpha_{s,\text{short}}$ with preparation 1 was consistently lower than that with preparation 2, and the opposite was true for $\alpha_{s,\text{long}}$. Reproducible differences between the two $\beta\gamma$ preparations in the recoveries of $\alpha_{i,2}$, $\alpha_{i,3}$, and α_o were also observed. However, the abilities of the two preparations to confer receptor–α-subunit coupling were not noticeably different. The recovery of $\beta\gamma$ during reconstitution was typically 15–40% and was not altered by the presence of either receptor or α subunit (data not shown).

G-Protein $\beta\gamma$ subunits are required to observe the regulation of α subunits by receptor (Fung, 1983; Florio & Sternweis, 1989). Agonist-liganded receptor did not stimulate GTPγS
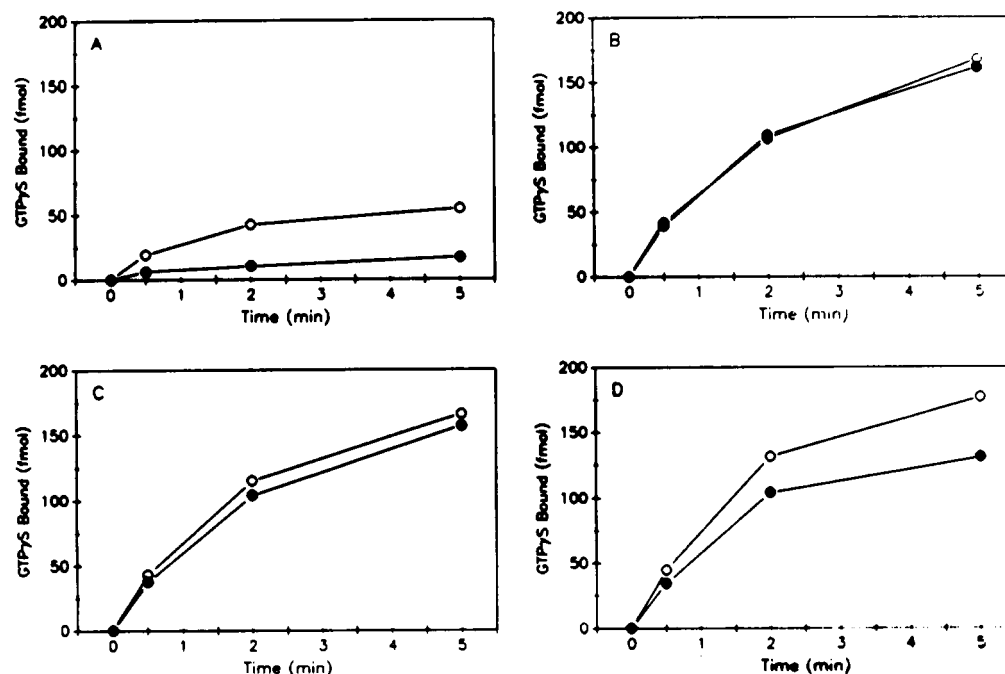
FIGURE 2: Regulation of GTPγS binding by receptor depends on association of α_s with vesicle-bound $\beta\gamma$. Recombinant $\alpha_{s,long}$ was combined with β-adrenergic receptor containing vesicles using four different protocols described below. GTPγS binding was measured in the presence of 10 μM isoproterenol (O) or 100 nM propranolol (●). (A) β-Adrenergic receptor, $\alpha_{s,long}$, and $\beta\gamma$ (preparation 1) were reconstituted as described under Experimental Procedures. Each assay contained 2.0 fmol of receptor, 180 fmol of total $\alpha_{s,long}$, and 236 fmol of $\beta\gamma$. (B) Receptor alone was reconstituted as described, and soluble $\alpha_{s,long}$ was added to the vesicles. Each assay contained 2.9 fmol of receptor and 200 fmol of α_s. (C) Receptor and $\beta\gamma$ were reconstituted as described, and soluble $\alpha_{s,long}$ was added to the vesicles. Assays contained 2.3 fmol of receptor, 200 fmol of $\alpha_{s,long}$, and 192 fmol of $\beta\gamma$. (D) Receptor alone was reconstituted, and both $\alpha_{s,long}$ (200 fmol/assay) and $\beta\gamma$ (230 fmol/assay) were added to vesicles that contained 2.9 fmol of receptor per assay. For each system (A–D), Lubrol 12A9 was added to give a final concentration in the assays of 10 ppm to account for Lubrol that was added with the $\beta\gamma$ subunits.

binding if recombinant α_s was added to preformed receptor-containing vesicles in the absence of $\beta\gamma$ (Figure 2B). If soluble α was added to vesicles that contained both receptor and $\beta\gamma$ subunits or if soluble α and Lubrol-solubilized $\beta\gamma$ were added to preformed vesicles that contained receptor alone (Figure 2C,D), slight stimulation of GTPγS binding was consistently observed during multiple exploratory experiments. However, the basal rate of GTPγS binding was much higher than when all three protein components were coreconstituted by gel filtration and accurate quantitation of coupling was not feasible. Free α subunits are known to display a relatively high nucleotide exchange rate that is suppressed by $\beta\gamma$ (Northup et al., 1982; Higashijima et al., 1987). As predicted by this explanation, the basal rate of GTPγS binding to a mixture of free $\alpha_{s,long}$ and receptor/$\beta\gamma$ vesicles was markedly reduced by chromatography on Ultrogel AcA34 although the agonist-stimulated binding rate was essentially unchanged (not shown).

Regulation of Different α Subunits by the β-Adrenergic Receptor. To study the selectivity of the β-adrenergic receptors among different G proteins, purified recombinant α subunits were mixed with $\beta\gamma$ subunits and β-adrenergic receptor and reconstituted into vesicles as described above. Measurements of agonist-stimulated GTPγS binding and agonist-stimulated GTPase yielded roughly similar patterns of selectivity. As shown in Figure 3, isoproterenol accelerated GTPγS binding to both forms of α_s and to all three α_i's. Stimulation of GTPγS binding to recombinant $\alpha_{i,1}$ or $\alpha_{i,3}$ from *E. coli* was about equal to the stimulation of a preparation of bovine brain α_i that was similarly reconstituted. These data are consistent with the finding of Asano et al. (1984) that the β-adrenergic receptor can regulate rabbit hepatic G$_i$. The β-adrenergic receptor also stimulated the steady-state GTPase activities of the α_s's and α_i's in an agonist-dependent manner (Figure 4, Table II).

Table II: Receptor-Stimulated GTPase Activities of Receptor-α-$\beta\gamma$ Vesicles[a]

α subunit	GTPase (min^{-1})	
	INE	DTT/INE
$\alpha_{s,long}$	0.088	0.220
$\alpha_{s,short}$	0.121	0.363
$\alpha_{i,1}$	0.014	0.092
$\alpha_{i,2}$	0.008	0.039
$\alpha_{i,3}$	0.018	0.125
α_o	0.002	0.013
bovine α_i	0.008	0.005

[a] Vesicles that contained receptor, $\beta\gamma$, and each of the recombinant α subunits or the bovine 41-kDa α subunit (prepared as described for Figure 3) were assayed for GTPase activity as described in the legend to Figure 4. Activities in a 10-min assay (15 min for the bovine α_i) are expressed as moles of GTP hydrolyzed per minute per mole of total α subunit. DTT, treated with dithiothreitol (see Figure 4); INE, 10 μM isoproterenol. Activities measured in the presence of propranolol alone have been subtracted from all of the values shown.

Again, receptor stimulated the α_s's more than the α_i's. Slight stimulation of α_o was sometimes detected.

This general rank order of selectivity among the different α subunits (s > $i_{1,3}$ > i_2 ≥ o) was maintained throughout a large number of GTPase and GTPγS-binding experiments over several months, although quantitative variation in selectivity ratios was frequently greater than 2-fold. The variability did not appear to result from the choice of $\beta\gamma$ preparation used in a specific experiment. A more reproducible assay is introduced below.

Treatment of the reconstituted vesicles with pertussis toxin inhibited isoproterenol-stimulated GTPγS binding to the α_i's by 80–90% in several experiments (not shown). Inhibition was NAD-dependent. Such inhibition is characteristic of regulation of G$_i$ by receptors that are generally considered to be G$_i$-coupled. There is no ADP-ribosylation site for pertussis
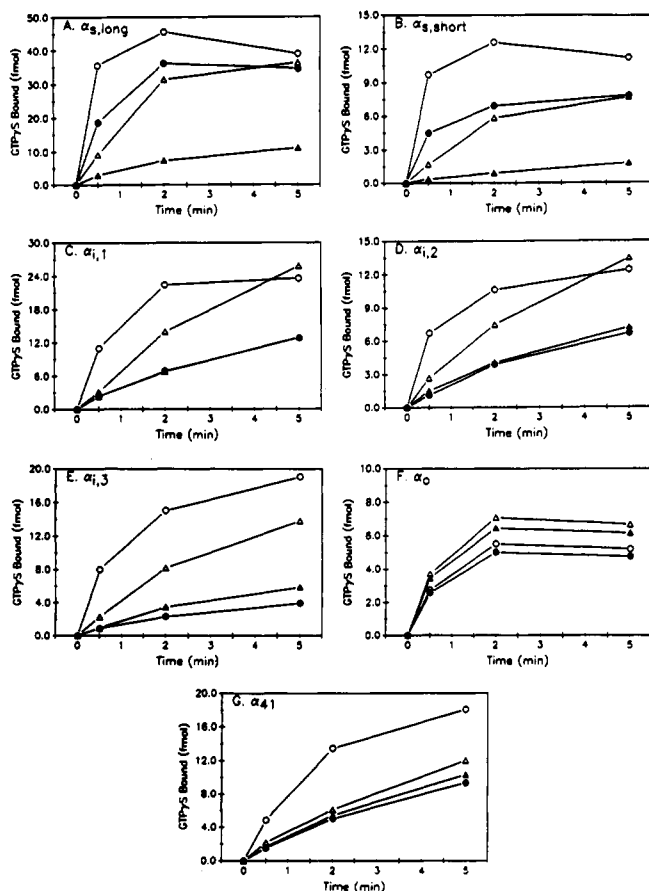
FIGURE 3: Stimulation of GTPγS binding to different α subunits by the β-adrenergic receptor. β-Adrenergic receptor (4–8 pmol) and βγ subunits (preparation 1, 46 pmol) were reconstituted with 10–20 pmol of the indicated α subunit as described under Experimental Procedures. The resulting vesicles were incubated for 2 h at 0 °C with 0.1 mM ascorbate and 0.1 μM propranolol in either the presence (O, ●) or the absence (▲, △) of 5 mM DTT. Propranolol was added to stabilize thiol-reduced receptors (Pedersen & Ross, 1986). Vesicles were diluted 12-fold, and GTPγS binding was assayed at the indicated times in the presence of 10 μM (−)-isoproterenol (O, △) or 0.1 μM (−)-propranolol (●, ▲). The assays contained total concentrations of (A) 1.05 fmol of receptor and 111 fmol of $\alpha_{s,long}$, (B) 0.81 fmol of receptor and 33 fmol of $\alpha_{s,short}$, (C) 1.2 fmol of receptor and 78 fmol of $\alpha_{i,1}$, (D) 1.02 fmol of receptor and 27 fmol of $\alpha_{i,2}$, (E) 1.8 fmol of receptor and 38 fmol of $\alpha_{i,3}$, (F) 2.25 fmol of receptor and 22 fmol of α_o, and (G) 0.54 fmol of receptor and 72 fmol of 41-kDa α_i from bovine brain.

toxin on α_s, and treatment of α_s-containing vesicles was without effect.

Selectivity of the β-Adrenergic Receptor among G-Protein α Subunits. We routinely monitored the efficiency with which the β-adrenergic receptor regulates individual α subunits by assaying receptor-catalyzed exchange of bound and free GDP at equilibrium. This assay yielded more reproducible data than did the GTPase or GTPγS-binding assay (Table III). Because GDP/GDP exchange and the binding of radiolabeled GTPγS monitor the same rate-limiting reaction, the results of both assays should be comparable [see Ross (1989)], and this prediction was confirmed by the data shown in Figure 5. The release of prebound [α-^{32}P]GDP mirrored both the rate and the extent of GTPγS binding to similar vesicles that had been prepared with unlabeled α subunit. Similar initial rates of receptor-catalyzed nucleotide exchange and a similarly sized pool of receptor-coupled G protein were observed using either protocol. The release of [α-^{32}P]GDP did not depend on the concentration of the reconstituted α subunit over a 20-fold range (Rubenstein, 1990). Neither the rate nor the extent of
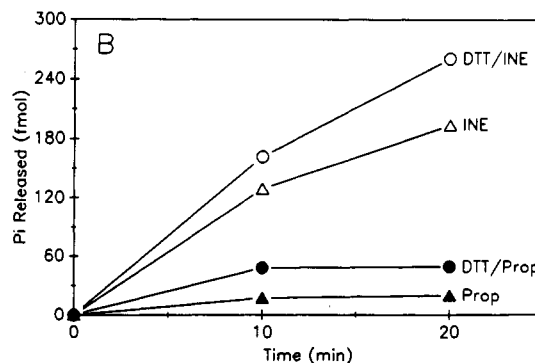

FIGURE 4: Regulation of the steady-state GTPase activity of reconstituted $\alpha_{s,long}$ by the β-adrenergic receptor. The same vesicles used in the experiment shown in Figure 3A were similarly incubated in the presence or absence of DTT. GTPase activity was assayed in the presence of either 10 μM isoproterenol or 0.1 μM propranolol. Each assay contained 0.7 fmol of receptor, 74 fmol of $\alpha_{s,long}$, and 104 fmol of βγ.

Table III: β-Adrenergic Receptor Stimulated GDP Release from Reconstituted α Subunits[a]

	basal k_{obs} (min^{-1})	$dk_{obs}/d[R]$ (min^{-1} nM^{-1})	r	$dk_{obs}/d[R]$ + DTT (min^{-1} nM^{-1})	r
$\alpha_{s,long}$	0.39	1.19	0.96	2.75	0.99
$\alpha_{s,short}$	0.32	1.44	0.98	nd[b]	
$\alpha_{i,1}$	0.18	0.39	0.97	0.69	0.99
$\alpha_{i,2}$	0.26	0.13	0.94	nd	
$\alpha_{i,3}$	0.19	0.34	0.93	nd	
α_o	0.84	0.13	0.77	0.25	0.96

[a] Vesicles that contained each α subunit, βγ (preparation 2), and increasing concentrations of β-adrenergic receptor were prepared and assayed for equilibrium GDP exchange in the presence of isoproterenol and propranolol as described under Experimental Procedures. Each value of the first-order exchange rate constant, k_{obs}, was derived from a nonlinear, one-component fit of duplicate four-point dissociation time courses, with the zero time value allowed to float during the fit. Each value of $dk_{obs}/d[R]$, the slope of the plot of k_{obs} vs the concentration of agonist-liganded receptor, was derived from a linear fit for six to eight values of k_{obs}, each obtained with a separate batch of vesicles that contained varying amounts of receptor (r, correlation coefficient). Separate batches of vesicles that contained $\alpha_{s,long}$, $\alpha_{i,1}$, and α_o were incubated with DTT ("+DTT" in table) as described in the legend to Figure 3, and values of k_{obs} were obtained from duplicate four-point time courses at 20 °C. For the DTT-treated vesicles, values of $dk_{obs}/d[R]$ were determined from four values of k_{obs}. [b] nd, not determined.

[α-^{32}P]GDP release depended on whether GTPγS or GDP was used as the unlabeled exchanging nucleotide or on the concentration of unlabeled GDP (0.1–10 μM).

Guanine nucleotide exchange reactions in reconstituted systems indicate the existence of two pools of G protein, one that is regulated by receptor ("coupled") and another that is apparently inaccessible to receptor ("uncoupled"). The ratio of the two pools varies among preparations (Asano et al., 1984b; Brandt & Ross, 1986). Such behavior predicts biexponential kinetics in the presence of agonist: a slow rate displayed by the uncoupled G proteins and a faster rate displayed by the coupled G proteins. Coupled G proteins are predicted to display single-component, first-order kinetics regardless of the extent of stimulation (e.g., at different agonist concentrations) with the extent of stimulation reflected only in the magnitude of the single rate constant [see Chau et al. (1981)]. The receptor–G-protein vesicles used in this study also displayed a small amount of initial rapid GDP/GDP exchange in the presence or absence of agonist. This burst was observed for both equilibrium GDP/GDP exchange and GTPγS binding (Figure 5 and 6A). Its interference in the
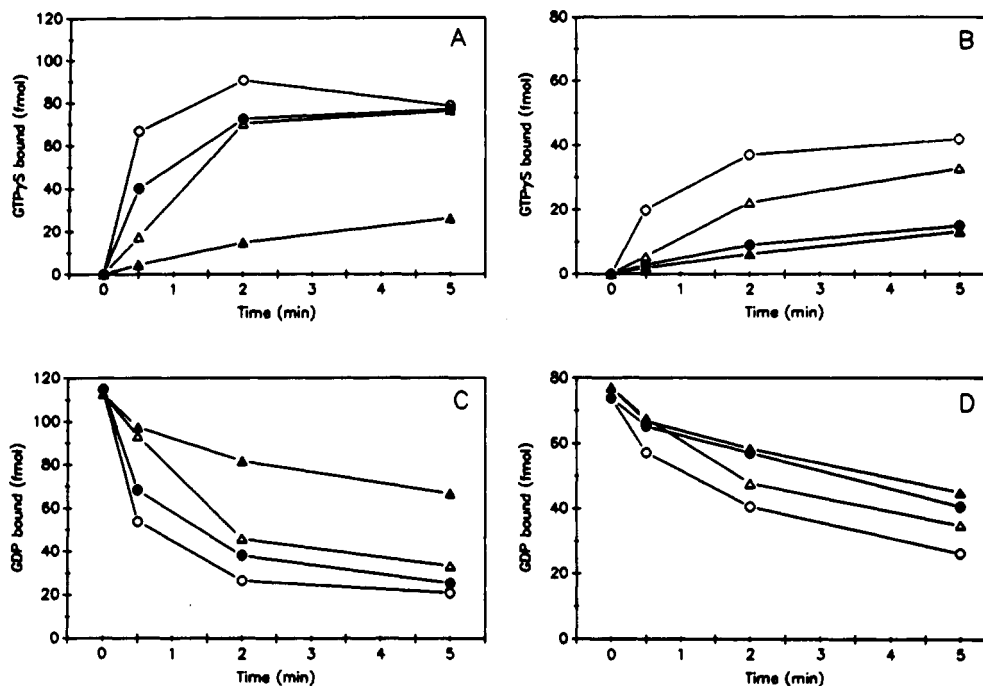
FIGURE 5: Comparison of receptor-stimulated [^{32}P]GDP release and [^{35}S]GTPγS binding. Either $\alpha_{s,long}$ (A and C; 30 pmol) or $\alpha_{i,3}$ (B and D; 17.5 pmol) was incubated with either [α-^{32}P]GTP (C and D) or equimolar unlabeled GTP (A and B) as described under Experimental Procedures. The α subunits were then reconstituted with β-adrenergic receptor (8.6 pmol) and $\beta\gamma$ subunits (preparation 2, 80 pmol), and the vesicles were incubated either with (O, ●) or without (▲, △) DTT as described in the legend to Figure 3. The vesicles were assayed either for [^{35}S]GTPγS binding (A and B) or for [^{32}P]GDP release (C and D) in the presence of 10 μM isoproterenol (O, △) or 0.1 μM (-)-propranolol (●, ▲) after 10-fold dilution. The concentration of GTPγS, either ^{35}S-labeled (A and B) or unlabeled (C and D), was 200 nM. Each assay contained (A) 3.2 fmol of receptor and 116 fmol of $\alpha_{s,long}$, (B) 3.7 fmol of receptor and 64 fmol of $\alpha_{i,3}$, (C) 3.2 fmol of receptor and 124 fmol of $\alpha_{s,long}$, and (D) 4.4 fmol of receptor and 80 fmol of $\alpha_{i,3}$.

assay could be avoided by measuring exchange after the first 2 min of incubation. After the initial rapid release of GDP, exchange was monophasic in the absence of agonist and proceeded with a rate similar to that previously reported for hepatic G$_s$ (Asano & Ross, 1984; Brandt & Ross, 1986). Agonist-stimulated exchange was biphasic, with a stimulated component (primarily coupled G protein) followed by a slower component (primarily uncoupled G protein). The slow component displayed a rate constant equal to that observed in the absence of agonist. Both basal and receptor-stimulated exchange could be fit to a two-component first-order model:[2]

$$\frac{[\alpha \cdot D^*]_t}{[\alpha \cdot D^*]_{t=0}} = Fe^{-k_r t} + (1 - F)e^{-k_b t} \qquad (1)$$

where $\alpha \cdot D^*$ is [^{32}P]GDP-liganded α subunit, k_r is the apparent first-order rate constant for receptor-stimulated exchange, k_b is the rate constant for basal exchange, and F is the fraction of α subunit that is accessible to receptor.

When GDP exchange was measured in vesicles that contained increasing amounts of β-adrenergic receptor, it was found that k_r, the rate constant for receptor-stimulated exchange, increased linearly with the concentration of receptor (Figure 6B). The values of k_b that were determined from a one-component fit of exchange in the absence of agonist or from a two-component fit in the presence of agonist were equal and did not vary with the concentration of receptors in the vesicles (Figure 6B). The fraction of α subunit that was accessible to receptor (F) was not dependent on the concentration of receptor over the range used in these experiments (Figure 6C). This suggests that the dependence of k_r on the concentration of receptor does not in some way reflect a

variation in the number of G proteins that have access to receptors. The coupled fraction also did not differ by more than 2-fold among vesicles prepared with different α subunits.

Although the approach to measuring exchange shown in Figure 6 is analytically valid, it depends on initiating receptor-catalyzed nucleotide exchange after significant dissociation of labeled GDP has occurred. It also depends on a multiparameter fit in which errors in estimates of single parameters may be large and heavily dependent on the fits of other parameters. Therefore, we routinely compared the regulation of different α subunits by receptor by analyzing only the initial rates of dissociation according to a single-component model. The initial rate of GDP/GDP exchange is described by a single rate contant, k_{obs}, that is a weighted combination of k_r (defined as described above), k_b' (a composite description of all non-receptor-promoted exchange), and F (still defined as the fraction of G protein that undergoes receptor-stimulated exchange). If k_b' and F remain constant within an experiment, then values of k_{obs} obtained at an arbitrary early time can be related to k_r by the equations (see Discussion):

$$e^{-k_{obs}} = Fe^{-k_r} + (1 - F)e^{-k_b'} \qquad (2)$$

$$k_r = -\ln \left[\frac{e^{-k_{obs}} - e^{-k_b'}(1 - F)}{F} \right] \qquad (3)$$

The values of k_{obs} that were determined by analysis of initial rates of GDP exchange increased linearly with the concentration of β-adrenergic receptor, as was found using the more complex fitting protocol described above (Figure 6B). There was a negligible effect of receptor on the rate in the absence of agonist, as would be expected if only the agonist-liganded receptor acts as a catalyst of nucleotide exchange.

The linear dependence of k_{obs} on the concentration of receptor (dk_{obs}/d[R]; as in Figure 6B) is an index of the intrinsic efficiency with which the receptor regulates different α sub-

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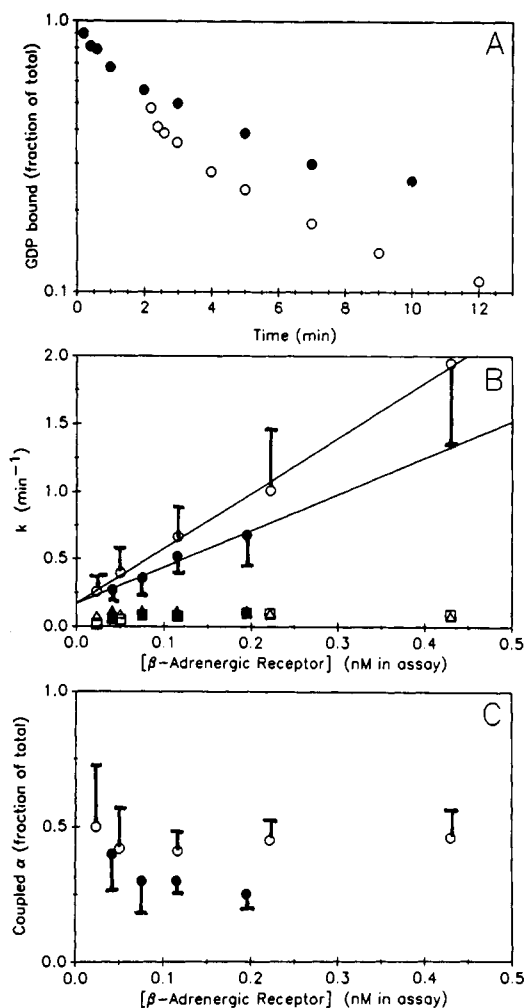[2] Derivation of eq 1–3 and supporting data for this analysis are given by Rubenstein (1990).

FIGURE 6: Dissociation of [^{32}P]GDP from $\alpha_{s,long}$ and $\alpha_{i,1}$. (A) Dissociation time courses for α_s. Receptor-stimulated dissociation of [α-^{32}P]GDP from $\alpha_{s,long}$ was measured as described under Experimental Procedures (O). Isoproterenol (10 μM) was added after 2 min of incubation in the absence of β-adrenergic ligand. The basal rate of dissociation (●) was also assayed in medium that contained 100 nM (−)-propranolol. (B) Dissociation of [α-^{32}P]GDP from vesicles that contained $\beta\gamma$ subunits, varying amounts of receptor, and either $\alpha_{s,long}$ (open symbols) or $\alpha_{i,1}$ (closed symbols). Dissociation was measured after 2 min as described for panel A. Data were fit to the two-component model described in the text (eq 1). The dissociation rate constants k_r (receptor-stimulated; O, ●) and k_b (basal; ▲, △) are shown for each batch of vesicles. Values of k_b were also obtained from one-component fits of dissociation in the presence of propranolol (□, ■). The slopes of the plots of k_r vs receptor concentration were 4.2 min^{-1} nM^{-1} for $\alpha_{s,long}$ and 2.7 min^{-1} nM^{-1} for $\alpha_{i,1}$. (C) The fractions of $\alpha_{s,long}$ (O) and $\alpha_{i,12}$ (●) that were stimulated by receptor (F in eq 1) in the experiments described in panel B were also obtained from two-component fits. Error bars indicate standard errors.

units. To determine the efficiency of coupling of the β-adrenergic receptor with different G-protein α subunits, we assayed the initial rates of [^{32}P]GDP release from multiple batches of vesicles that contained different concentrations of β-adrenergic receptor and constant amounts of each of the various [^{32}P]GDP-liganded α subunits. For each G protein, dk_{obs}/d[R] was determined using values of k_{obs} obtained for six to eight preparations of vesicles that contained different concentrations of receptor. The results of several such titrations are summarized in Table III. For the β-adrenergic receptor, dk_{obs}/d[R] was about 3–4-fold higher for either form of α_s than for either $\alpha_{i,1}$ or $\alpha_{i,3}$, which is consistent with our previous studies of hepatic G$_i$ (Asano et al., 1984). β-Adrenergic stimulation of $\alpha_{i,1}$ and $\alpha_{i,3}$ was observed in all experiments. The receptor regulated both $\alpha_{i,2}$ and α_o much less

efficiently than the α_s's; dk_{obs}/d[R] was about 10-fold lower for both G$_{i,2}$ and G$_o$. This level of regulation of $\alpha_{i,2}$ was a consistent observation and might be physiologically important. The stimulation of α_o, which was undetectable in assays of GTPγS binding and steady-state GTPase, is probably not meaningful. The nonstimulated rate of GDP exchange for α_o was already quite high, and our ability to quantitate stimulation over this basal rate was in fact marginal. We do not believe that the differences in dk_{obs}/d[R] between the long and short forms of α_s or between $\alpha_{i,1}$ and $\alpha_{i,3}$ are meaningful; they are within the range of experimental variability.

Effects of Thiols on the Selectivity of the β-Adrenergic Receptor. Reduction of disulfide bridges in the β-adrenergic and muscarinic cholinergic receptors potentiates the ability of the receptors to stimulate G proteins (Pedersen & Ross, 1985; Florio & Sternweis, 1989; Moxham et al., 1988; Parker et al., 1991). Treatment of the reconstituted β-adrenergic receptor with dithiothreitol potentiated its ability to regulate all of the recombinant α subunits when assayed in the presence of isoproterenol (Figures 3–5; Tables II and III). In the absence of agonist or in the presence of antagonist, however, the DTT-treated receptor stimulated the α_i's slightly, if at all. Values of dk_{obs}/d[R] for DTT-treated, agonist-liganded receptor were compared for $\alpha_{s,long}$, $\alpha_{i,1}$, and α_o (Table III). Incubation with dithiothreitol did not alter the selectivity of the agonist-liganded receptor among different α subunits.

DISCUSSION

Extensive sequence similarity among the G-protein α subunits and the G-protein-coupled receptors suggests that a single receptor is able to regulate more than one G protein or that a single G protein could be regulated by more than one receptor. For some receptors, such divergent coupling has been relatively easy to demonstrate. For example, the M1 muscarinic cholinergic receptor expressed in CHO cells stimulates inositol triphosphate release through the action of two G proteins, only one of which is sensitive to inhibition by pertussis toxin (Ashkenazi et al., 1989). The number and identity of the G proteins that convey a receptor's signal are usually more difficult to determine.

The initial observation that the β-adrenergic receptor can stimulate G$_i$ (Asano et al., 1984a) was puzzling because β-adrenergic stimulation has been consistently associated only with the G$_s$-mediated stimulation of adenylyl cyclase and the only known activity of G$_i$ was to inhibit the cyclase. Because G$_s$ and G$_i$ are now known to regulate several effector proteins, the β-adrenergic stimulation of G$_{i,1}$ and G$_{i,3}$ can be interpreted as a mechanism for generating multiple second messengers from a single β-adrenergic signal.

The data reported here suggest that signaling from the β-adrenergic receptor is primarily mediated by G$_s$ but that it is also significantly routed through G$_{i,1}$ and G$_{i,3}$. Regulation of these G$_i$'s predicts the occurrence of β-adrenergic stimulation of a phospholipase A$_2$, a K$^+$ channel, or another physiological target of G$_i$, and we are attempting to measure such regulation. From the present data, it seems less likely that the β-adrenergic receptor regulates G$_{i,2}$ to a significant extent, and significant regulation of G$_o$ seems improbable. G$_{i,2}$, which is more widespread among animal tissues than G$_{i,1}$ or G$_{i,3}$ (Mumby et al., 1988), may respond to a more restricted or different range of receptors than do G$_{i,1}$ and G$_{i,3}$. The range of targets of the β-adrenergic receptor may actually be broader than G$_s$, G$_{i,1}$, and G$_{i,3}$. We have not observed significant β-adrenergic regulation of G$_z$ (Parker et al., 1991; S. K.-F. Wong, unpublished data), but there is some evidence that the receptor can modulate an Na/H exchange activity through

a G protein that is neither G_s nor G_i (Barber et al., 1989).

These data and those of Asano et al. (1984a) indicate that the β-adrenergic receptor requlates G_i more efficiently than was observed by Cerione et al. (1985), who also used a re-constituted assay system. Neither group determined which α_i's were in their G_i preparations. Asano et al. (1984a) used G_i prepared from rabbit liver, which can contain variable ratios of $\alpha_{i,2}$ to $\alpha_{i,1}$ plus $\alpha_{i,3}$. Cerione et al. may have used a prep-aration that contained relatively more $G_{i,2}$, although $G_{i,2}$ does not appear to be the major α_i in purified human erythrocyte G_i (Carty & Iyengar, 1990). Reduction of disulfides in the receptor, which probably occurred in the experiments of Asano et al. (1984a), is not a determinant of the receptor's selectivity (Table II) and therefore does not account for receptor–G_i coupling. Treatment of the receptor with DTT simply makes the less efficient regulation of G_i easier to observe.

The parameter that was used to compare the efficiency of regulation of different G proteins, $dk_{obs}/d[R]$, was reproducible among multiple sets of data and intuitively provides a good measure of coupling. A more concrete comparison and more basic mechanistic information could have been derived from a study of $dk_r/d[R]$, the actual efficiency of the receptor as a catalyst of nucleotide exchange. Significant non-receptor-mediated GDP exchange precluded our reliably extracting this constant from the data with acceptable precision. Because k_{obs} increased linearly with receptor concentration and because the fraction of each G protein that was regulated by receptor did not vary widely (0.2–0.5), the dependence of k_{obs} on receptor was a valid basis for comparison of coupling.[3] We did not observe the interesting saturable dependence of coupling on the concentration of G protein that was reported by Senogles et al. (1990) for the D_2 dopaminergic receptor. There is no theoretical reason either to support or to question such kinetic behavior over a given range of G-protein concentrations. The lack of dependence of k_{obs} on the concentration of G protein found in the present study is consistent with the relatively low affinity of the turkey erythrocyte β-adrenergic receptor for G_s that was predicted by Levitzki and co-workers from kinetic analysis of adenylyl cyclase activation [Tolkovsky & Levitzki, 1978, 1981; Tolkovsky et al., 1982; discussed by Rubenstein (1990)].

Reconstituted Systems for Assaying the Selectivity of Receptors and G Proteins. The analysis of receptor–G-protein selectivity in a purified and reconstituted system has both strengths and weaknesses. The most obvious advantage is that a reconstituted system can use a clearly identified G protein. Measurements of second-messenger responses in intact cells or membranes often do not indicate which G protein (or proteins) may be conveying the signal from a receptor. The use of purified recombinant α subunits further clarifies matters. Because α subunits are structurally similar, it is difficult to purify either a single α subunit or a trimeric G protein free of contamination by others. It is also difficult to demonstrate such purification conclusively. Reconstitution using different recombinant α subunits and a single preparation of $\beta\gamma$ avoids any selective influence on coupling that may be exerted by the different $\beta\gamma$ subunits that are found in preparations of trimeric G proteins (see below). Reconstitutive assays also allow a

quantitative comparison of the regulation of different G pro-teins by receptors. Interpreting G-protein activation according to signaling in intact systems is complicated by our ignorance of the efficiency with which G proteins regulate effectors.

The assay system described here is applicable to any G-protein-coupled receptor, including recombinant wild-type and mutant receptors that have been expressed in heterologous cells. The receptor need only be relatively concentrated and free of substantial G-protein contamination. We have used this system to study the G-protein selectivity of muscarinic cholinergic receptors (Parker et al., 1991), to study the se-lectivity of chimeric receptors (S. K.-F. Wong and E. M. Ross, in preparation), and to study the regulation of site-directed mutants in α_s.

The recombinant α subunits from *E. coli* appear to interact normally with the β-adrenergic receptor. The behavior of recombinant $\alpha_{i,1}$ and $\alpha_{i,3}$ was similar to that of the bovine 41-kDa α subunit (Figure 3 and other data not shown), and the behavior of recombinant α_s was similar to that observed previously with purified rabbit hepatic G_s [see Brandt and Ross (1985) for example]. However, α subunits expressed in *E. coli* do differ in some respects from the same proteins in mam-malian cells. Both α_i and α_o in animal cells are myristoylated at their amino termini, but these subunits are not myristoylated when expressed in *E. coli* (Linder et al., 1991). Although mammalian α_s is not myristoylated, its amino terminus is blocked by an unknown modification, and it is more hydro-phobic than would be predicted from its amino acid sequence (Sternweis, 1986). G-Protein α subunits from *E. coli* regulate adenylyl cyclase and ion channels with lower potency than do the same subunits from mammalian cells (Graziano et al., 1989; Mattera et al., 1989) and bind to $\beta\gamma$ with lower affinity (Linder et al., 1991) Non-myristoylated α_o does not bind tightly to the plasma membrane of mammalian cells (Mumby et al., 1990). Data on protein–protein interactions of recom-binant subunits from *E. coli* must therefore be interpreted carefully.

Role of the $\beta\gamma$ Subunits. The G-protein $\beta\gamma$ subunits are required for the association of α subunits with phospholipid bilayers (Sternweis, 1986; this study) and for receptor–α-subunit coupling (Fung, 1983; Florio & Sternweis, 1989; this study). They also regulate nucleotide binding to the α subunit (Higashijima et al., 1987). Non-retinal tissues express at least two species of β subunit (Evans et al., 1987) and at least three γ subunits (Tamir et al., 1991), and preparations of isolated $\beta\gamma$ subunits or of trimeric G proteins generally contain mix-tures of β's and γ's. No previous evidence suggested that non-retinal $\beta\gamma$ subunits were selective among either receptors or α subunits. However, the two preparations of $\beta\gamma$ used here differed reproducibly in their ability to efficiently reconstitute $\alpha_{s,short}$. A third preparation of $\beta\gamma$ behaved essentially iden-tically to preparation 2. More recently, we have noticed that different $\beta\gamma$-subunit preparations also alter the reconstitution of other receptors and α subunits (T. Higashijima, G. Berstein, E. M. Parker, and E. M. Ross, unpublished data). We do not know the basis of these differences among preparations of $\beta\gamma$. It is likely that we will need to purify dimers composed of known β and γ species, expressed in an appropriate cellular background, in order to sort out these effects. The recon-stituted system described here may provide a good assay medium for probing specific functions of β and γ when these defined $\beta\gamma$ dimers become available.

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[3] The relationship between $dk_{obs}/d[R]$ and $dk_r/d[R]$ is complex and dependent upon the fraction of G protein that is regulated by receptor (F or F'). k_r is predicted to increase linearly with the concentration of receptor ($dk_r/d[R]$ is constant). In an incompletely coupled system, $dk_{obs}/d[R]$ will only be constant at low receptor concentrations. Deriv-ations of eq 1–3 and a more complete discussion of their applicability to these data have been presented elsewhere (Rubenstein, 1990).

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