Reconstitution of Catecholamine-stimulated Adenylate Cyclase Activity Using Three Purified Proteins*

David C. May†, Elliott M. Ross‡, Alfred G. Gilman, and Murray D. Smigel

From the Department of Pharmacology, Southwestern Graduate School of Biomedical Sciences, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

β-Adrenergic receptors, the GTP-binding regulatory protein that stimulates adenylate cyclase (G), and adenylate cyclase were each purified and reconstituted into unilamellar vesicles composed of phosphatidylethanolamine and phosphatidylserine (3:2, w/w). The molar ratio of receptor:adenylate cyclase was estimated to be about 1:10:1. Adenylate cyclase activity in the vesicles was stimulated up to 2.6-fold by β-adrenergic agonists. Stimulation was dependent on the presence of guanine nucleotide, displayed appropriate β-adrenergic selectivity and stereoselectivity for agonists, and was blocked appropriately by β-adrenergic antagonists. Therefore, while additional proteins may modulate adenylate cyclase activity in native membranes, these results show that these three proteins are sufficient for the expression of hormone-stimulated adenylate cyclase.

Hormone-sensitive adenylate cyclase is a membrane-associated multiprotein system that is responsible for regulating the synthesis of cyclic AMP in response to a variety of stimulatory and inhibitory hormonal signals. Stimulatory and inhibitory receptors modulate the activity of adenylate cyclase indirectly, via the guanine nucleotide-binding regulatory proteins, G, and G, (see Refs. 1 and 2 for review). G, the proximal activator of adenylate cyclase, has been purified from several sources. Its subunit structure, activation by guanine nucleotides, and ability to hydrolyze GTP to GDP have been described in some detail (2–4).

The β-adrenergic receptor is the only receptor purified to date that acts by stimulating adenylate cyclase (5, 6). It is a single, glycosylated polypeptide (6) that probably assumes a transmembrane orientation in its native state. In plasma membranes, receptor does not promote the activation of adenylate cyclase in the absence of G, (7); when G, is present, receptor interacts directly with it. Thus, when purified receptors and G, are reconstituted together into phospholipid vesicles, the addition of agonist stimulates the binding of guanine nucleotides and the hydrolysis of GTP by G, (8–11), and the addition of guanine nucleotide decreases the affinity of receptor for agonist (9). Reconstitution of the proteins into vesicles is necessary for these experiments because receptor and G, do not interact functionally in detergent solution (see Ref. 2 for references).

Adenylate cyclase (previously referred to as the catalyst, C, or the catalytic unit) catalyzes the synthesis of cyclic AMP. G, can bind to adenylate cyclase and stimulate its activity (12); Mn⁴⁺ (7) and forskolin (12–15) also stimulate its activity. The synthesis of forskolin-agarose (14) has facilitated the purification of adenylate cyclase (14, 15), and all three postulated components of the system are now available in pure form.

The complete reconstitution of hormone-sensitive adenylate cyclase activity has not yet been achieved using purified components. Cerione et al. (16) reported the reconstitution of agonist-stimulated synthesis of cyclic AMP using purified β-adrenergic receptor, G, and a detergent extract of cerebral cortex that contained adenylate cyclase activity from which G, activity had been removed. However, the number of relevant components in this fraction was not clear. Given the recent purification of adenylate cyclase (15), we have now undertaken the reconstitution of hormone-stimulated adenylate cyclase activity by incorporating the three purified proteins, receptor, G, and adenylate cyclase, into unilamellar vesicles. The appropriate regulation of cyclic AMP synthesis that is displayed in these vesicles demonstrates that only these proteins are required for hormone-stimulated adenylate cyclase activity. The receptor-G, -cyclase vesicles will allow the direct biochemical study of the interaction of these three proteins in a chemically defined bilayer in the absence of contaminating protein.

**EXPERIMENTAL PROCEDURES**

Materials—[³H]DHA was purchased from New England Nuclear. [³H]iodocyanopindolol was synthesized according to Engel et al. (17); (+)-cyanopindolol was a gift of G. Engel, Sandoz Pharmaceuticals. [³²P]GTP-S was purchased from New England Nuclear. Unlabeled GTPyS (Boehringer Mannheim) was repurified as described (11). [³²P]GTP and [α-³²P]ATP were prepared according to Johnson and Wallach (18). The purity of all guanine nucleotides was at least 97 %. Forskolin was purchased from Calbiochem-Behring, and 7-hemisuccinyl forskolin-agarose was prepared essentially as described by Pfenniger et al. (14, 15). CHAPS was purchased from Polyscience, Inc., or synthesized by the method of Hjelmelund (19). Lubrol 12A9 was a gift of ICI, Ltd., and was deionized by passage as a 10% solution over Dowex AG 501 (Bio-Rad) prior to use. Digitonin was purchased from Sigma. Lipids were purchased from Avanti Polar Lipids and were at least 95% pure by thin layer chromatography. Alprenolol was a gift of Hässle Pharmaceuticals. The sources of other reagents are listed elsewhere (8, 11, 15, 20, 21).

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‡ The abbreviations used are: G, G, and G, the stimulatory and inhibitory GTP-binding proteins, respectively, of the adenylate cyclase system; GTP-S, guanosine 5'-O-(3-thiotriphosphate); CHAPS, 3-(cholamidopropyl)dimethylammonio)-1-propanesulfonate; SDS, sodium dodecyl sulfate; DHA, dihydralprinolol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA, ethylene glycol bist(β-aminooethyl) ether)-N,N,N',N'-tetraacetic acid.
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Adenylate cyclase was purified from bovine cerebral cortex as described (15) using sequential affinity chromatography on homosuccinylforskolin-agarose and wheat germ lectin-agarose.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein</th>
<th>Specific activity*</th>
<th>Total activity</th>
<th>Recovery</th>
<th>Relative purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>Extract</td>
<td>2,240</td>
<td>2,970</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Forskolin-agarose</td>
<td>Lectin-agarose</td>
<td>0.067</td>
<td>310</td>
<td>21</td>
<td>7,020</td>
</tr>
</tbody>
</table>

*Adenylate cyclase was assayed in the presence of 5 mM Mn⁴⁺ and 0.1 mM forskolin.

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were mixed with phospholipid and chromatographed on Sephacryl of the applied phospholipid were eluted in the void volume. Activities in preparations of vesicles are shown in Table I. The absolute activities were dependent on the amounts of each protein added to the mixture used for the reconstitution. The reconstitution of these three proteins into vesicles resulted in the ability of β-adrenergic agonists to stimulate adenylate cyclase activity (Fig. 3 and Table II). The relative selectivity for the (+)- isoproterenol (INE/GTP), or 10 mM NaF plus 50 μM AICl3 (NaF). Assayed by [3H]DHA binding. Assayed by maximal [35S]GTPγS binding in the presence of 50 mM MgCl2 and Lubrol. Assayed in the presence of 6 mM MnCl2 plus 0.1 mM forskolin. *NA, not assayed.

### RESULTS

When β-adrenergic receptors, Gα, and adenylate cyclase were mixed with phospholipid and chromatographed on Sephadex G-50 as described above, each activity and essentially all of the applied phospholipid were eluted in the void volume fraction that contained unilamellar vesicles of diameters 200–500 nm (Fig. 2). Enzymatic and β-adrenergic ligand binding activities in preparations of vesicles are shown in Table II. The absolute activities were dependent on the amounts of each protein added to the mixture used for the reconstitution.

The reconstitution of these three proteins into vesicles resulted in the ability of β-adrenergic agonists to stimulate adenylate cyclase activity (Fig. 3 and Table II). The relative stimulation caused by isoproterenol in the experiment shown in Fig. 3, 2.3-fold, was typical of that seen in 12 reconstitutions using different preparations of receptor, Gα, and adenylate cyclase. The stimulation of activity by (+)-isoproterenol required the presence of a guanine nucleotide (Fig. 3) and was blocked by the β-adrenergic antagonist (−)-propranolol (Fig. 4). Propranolol by itself had no effect on activity in the absence of agonist. Stimulation of activity by isoproterenol displayed a 100-fold selectivity for the (+)- over the (−)-isomer, and (−)-epinephrine and terbutaline were 10- and 100-fold less potent, respectively, as agonists than was (+)-isoproterenol (Fig. 4).

The data above indicate that only three proteins (a receptor for a stimulatory agonist, Gα, and adenylate cyclase) are sufficient to display guanine nucleotide-dependent, agonist-
stimulated adenylate cyclase activity. In the experiments described in Table II, vesicles were prepared with only one or two of the three proteins to test the requirements for each. As shown, the absence of any one of the three components resulted in the predictable loss of this regulatory pattern (Table II). Omission of receptor caused the loss of response to agonist but did not alter the Go-mediated response to ATP$^+$ plus F$, and the omission of added Go eliminated stimulation by these compounds. The small stimulation (15%) by isoproterenol plus forskolin that was observed in the vesicles that contained only adenylate cyclase and Go was not a reproducible observation. It may reflect contamination of the adenylate cyclase by a small amount of the $\alpha$ subunit of Go (15).

The recovery of receptor in the vesicles was generally 20–45% based on [3H]DA binding, and the usual concentration in the eluted volume was 1–8 nM. The recovery of Go was usually 75–90%, yielding a typical concentration 10–50 nM. The recovery and molar concentration of adenylate cyclase are more difficult to quantitate because of the dependence of this activity on the lipid or detergent present during the assay. We have defined 100% recovery as equal to the amount of activity present when stock solutions of adenylate cyclase were diluted 250-fold into a detergent-free assay mixture. When normalized to this value, the recovery of activity in the vesicles was typically 20–50%. When the adenylate cyclase was diluted into buffer containing 0.1% Tween 60 (15), its activity was 25–75% higher than that observed in buffer alone. Based on the amount of protein initially present and the molecular weight of 120,000 and using the recovery of activity as a reference, we estimate that the vesicles typically contained about 1–3 nM adenylate cyclase.

The recoveries of receptor and Go activities were relatively constant in multiple preparations of vesicles and were not altered by the presence or absence of adenylate cyclase. The recovery of adenylate cyclase activity, assayed in the presence of Mn$^{2+}$ plus forskolin, was increased up to 2-fold when the vesicles also included Go (data not shown). This may reflect potentiation of the effects of forskolin by Go, or stabilization of adenylate cyclase.

Stimulation of adenylate cyclase activity by agonist in the receptor-Go-cyclase vesicles required the presence of Mg$^{2+}$, with maximum response to agonist occurring at about 3 mM free Mg$^{2+}$ (data not shown). Increasing the concentration to 10 mM decreased stimulation slightly. The presence of a guanine nucleotide was also required for hormonal stimulation. GTP was half-maximally effective in allowing stimulation by agonist at approximately 0.1 $\mu$M. GTP alone stimulated activity only slightly (Table II). GTP$^\gamma$S activated adenylate cyclase in the absence of agonist, usually to a maximal activity slightly greater than that caused by isoproterenol plus GTP at their optimal concentrations. We have observed a modest stimulation by isoproterenol in addition to GTP$^\gamma$S, usually to 20–30% above the activity observed in the absence of the agonist.

The receptor-Go-cyclase vesicles displayed relatively low GTPase activity in the absence of agonist, and activity was stimulated approximately 6-fold by the addition of isoproterenol. The molar turnover number of the GTPase in the presence of agonist, normalized according to the amount of agonist-stimulated [3H]GTP binding, was approximately 0.4 mol of GTP hydrolyzed per min-mol of Go. This is similar to the turnover number of 0.3 mol of GTP hydrolyzed per min-mol of Go observed in receptor-Go vesicles assayed under the same conditions (8, 21). This is not unexpected because the molar ratio of Go to cyclase in the vesicles was high (approximately 10:1), and any effect of adenylate cyclase on the GTPase activity would probably be too small to detect.

**Discussion**

This report demonstrates the functional reconstitution of hormone-sensitive adenylate cyclase activity using purified proteins. During this series of experiments, we have used three preparations of $\beta$-adrenergic receptor, two preparations of Go, and five preparations of adenylate cyclase in 12 successful reconstitutions. As expected, vesicles deficient in any protein component demonstrated a predictable loss of hormone-stimulated activity, with the interruption localized to the functional event mediated by the absent protein (Table II). Although any of these preparations may have contained 3–10% impurities, it is unlikely that any specific contaminating protein was required for the hormone-stimulated cyclic AMP synthesis that was seen in each case. The use of less pure preparations during the development of these procedures did not yield higher levels of activity or stimulation by agonists. These data indicate that the three purified proteins used in these reconstitutions are the only proteins required for hormone-stimulated adenylate cyclase activity.

The relative extent of the receptor-mediated stimulation of adenylate cyclase in the reconstituted system requires comment. The extent of stimulation by agonist plus GTP was typically 10–12% (range 8–15%) of the maximum assayed in the presence of Mn$^{2+}$ plus forskolin; the fluoride-stimulated activity was 18–20% (range 11–22%) of the maximum (Table II). This indicates that the hormone-stimulated adenylate cyclase activity represented a significant portion of the enzyme that was available to Go. In addition, the extent of stimulation by isoproterenol was similar to that seen in a variety of membrane preparations relative to their maxima assayed with forskolin (see Ref. 29, for example). The reconstituted system compares particularly well with cerebral cortical adenylate cyclase, which is relatively unresponsive to agonists in membrane preparations (see Ref. 30, for example). However, the extent of stimulation relative to basal activity, less than 3-fold in the vesicles, is low when compared to membranes from many other tissues. This may reflect the use of proteins from three different tissues and species (two
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phylogenetic families. The heterologous nature of this reconstituted system is further confirmation that there is essential structural conservation of this system throughout the vertebrates.

Other proteins are of course involved in the cellular regulation of the synthesis of cyclic AMP. These include receptors for inhibitory hormones, G, calmodulin, cytoskeletal components, and perhaps others. Regardless, study of this purified system, which contains the essential elements of the stimulatory pathway, should provide basic information on the biochemical control of cyclic AMP synthesis and form the basis for the study of the regulatory roles of the other proteins.

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REFERENCES