Activation of the Inhibitory GTP-binding Protein of Adenylate Cyclase, G\textsubscript{i}, by \( \beta \)-Adrenergic Receptors in Reconstituted Phospholipid Vesicles*

(Received for publication, March 23, 1984)

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\( \beta \)-Adrenergic receptors and the inhibitory GTP-binding protein, G\textsubscript{i}, of the adenylate cyclase system were reconstituted into phospholipid vesicles by the method described previously for reconstituting receptors and the stimulatory GTP-binding protein, G\textsubscript{s} (Brandt, D. R., Asano, T., Pedersen, S. E., and Ross, E. M. (1983) Biochemistry 22, 4357–4362). In the receptor-G\textsubscript{i} vesicles, \( \beta \)-adrenergic agonists stimulated both the high-affinity binding of guanosine 5'-O-(3-thiotriphosphate) (GTP\textsubscript{yS}) to G\textsubscript{i} and GTPase activity to an extent similar to that observed in vesicles containing \( \beta \)-adrenergic receptors and G\textsubscript{s}. Stimulation required receptors and displayed appropriate \( \beta \)-adrenergic specificity. The prior treatment of receptor-G\textsubscript{i} vesicles with islet-activating protein (pertussis toxin) plus NAD markedly inhibited both the isoproterenol-stimulated binding of GTP\textsubscript{yS} and the isoproterenol-stimulated GTPase activity. No contamination of G\textsubscript{i} by G\textsubscript{s} was found in the preparations after exhaustive M\textsubscript{F}-promoted denaturation of the proteolytically cleaved subunits to dissociate. This has suggested that G\textsubscript{s}-mediated inhibition primarily reflects an increased concentration of free G\textsubscript{s} that promotes the reassociation of G\textsubscript{s} with the \( \alpha \) subunit of G\textsubscript{s} to cause its deactivation (7, 8). It is also likely that the \( \alpha \) subunit of G\textsubscript{s} causes inhibition of adenylate cyclase, although not necessarily directly (8–10).

Given the homology of stimulatory and inhibitory G proteins, it was of interest to determine whether G\textsubscript{i} or G\textsubscript{s} could interact with a receptor from the opposite pathway. We have recently reconstituted purified G\textsubscript{i} and partially purified \( \beta \)-adrenergic receptors into unilamellar phospholipid vesicles in order to study their interactions directly (11, 12). Reconstitution efficiently restores catecholamine-stimulated GTP\textsubscript{yS} binding to G\textsubscript{s} and G\textsubscript{i}, allowing relatively detailed studies of these processes. More recently, we have combined pure (>95%) receptors from turkey erythrocytes and pure G\textsubscript{s} from rabbit liver into phosphatidylinerine-phosphatidylethanolamine:cholesterol vesicles (12). Receptor-G\textsubscript{i} coupling in this system indicates that the single receptor polypeptide (Mr = 43,000) can interact directly with G\textsubscript{i}. We have now used these procedures to probe the coupling of \( \beta \)-adrenergic receptors, which generally activate adenylate cyclase, to G\textsubscript{i}. We report here the efficient interaction of these proteins in reconstituted lipid vesicles.

Adenylate cyclase activity in animal cells is controlled by receptor-mediated hormonal stimulation as well as receptor-mediated inhibition (see Ref. 1 for review). The mechanisms of these events are homologous. Stimulatory hormones act via their receptors to promote the binding of G\textsubscript{s} to a stimulatory GTP-binding regulatory protein, G\textsubscript{s}. The binding of GTP "activates" G\textsubscript{s} to a form in which it can stimulate the activity of the catalytic unit of adenylate cyclase. Hydrolysis of G\textsubscript{s}, bound GTP to G\textsubscript{s} terminates activation of G\textsubscript{s}. In the presence of a nonhydrolyzable analog of GTP, such as GTP\textsubscript{yS},\textsuperscript{1} activation of G\textsubscript{s} is reversed only slowly by the dissociation of nucleotide.

G\textsubscript{s} is composed of three subunits. The \( \alpha \) subunit (M\textsubscript{r} = 45,000 or 52,000) binds GTP and is capable of stimulating adenylate cyclase. The \( \beta \gamma \) subunit (M\textsubscript{r} = 35,000) regulates the binding of nucleotide to the \( \alpha \) subunit and dissociates from \( \alpha \) when G\textsubscript{s} is activated (2, 3). A \( \gamma \) subunit (M\textsubscript{r} ~ 8000) sometimes copurifies with G\textsubscript{s}. It is of unknown function and appears to remain associated with the \( \beta \gamma \) subunit when G\textsubscript{s} is activated (4).

Hormonal inhibition of adenylate cyclase is a strikingly similar process. Inhibitory receptors promote the binding of GTP to an inhibitory regulatory protein, G\textsubscript{i}, thereby activating it (1). G\textsubscript{i} is structurally homologous to G\textsubscript{s}, having a homologous GTP-binding \( \alpha \) subunit (M\textsubscript{r} = 41,000), a very similar or identical \( \beta \) subunit (5, 6), and a presumably identical \( \gamma \) subunit (4). The activation of G\textsubscript{i} also causes the \( \alpha \) and \( \beta \gamma \) subunits to dissociate. This has suggested that G\textsubscript{s}-mediated inhibition primarily reflects an increased concentration of free \( \beta \gamma \) that promotes the reassociation of \( \beta \gamma \) with the \( \alpha \) subunit of G\textsubscript{s} to cause its deactivation (7, 8). It is also likely that the \( \alpha \) subunit of G\textsubscript{s} causes inhibition of adenylate cyclase, although not necessarily directly (8–10).

MATERIALS AND METHODS

Most of the procedures used in this study have been described in detail previously (11). G\textsubscript{i} and G\textsubscript{s} were purified from rabbit liver according to Bokoch et al. (13) and Sternweis et al. (14), respectively. Purified G\textsubscript{i} was free of the \( \alpha \) subunit of G\textsubscript{s} according to silver-stained sodium dodecyl sulfate-polyacrylamide gels. No G\textsubscript{s} regulatory activity was found in the G\textsubscript{i} preparations after exhaustive Mg\textsuperscript{2+}-promoted activation in detergent solution by 50 \( \mu \)M GTP\textsubscript{yS} when assayed by reconstitution into G\textsubscript{s}-deficient membranes. 2,3,5,6 Reconstitution of receptor-G\textsubscript{i} coupling in this system indicates that the single receptor polypeptide (M\textsubscript{r} = 43,000) can interact directly with G\textsubscript{i}. We have now used these procedures to probe the coupling of \( \beta \)-adrenergic receptors, which generally activate adenylate cyclase, to G\textsubscript{i}. We report here the efficient interaction of these proteins in reconstituted lipid vesicles.
that catalyzes the ADP-ribosylation of G{sub i} was a gift of Dr. Michio Ui (Hokkaido University). [35S]GTP{sub i}S was purchased from New England Nuclear, and unlabeled GTP{sub i}S was purified as described (11).

β-Adrenergic receptors and either G{sub i} or G{sub s} were reconstituted into vesicles composed of dimyristoylphosphatidylcholine and turkey erythrocyte polar lipids as described previously (11).{sup 2} The recovery of G{sub s} was 45 ± 10% of that added to the original mixture (n = five preparations). The recovery of receptor was 27 ± 2%. The molar ratio of G{sub s} to receptor was 2-6 in the vesicle preparations used here.

The assays for [35S]GTP{sub i}S binding (11),{sup 3} GTPase (11), and [125I]iodocyanopindolol binding (11) have been described, as has the assay for the activation of G{sub i} by GTP{sub i}S (16). Details are also provided in the figure legends. Other ancillary assays and procedures are described elsewhere (11, 16).{sup 3} To ADP-ribosylate G{sub i}, reconstituted vesicles were incubated for 10 min at 30 °C with 25 µg/ml of IAP and 1 mM NAD in 20 mM NaHepes (pH 8.0), 1 mM EDTA, 2 mM MgCl{sub 2}, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.1% Lubrol, and 1 mM diethiothreitol.

RESULTS

When either G{sub i} or G{sub s} was reconstituted into vesicles with β-adrenergic receptors, the β-adrenergic agonist isoproterenol markedly stimulated the rate of quasi-irreversible binding of [35S]GTP{sub i}S to each G protein (Fig. 1). In both cases, binding was rapid and the agonist-stimulated reaction displayed an apparent first-order rate constant of 1.5-2 min{sup -1} under the conditions shown. These rates are typical of those observed in this study and those reported elsewhere for receptor-G{sub i} coupling or is a consequence of the reconstitution protocol.

The data of Fig. 2 indicate that the stimulation of GTP{sub i}S binding to G{sub s} displays β-adrenergic specificity. Selectivity among catecholamine agonists was appropriate for β- rather than α-adrenergic effects, and appropriate stereoselectivity was observed. (−)-Propranolol was a potent antagonist, but α-adrenergic blockers were ineffective at inhibiting the stimulation of binding by isoproterenol.

Receptor-mediated stimulation of the binding of GTP{sub i}S to G{sub s} was blocked efficiently if the vesicles were treated with

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Fig. 1. Isoproterenol-stimulated binding of [35S]GTP{sub i}S in receptor-G{sub s} vesicles (A) or receptor-G{sub i} vesicles (B). Vesicles were incubated at 30 °C in medium containing 20 mM Hepes (pH 8.0), 1 mM EDTA, 2 mM MgCl{sub 2}, 0.1 M NaCl, 1 mM diethiothreitol, 0.1 mM ascorbic acid, 0.1 µM [35S]GTP{sub i}S, and either 1 µM (−)-isoproterenol (●) or 0.1 µM (−)-propranolol (O). At the time indicated, aliquots were withdrawn into 2 volumes of cold quenching solution containing 20 mM Hepes (pH 8.0), 10 mM MgCl{sub 2}, 0.1 M NaCl, 0.1 mM GTP{sub i}S, 0.1 mM propranolol, 0.1% Lubrol 12A9, and 1 mM 2-mercaptoethanol (12). Nucleotide bound to the vesicles was separated by filtration on Schleicher & Schuell BA85 filters. The increments in binding caused by isoproterenol are also shown (△). The total amount of G protein in the vesicles was 85 fmol of G{sub i} (A) and 150 fmol of G{sub s} (B).

Fig. 2. Effect of adrenergic agents on the binding of [35S]GTP{sub i}S to receptor-G{sub s} vesicles. A, stimulation of binding by β-adrenergic agonists. Receptor-G{sub s} vesicles were incubated for 3 min at 30 °C as described in the legend to Fig. 1 and then assayed for bound nucleotide. The incubation volumes contained increasing concentrations of either (−)-isoproterenol (O), (+)-isoproterenol (●), (−)-epinephrine (△), or (−)-epinephrine (●) as shown. B, effect of α- and β-adrenergic antagonists on isoproterenol-stimulated binding of GTP{sub i}S to G{sub s}. Receptor-G{sub s} vesicles were incubated for 3 min at 30 °C with antagonist in assay medium containing 0.1 µM (−)-isoproterenol and the indicated concentrations of (−)-propranolol (O), phentolamine (●), or yohimbine (△).
NAD and IAP, a toxin from Bordetella pertussis that specifically ADP-ribosylates and inactivates $G_i$ (17) (Fig. 3). The effect of IAP was greatest on the agonist-stimulated binding reaction, where inhibition was 82% of the isoproterenol-stimulated increment above basal. This is in contrast to results obtained using soluble $G_i$, where little or no effect of IAP on treatment on GTP$\gamma$S binding was observed. Inhibition of the basal GTP$\gamma$S binding reaction in the vesicles was relatively slight. Treatment with IAP had no effect on the ability of the receptors to bind the $\beta$-adrenergic ligand $[^{3}H]$iodoacetylindolol. IAP is extremely selective for $G_i$ over $G_s$ (Ref. 17; also Ref. 18 and references in Ref. 1). Thus, the results of Fig. 3 indicate that the $\beta$-adrenergic stimulation of GTP$\gamma$S binding in the receptor-$G_i$ vesicles is not to contaminating $G_s$ in the $G_i$, or receptor preparations. As a further control, receptor-$G_i$ vesicles were assayed for the adenylate cyclase stimulatory activity that is characteristic of $G_s$. Receptor-$G_i$ vesicles were incubated with isoproterenol plus 0.1 $\mu$M GTP$\gamma$S at 30°C for 5 min and then reconstituted into the $G_i$-deficient plasma membranes of cyc- S49 lymphoma cells (16). In such experiments, the receptor-$G_i$ vesicles contained no $G_s$ at a lower level of detection of 1.6 mol% of the $G_i$ present, consistent with the results of dodecyl sulfate-polyacrylamide gel electrophoresis of the $G_i$ used to prepare the vesicles. Receptor-$G_i$ vesicles have been shown to display $\beta$-adrenergic catecholamine-stimulated GTPase activity (11), and the receptor $G_i$ vesicles display this activity as well (Fig. 4). The molar turnover number of $G_i$-catalyzed GTP hydrolysis was 0.33 min$^{-1}$ in this experiment, somewhat lower than that observed in receptor-$G_i$ vesicles. In two other experiments, turnover numbers were calculated to be 0.27 and 0.29 min$^{-1}$. The agonist-stimulated GTPase activity was inhibited 90% by treating the vesicles with IAP plus NAD. The basal GTPase activity was inhibited only slightly by IAP, as was observed in the GTP$\gamma$S binding experiment shown in Fig. 3. Thus, at a descriptive level, IAP appears to uncouple receptor from $G_i$ in the vesicles, as is the case in native membranes (see Ref. 18).

**DISCUSSION**

The data presented indicate that $\beta$-adrenergic receptors, which generally stimulate adenylate cyclase via the GTP-binding protein, $G_s$, can also stimulate the activation of the inhibitory GTP-binding protein, $G_i$. Stimulation was demonstrated as an increased rate of GTP$\gamma$S binding and an increased GTPase activity caused by $\beta$-adrenergic agonists. The blockade of these effects by treatment with IAP and NAD, the purity of the $G_i$ preparation, and the absence of any $G_s$ activity in the receptor-$G_i$ vesicles indicate that the GTPase activity and GTP$\gamma$S binding are due to $G_i$. The pharmacological specificity of stimulation indicates that observed effects are mediated via the $\beta$-adrenergic receptor. It is tempting to speculate on the possible physiological significance of $\beta$-adrenergic activation of $G_i$. However, it is possible that the unexpected interaction of $\beta$-adrenergic receptors and $G_i$ might reflect the partial denaturation of one or both proteins, the nonphysiological phospholipid environment, the phylogenetic distance between turkeys and rabbits, or some other oddity of our reconstituted system that minimizes the differences between two highly homologous but not totally identical proteins. Alternatively, the activation of $G_i$ by receptors that are normally considered to stimulate adenylate cyclase might represent an important regulatory mechanism. Murayama and Ui (19) have presented data suggesting that $\beta$-adrenergic stimulation of rat adipocyte membranes can lead to the inhibition of adenylate cyclase and that this inhibition is blocked by treatment with IAP. Similar data have been presented recently for platelets by Jakobs et al. (20). Both reports are consistent with the $\beta$-adrenergic stimulation of $G_i$. A different corollative experiment has been performed by Abramson and Molinoff. These authors found that $\beta$-adrenergic receptors in cyc- S49 lymphoma cell membranes bind the agonist $[^{3}H]$hydroxybenzylisopropenoterol with the high affinity that is usually ascribed to the receptor-$G_i$ complex (see Ref. 1 for review). This high-affinity binding was inhibited by the addition of GTP. However, cyc- cells lack functional $G_s$ and have normal levels of $G_i$ (Refs. 9, 10, 21, and 22; see Ref. 1 for review), and $G_i$ may promote the high-affinity agonist binding observed by these authors. The finding that cyc- cells can become desensitized either to $\beta$-adrenergic agonists or to prostaglandin E, (20) may also reflect an interaction of $G_i$ with stimulatory receptors. Thus, the interaction of $\beta$-adrenergic receptors with $G_i$ may be of importance in native membranes, perhaps acting as a mechanism to attenuate hormonal stimulation of the adenylate cyclase system. Such attenuation might be rapid and act as a buffer on stimulation, or it might be delayed and form the basis for a novel form of heterologous desensitization. $G_i$ might also act to bind receptors in a nonproductive complex, $4^S$. Abramson and P. Molinoff, personal communication.
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thereby causing receptor-specific, or homologous, desensitization. These questions can be addressed quantitatively in vesicles containing receptors and either G or Gα, or both G proteins. IAP and cholera toxin will be useful in the analysis of these effects in native membranes.

Acknowledgments—We are grateful to Professor Michio Ui for his generous gift of IAP. We thank E. A. Lawson for technical assistance and B. Moore for preparation of the manuscript.

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Activation of the inhibitory GTP-binding protein of adenylate cyclase, Gi, by beta-adrenergic receptors in reconstituted phospholipid vesicles.

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