Purification and Characterization of a Soluble Form of Mammalian Adenylyl Cyclase*

An engineered, soluble form of mammalian adenylyl cyclase has been expressed in Escherichia coli and purified by three chromatographic steps. The enzyme utilizes one molecule of ATP to synthesize one molecule of cyclic AMP and pyrophosphate at a maximal specific activity of 12.8 mol/min/mg, corresponding to a turnover number of 720 min⁻¹. Although devoid of membrane spans, the enzyme displays all of the regulatory properties that are common to mammalian adenylyl cyclases. It is activated synergistically by Gsα and forskolin and is inhibited by adenosine (P-site) analogs with kinetic properties that are identical to those displayed by the native enzymes. The purified enzyme is also inhibited directly by the G protein βγ subunit complex. After adenovirus-mediated expression in adenyl cyclase-deficient HC-1 cells, the enzyme can be stimulated synergistically by Gα-coupled receptors and forskolin.

Ten isoforms of mammalian adenylyl cyclase have been identified during the past 7 years, and these enzymes display a complex array of both shared and distinct regulatory properties. All members of the family are activated by the α subunit of the heterotrimeric G protein Gsα, and receptor-mediated liberation of the GTP-bound form of Gsα is the dominant mechanism for stimulation of cyclic AMP synthesis. Furthermore, all of the characterized mammalian adenylyl cyclases are activated by the diterpene forskolin (3); stimulation of catalysis by forskolin and Gsα is highly synergistic in many cases (type II and IV–VI adenylyl cyclases). Finally, all family members are also inhibited by adenosine analogs known as P-site inhibitors (4). The physiological significance of P-site inhibition is not clear, but at least some relevant compounds appear to be present at sufficient concentrations in vivo to affect adenylyl cyclase activity (5).

Each mammalian adenylyl cyclase is thought to contain a short and variable amino terminus, followed by two repeats of a unit composed of six transmembrane spans and a large (∼40 kDa) cytosolic domain (6). Although there is little sequence homology among the various enzymes within the transmembrane spans, the overall identity of the different adenylyl cyclases within the cytosolic regions (denoted C1a and C2a) ranges from 50 to 90%. The most highly conserved sequences are within the amino-terminal half of each cytosolic domain (C1a and C2a). Furthermore, these domains are ∼50% similar to each other (within a single adenylyl cyclase) and to the catalytic domains of the guanylyl cyclases.

Attempts to understand the mechanisms of regulation of adenylyl cyclases and to relate their common and distinct features to structure have been frustrated by both low levels of expression and the requirement for detergent for solubility. To overcome these hurdles, Tang and Gilman (7) constructed a soluble adenylyl cyclase by linkage of the C1a domain of the type I enzyme with the C2 domain of the type II enzyme. Expression of this molecule in Escherichia coli complemented the catabolic defect in an adenylyl cyclase-deficient strain of the bacterium. The crude lysate of such bacteria displayed adenylyl cyclase activity that was activated synergistically by Gsα and forskolin and inhibited by 2′,3′-AMP,1 a potent P-site analog. To characterize this molecule further, we have developed methods to improve its expression and to purify it in a hexahistidine-tagged form. The basic biochemical properties of this protein are described herein.

**MATERIALS AND METHODS**

Plasmid Construction—The expression vector pTrc6h6 was created by ligation of phosphorylated linkers (5'-CATGATCCACCATCCACCATCAGCCTGCAAGA and 5'-CATCTCCATCCGCCTACGATGG-GATGTGATGGTGAGTGATGGTAGGAT) with Nco1- and BamHI-digested pTrcHisA (Invitrogen, San Diego, CA). To produce a construct in frame with the hexahistidine tag, the 1.64-kilobase BspHI-HindII fragment of pTrc6h6 was ligated with pTrcHisA (Invitrogen, San Diego, CA) to produce a construct in frame with the hexahistidine tag. The 1.64-kilobase BspHI-HindII fragment of pTrc6h6 was also cloned into the Ncol- and HindII-digested H6pQE60 vector (8). The adenovirus shuttle vector pH273111L3-ACCMV was created by ligation of the 1.6-kilobase EcoRI-KpnI fragment from H6pQE271111L3 with the vector ACCMV after digestion with the same enzymes.

Antibodies—Two peptides were synthesized corresponding to amino acid sequences in the C1a domain of type I adenylyl cyclase and the C2a domain of type II adenylyl cyclase: IC1-452 (CGDYEVEPGHGHERNSF) and IC2-570 (CSRLKNEELHOSYD). The peptides were coupled to the purified protein derivative of tuberculin (Stantens Seruminstitut, Copenhagen) (9). Antibodies were produced in New Zealand White rabbits and affinity-purified as described (10).

G Protein Subunits—Recombinant Gsα was purified from E. coli as described (8) and activated by incubation with 50 mM NaHepes (pH 8.0), 10 mM MgSO4, 1 mM EDTA, 2 mM dithiothreitol, and 400 μM GTP-S at 30 °C for 30 min. Free GTP-S was removed by gel filtration. Recombinant β1γ2 and nonrecombined β1γ2 Cy5-labeled Ser were purified from Sf9 cells as described (11, 12).

Purification of the Soluble Form of Adenylyl Cyclase—Luria broth medium (18 liter) was inoculated with 180 ml of log-phase E. coli BL21(DE3) transformed with H6pTrc271111L3 cells were grown for 1 h at 30 °C and then at room temperature until they reached an A600 of 0.4. Isopropyl-1-thio-β-galactopyranoside (20 μM) was added, and cells were harvested 15 h later. The cells were washed with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and mixed protease inhibitors and then

1 The abbreviations used are: 2′,3′-AMP, 2′-deoxyadenosine 3′-monophosphate; GTP-S, guanosine 5′-O-(thio)triphosphate; NTA, nickel tridentate acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid.

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fractioned in liquid N2. The mixed protease inhibitors used were 22 mg/liter each L-1-tosylamide-2-phenylthyl chloromethyl ketone, 1-choro-3-to- sylamido-7-amin-2-heptanone, and phenylmethylsulfonyl fluoride, plus 3.2 mg/liter each leupeptin and lima bean trypsin inhibitor.

The frozen cells were resuspended with a Polytron in 1.5 liters of lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 50 mM NaCl, 0.5 mM apotinin, and a concentration of medium and addition of 0.2 mg/ml lysozyme while stirring. After 30 min at 4°C, 32 mg of DNase and 5 mM MgCl2 were added, and the suspension was incubated for 30 min. The membrane and cellular debris were pelleted by centrifugation for 30 min at 100,000 × g. The supernatant was supplemented with NaCl (250 mM final concentration) and loaded onto a Mono Q HR 10/10 fast protein liquid chromatography column. The Ni2+-NTA eluate was filtered and loaded onto a Mono Q HR 10/10 fast protein liquid chromatography column. The column was washed with 40 ml of buffer C (20 mM NaHepes (pH 8.0), 2 mM MgCl2, 1 mM EDTA, and 2 mM diithiothreitol) and then eluted with a gradient of 0–400 mM NaCl.

Adenylyl Cyclase Assays—Adenylyl cyclase activity was measured as described by Smigel (13). All assays were performed for 15–20 min at 30°C in a final volume of 100 μl unless stated otherwise. The final concentration of MgCl2 was 10 mM. Adenylyl cyclase and G proteins were first incubated on ice in a total volume of 40 μl. Incubations involving the kinase P-site inhibitors and determination of Km were initiated by the addition of enzyme. Assays that contained G protein β3y subunits were performed in the presence of 1.2 mM CHAPS.

To quantitate both the reactants and products of the adenylyl cyclase reaction, incubations were performed in the absence of an ATP-regenerating system with either α-32P- or γ-32P-labeled ATP. γ-32P-ATP was used to measure production of pyrophosphate using a modification of the method of Hayaishi et al. (21) in which charcoal was equilibrated with 50 mM sodium phosphate and 50 mM pyrophosphate; this permitted the complete recovery of both molecules. α-32P-ATP was used to quantitate loss of ATP and production of cyclic AMP after their separation by Dowex and alumina chromatography (15). A portion of each reaction mixture and the separated products were analyzed by thin layer chromatography in TB buffer to determine their purity and quantify contaminating ATP or the labeled ATP. Thin layer chromatography demonstrated no production of phosphate during the course of the reaction. Recovery of cyclic AMP during chromatography was determined by quantitation of [γ32P]AMP, which was added at the end of the reaction.

Production of Recombinant Adenovirus—The HC-1 and 293 cell lines were maintained in Dulbecco’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated calf serum. To transfect the HC-1 and 293 cell lines, the adenovirus vector (pAd5ΔE1ΔE3) containing the IC1 domain were not detected with antibody IIC2-870 (Fig. 1A, B), which contains the IC1 domain were not detected with antibody IC1-454 (data not shown).

Expression and Purification of the Soluble Adenylyl Cyclase—Expression of the hexahistidine-tagged soluble adenylyl cyclase (H6(271L),I1,L,L3) in E. coli results in the accumulation of ~2–5 nmol of adenylyl cyclase activity/min/mg in the cytosol. However, >90% of the adenylyl cyclase protein is found in inclusion bodies and cannot be solubilized in an active form. Growth of the bacteria at room temperature permitted the maximal accumulation of soluble and active protein. Concurrent expression of thioredoxin or the chaperonin GroEL and GroS increased the amount of adenylyl cyclase protein in the cytosol (detected by immunoblotting), but failed to increase adenylyl cyclase activity in these cells (data not shown). A proteolysis product representing the IC1b domain (detected by antibody IIC2-870) also accumulated (Fig. 1A), but fragments containing the IC3 domain were not detected with antibody IC1-454 (data not shown).

The soluble adenylyl cyclase activity in the lysate can be enriched 50-fold using Ni2+-NTA chromatography (Table I). The yield is only ~30%, and much of the lost activity is not found in the flow-through or washes of the column. The protein is susceptible to proteases in crude extracts, and rapid manipulation at this stage improves recovery. Chromatography over the Mono Q column results in only a 3-fold purification, but several major contaminants are removed. The low enrichment is due to a broad elution profile on this and many other columns (Fig. 1C). The protein is eluted from the final (phenyl-Superose) column with CHAPS in the absence of salt. Although the detergent can be removed with retention of enzymatic activity, the stability of the protein is greatly enhanced by CHAPS during freezing and thawing. The soluble adenylyl cyclase might be a chimera and lacks its integral cathodic electrophoretic mobility (data not shown). The yield is only ~30%, and much of the lost activity is not found in the flow-through or washes of the column. The protein is susceptible to proteases in crude extracts, and rapid manipulation at this stage improves recovery. Chromatography over the Mono Q column results in only a 3-fold purification, but several major contaminants are removed. The low enrichment is due to a broad elution profile on this and many other columns (Fig. 1C). The protein is eluted from the final (phenyl-Superose) column with CHAPS in the absence of salt. Although the detergent can be removed with retention of enzymatic activity, the stability of the protein is greatly enhanced by CHAPS during freezing and thawing. The soluble adenylyl cyclase might be a chimera and lacks its integral cathodic electrophoretic mobility (data not shown).
membrane domains, it displays most of the regulatory features that are characteristic of its native counterparts. The enzyme has very low basal activity (20–50 nmol/min/mg), but can be stimulated 100-fold by GTP$^g$$S$-Gs or forskolin. The $K_m$ for Mn$^{2+}$-ATP is 50 $\mu$M and is little affected by the presence of enzyme activators. However, the $K_m$ for Mg$^{2+}$-ATP remains relatively low when the enzyme is stimulated by GTP$^g$$S$-Gs (65 $\mu$M), but increases to 620 $\mu$M in the presence of forskolin (Fig. 3). When both GTP$^g$$S$-Gs and forskolin are present, the $V_{max}$ is 12.8 $\mu$mol/min/mg, and the $K_m$ remains elevated (300 $\mu$M). Similar changes have been observed with adenylyl cyclase from bovine brain (22) or with type I adenylyl cyclase expressed in SF9 cells (23); the latter enzyme has a $K_m$ of 38 $\mu$M with Mn$^{2+}$ and forskolin and a $K_m$ of 440 $\mu$M with Mg$^{2+}$ and forskolin. Hill plots of the data in Fig. 3 were linear and gave a value of $n = 1.0$ with each activator (data not shown).

The turnover number for the soluble chimeric adenylyl cyclase is similar to the values for the native forms of the enzyme.
The turnover number is 360 min⁻¹ in the presence of forskolin and increases to 720 min⁻¹ with both forskolin and GTPγS-Gsα. The turnover numbers for the purified type I and II adenylyl cyclases are 890 and 260 min⁻¹, respectively, in the presence of forskolin and Mn²⁺ (24). The low Vₘₐₓ for the GTPγS-Gsα-stimulated soluble adenylyl cyclase (Fig. 3) is due only to the amount of GTPγS-Gsα used in the assay compared with the EC₅₀ for activation by Gsα (Fig. 4A). With maximal amounts of GTPγS-Gsα, the soluble adenylyl cyclase displays a Vₘₐₓ approaching that observed in the presence of both GTPγS-Gsα and forskolin (Fig. 4A).

The affinity of the soluble adenylyl cyclase for activated E. coli-derived Gsα is 100-fold lower than that of type I adenylyl cyclase (Fig. 4A) (25). However, forskolin potentiates the effect of low concentrations of GTPγS-Gsα, shifting the apparent affinity of the soluble adenylyl cyclase for GTPγS-Gsα by 2 orders of magnitude. For example, adenylyl cyclase activity was 9.2 µmol/min/mg in the presence of 225 nM GTPγS-Gsα and 2 µM forskolin, while the activities in the presence of these activators alone were 1.2 and 0.8 µmol/min/mg, respectively. This striking synergy is typical of type II adenylyl cyclase, but is not characteristic of the type I enzyme (25, 26). Half-maximal activation of the soluble adenylyl cyclase was observed with 3 µM forskolin (Fig. 4B), the same concentration required for type I and II adenylyl cyclases (22). Low concentrations of GTPγS-Gsα shift the apparent affinity of forskolin by 2 orders of magnitude. Unlike the situation with GTPγS-Gsα, maximal activity in the presence of forskolin is significantly less than that observed with GTPγS-Gsα or GTPγS-Gsα plus forskolin (Fig. 4B).

Ca²⁺-calmodulin had no stimulatory effect on the soluble adenylyl cyclase (data not shown); calmodulin activates type I adenylyl cyclase (27). The affinity of the soluble adenylyl cyclase for activated E. coli-derived Gsα is 100-fold lower than that of type I adenylyl cyclase (Fig. 4A) (25). However, forskolin potentiates the effect of low concentrations of GTPγS-Gsα, shifting the apparent affinity of the soluble adenylyl cyclase for GTPγS-Gsα by 2 orders of magnitude. For example, adenylyl cyclase activity was 9.2 µmol/min/mg in the presence of 225 nM GTPγS-Gsα and 2 µM forskolin, while the activities in the presence of these activators alone were 1.2 and 0.8 µmol/min/mg, respectively. This striking synergy is typical of type II adenylyl cyclase, but is not characteristic of the type I enzyme (25, 26). Half-maximal activation of the soluble adenylyl cyclase was observed with 3 µM forskolin (Fig. 4B), the same concentration required for type I and II adenylyl cyclases (22). Low concentrations of GTPγS-Gsα shift the apparent affinity of forskolin by 2 orders of magnitude. Unlike the situation with GTPγS-Gsα, maximal activity in the presence of forskolin is significantly less than that observed with GTPγS-Gsα or GTPγS-Gsα plus forskolin (Fig. 4B).
adenylyl cyclase. However, the C1β region of the type I enzyme that has been implicated in calmodulin binding is not present in the soluble protein studied here (27–29).

Inhibitors of the Soluble Adenylyl Cyclase—Type I and II adenylyl cyclases differ dramatically in their responses to Gα proteins and the G protein βγ subunit complex. Gα16, Gα20, and βγ all inhibit type I adenylyl cyclase (1, 30). The type II enzyme is not affected by Gαi, and is greatly stimulated by βγ in the presence of Gai (1, 30). The chimeric type IC1β-typeIIC2 soluble enzyme is unresponsive to Gαi, even at 10 μM concentrations (data not shown), but it can be inhibited almost completely by βγ (Fig. 5). This is surprising since the extent of inhibition of purified type I adenylyl cyclase by βγ is modest (30%) compared with the more dramatic inhibition of the membrane-bound protein (24). Inhibition of the soluble adenylyl cyclase is not due to interaction of βγ with Gai since βγ inhibits forskolin- and Gαi-activated enzymatic activity equally. The apparent affinity of the soluble adenylyl cyclase for βγ is substantially lower (10-fold or more) than that of type I or II adenylyl cyclase. However, inhibition is still dependent on prenylation of the γ subunit since the nonprenylated βγγγ-Cys68→Ser mutant was inactive at the highest concentrations tested (Fig. 5).

Chimeras have been used successfully to identify regions necessary for activation of type I and II adenylyl cyclases by calmodulin and protein kinase C, respectively (31). By coexpression of combinations of membrane-bound halves of type I and II adenylyl cyclases (noncovalent chimeras), Tang and Gilman (30) tentatively assigned the site for synergistic activation of adenylyl cyclase by βγ to the carboxy-terminal half of the molecule. Chen et al. (32) made a peptide corresponding to the sequence of a region in the C2 domain of type II adenylyl cyclase that blocks the interactions of βγ with several effectors, consistent with the assignment made by Tang and Gilman. It is thus perhaps surprising that the soluble adenylyl cyclase described herein is inhibited by βγ rather than activated. However, we are hesitant to draw further conclusions about this phenomenon until comparisons can be made with nonchimeric soluble forms of the type I and II enzymes and until binding interactions between βγ and these domains can be examined definitively.

It is not clear why Gai and βγ have reduced affinities for the soluble adenylyl cyclase relative to its membrane-bound counterparts or what role the membrane spans play in signal transduction. βγ is associated with the plasma membrane, at least in part because of prenylation of γ. Its apparent higher affinity for membrane-bound adenylyl cyclase may be due partially to the concentrating effect of membrane localization. It is also possible that βγ interacts directly with the membrane-spanning regions of adenylyl cyclase or normally interacts with both the C1 and C2 domains and that these interactions are constrained in the chimeric molecule. Despite these uncertainties, the fact that nonprenylated βγ failed to inhibit the soluble adenylyl cyclase points to specific interactions that are dependent on the lipid modification, rather than to simple concentration at the membrane. Similar arguments may apply to Gαi. However, the recombinant (E. coli-derived) Gαi used here does not contain lipid modifications and already has a lower affinity for membrane-bound adenylyl cyclases than does tissue-derived Gαi (33). All mammalian adenylyl cyclases are activated by Gαi in vitro with few quantitative differences. It thus seems unlikely that the chimeric nature of the soluble enzyme would contribute to the loss of affinity. Again, it seems most likely that domains of adenylyl cyclase removed in the engineered protein (e.g. loops between membrane spans, C1β, etc.) or conformational constraints imposed by linkage of C1 and C2 are responsible for the loss of affinity.

Natural, membrane-bound mammalian adenylyl cyclases are inhibited directly by adenosine analogs known as P-site inhibitors, so designated in reference to the requirement for an intact purine ring (4). The most potent inhibitors are 2'- or 5'-deoxy and 3'-phosphoryl compounds (34). P-site inhibition displays several unique features. Stimulated forms of the enzyme are substantially more sensitive to inhibition than nonactivated forms. Inhibition is dependent on metal and is characteristically noncompetitive or mixed noncompetitive with respect to metal-ATP (35–37). However, inhibition of the

FIG. 5. Inhibition of adenylyl cyclase by recombinant G protein βγγγ. The Soluble adenylyl cyclase (3.6 nM) was assayed for 20 min with 10 mM MgCl2, 1 mM ATP, and the indicated concentrations of βγ in the presence of 400 nM GTPγS-Gαi (●) or 50 μM forskolin (Fsk ▲). The effects of βγγγ-Cys68→Ser (△) were tested in the presence of GTPγS-Gαi. Activities are expressed as percentages of control values measured in the absence of βγ; 0.4 and 1.0 μmol/min/mg for GTPγS-Gαi and forskolin, respectively. All determinations were performed in duplicate and are representative of three experiments.

FIG. 6. Inhibition by the P-site inhibitor 2'-d3-AMP. Assays were performed with 0.5 mM ATP for 15 min plus the following: 400 nM GTPγS-Gαi (●), 50 μM forskolin (Fsk ▲), 100 nM GTPγS-Gαi plus 50 μM forskolin (▲), or 2 mM MnCl2 (▼). Control activities for these conditions were 1.1, 1.4, 4.5, and 0.05 μmol/min/mg protein, respectively. All determinations were performed in duplicate and are representative of two experiments.
Gs-activated enzyme is noncompetitive with respect to Mg2+-ATP (38). All of these features are preserved in the soluble adenylylcyclase. This enzyme is significantly more sensitive to 2'-d3'-AMP when activated by forskolin, GTPγS-Gsα, or the combination of the two regulators than when assayed with Mn2+ alone (Fig. 6). Thus, the GTPγS-Gsα- and forskolin-stimulated enzyme, which is 100-fold more active than the Mn2+-stimulated enzyme, is 300 times more sensitive to P-site inhibition (IC50 = 3 µM versus 1 mM). The IC50 of bovine brain adenylylcyclase for 2'-d3'-AMP is 2-3 µM in the presence of forskolin or GTPγS-Gsα (35).

P-site inhibition of the GTPγS-Gsα-stimulated soluble adenylylcyclase is noncompetitive with respect to Mg2+-ATP. This is also true of detergent-solubilized adenylylcyclase activity in rat brain (38). Noncompetitive inhibition is rare in unireactant systems and is characterized by a lower Vmax, a decreased apparent Kᵣ, and an unchanged Kᵣ/Vmax. The interpretation is that the inhibitor binds only to the enzyme-substrate complex, yielding an inactive enzyme. All other activated forms of the soluble adenylylcyclase show a mixed noncompetitive pattern of inhibition with respect to metal-ATP, where both 1/Vmax and Kᵣ/Vmax increase with increasing inhibitor concentrations (Fig. 7, B–D). True noncompetitive inhibition implies that the inhibitor binds with equal affinity to the free enzyme and the enzyme-substrate complex; inhibitor binding is thus independent of substrate concentration, and the Kᵣ remains constant. Vmax, Kᵣ, and Vmax/Kᵣ all decrease with increasing concentrations of inhibitor when the soluble adenylylcyclase is stimulated with forskolin (Mg2+ or Mn2+) or GTPγS-Gsα in the presence of Mn2+.

The x intercepts of replots of 1/Vmax or Kᵣ/Vmax versus the concentration of 2'-d3'-AMP represent the values of aKᵣ and Kᵣ, respectively, where Kᵣ is the dissociation constant of 2'-d3'-AMP from the enzyme-inhibitor complex and aKᵣ is the dissociation constant of 2'-d3'-AMP from the enzyme-substrate-inhibitor complex (Table II) (39). The calculated aKᵣ values correspond to the observed IC50 values for P-site inhibition and correlate well with those shown in Fig. 6. The factor α is equal
to 1 in the case of pure noncompetitive inhibition, >1 when the inhibitor favors binding to the free enzyme, and <1 when the inhibitor favors binding to the enzyme-substrate complex. The values of $\alpha$ determined here are <1 in all cases, showing a clear preference of the inhibitor for the adenyl cyclase-ATP complex compared with adenyl cyclase alone. Despite these observations, it has not been possible to make a simple assignment of regulatory or catalytic function to the C1 or C2 domain by a mutagenic approach (23).

The role of metals in influencing $K_m$, $V_{\text{max}}$, and the inhibition patterns is complex. $\text{Mn}^{2+}$ (compared with $\text{Mg}^{2+}$) decreases dramatically the $K_m$ for ATP in the presence of forskolin, but has no effect on the $K_i$ for a P-site inhibitor with this activator. When GTP/S-Ge, is the activator, the type of metal determines if $2'$,5'-diAMP binds solely to the adenyl cyclase-ATP complex or to both this complex and free adenyl cyclase. $\text{Mn}^{2+}$ can also influence the $V_{\text{max}}$, usually increasing activity by ~2-fold. Whether the metal causes each of these changes by working at the active site, an alternative metal-binding site, or both is still unclear.

**Adenovirus-mediated Synthesis of the Soluble Adenylyl Cyclase in HC-1 Cells**—The rat hepatoma HC-1 cell has no detectable adenylyl cyclase activity, but contains $G_s$ and binding activity characteristic of the $\beta$-adrenergic receptor (40). Although these cells constitute an ideal null background in which to express adenylyl cyclase, they are difficult to transfect. We thus constructed a recombinant adenovirus in which expression of the soluble adenylyl cyclase is driven by the cytomegalovirus early gene promoter. After infection of cells at virus/cell ratios of 10:1 to 40:1, adenylyl cyclase activity (0.7 nmol/min/mg) and immunoreactivity were detectable. We have used this system to study receptor-mediated cyclic AMP synthesis in vivo. An adenovirus encoding $\beta$-galactosidase was utilized as a control.

There was no detectable accumulation of cyclic AMP in noninfected HC-1 cells or in those expressing $\beta$-galactosidase after exposure to isoproterenol and/or forskolin (Fig. 8). Similarly, cyclic AMP did not accumulate in cells expressing the soluble adenylyl cyclase after treatment with isoproterenol alone. However, there was substantial accumulation of cyclic AMP in such cells after incubation with forskolin, and, for data averaged over five time points, isoproterenol further enhanced cyclic AMP accumulation by 50%. Despite the absence of all transmembrane-spanning sequences, the soluble adenyl cyclase can be activated by a G protein-coupled receptor when its apparent affinity for $G_s$ is increased by forskolin.

The failure to detect stimulation of cyclic AMP synthesis in the presence of isoproterenol alone was unexpected. The apparent affinities of the soluble adenylyl cyclase for the stimulator $G_s$ and the inhibitor $\beta Y$ are similar. However, in the presence of forskolin, the apparent affinity of the enzyme for $G_s$ is increased dramatically, while that for $\beta Y$ is not altered. Furthermore, the soluble adenylyl cyclase studied here is not unique in its failure to respond to $G_s$ in vivo in the absence of forskolin. When expressed by transfection, several membrane-bound adenyl cyclases have responded only to synergistic combinations of forskolin and appropriate receptor agonists (31, 41-43).

Since the soluble adenylyl cyclase studied here displays most of the regulatory features that are characteristic of the membrane-bound enzymes (and cytosolic domains necessary for other phenomena, such as activation by calmodulin and inhibition by $G_s$, may be missing), one wonders if the membrane spans of the native enzymes play only quantitative roles and are not important qualitatively for regulation of cyclic AMP synthesis. If so, what is the explanation for the complex topology of these enzymes, which is conserved in all of its known isoforms? The best current hypothesis is that of Reddy et al. (44), who have proposed that the activity state of at least certain mammalian adenylyl cyclases may be responsive to transmembrane potential.

In summary, a soluble form of mammalian adenylyl cyclase that displays all of the regulatory features that are common to the various isoforms of the enzyme can be synthesized in $E. coli$ and purified. This approach offers many advantages over study of the native enzymes, which can be obtained only in microgram quantities from native sources or after expression in heterologous systems. We hope that the availability of this
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