

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

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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 $\mu\text{mol}/\text{min}/\text{mg}$, 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 $G_{s\alpha}$ and forskolin and is inhibited by adenosine (P-site) analogs with kinetic patterns that are identical to those displayed by the native enzymes. The purified enzyme is also inhibited directly by the G protein $\beta\gamma$ subunit complex. After adenovirus-mediated expression in adenylyl cyclase-deficient HC-1 cells, the enzyme can be stimulated synergistically by G_s -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 (1, 2). All members of the family are activated by the α subunit of the heterotrimeric G protein G_s , and receptor-mediated liberation of the GTP-bound form of $G_{s\alpha}$ 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 $G_{s\alpha}$ 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 C_1 and C_2) ranges from 50 to 90%. The most highly conserved sequences are within the amino-terminal half of each cytosolic domain (C_{1a}

and C_{2a}). 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 both by 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 C_{1a} domain of the type I enzyme with the C_2 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 $G_{s\alpha}$ and forskolin and that was inhibited by 2'-d3'-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 pTrcH6 was created by ligation of phosphorylated linkers (5'-CATGCATCACCATCACCAT-CACGCCGCCATGGA and 5'-GATCTCCATGGCGCGGTGATGGTG-ATGGTGATG) with *Nco*I- and *Bam*HI-digested pTrcHisA (Invitrogen, San Diego, CA). To produce a construct in frame with the hexahistidine tag, the 1.64-kilobase *Bsp*HI-*Hind*III fragment of pTrcIC₁IIC₂L₃, which encodes the soluble adenylyl cyclase (7), was ligated with pTrcH6 that had been digested with *Nco*I and *Hind*III. The *Bsp*HI-*Hind*III fragment of pTrcIC₁IIC₂L₃ was also cloned into the *Nco*I- and *Hind*III-digested H6pQE60 vector (8). The adenovirus shuttle vector pH₆(271)I₁I₂L₃-ACCMV was created by ligation of the 1.6-kilobase *Eco*RI-*Kpn*I fragment from H6pQE(271)I₁I₂L₃ with the vector ACCMV after digestion with the same enzymes.

Antibodies—Two peptides were synthesized corresponding to amino acid sequences in the C_1 domain of type I adenylyl cyclase and the C_2 domain of type II adenylyl cyclase: IC₁-452 (CGDYEVPGHGH-ERNFSF) and IIC₂-870 (CRSLKNEELYHQSYD). The peptides were coupled to the purified protein derivative of tuberculin (Stantens Serum Institut, Copenhagen) (9). Antibodies were produced in New Zealand White rabbits and affinity-purified as described (10).

G Protein Subunits—Recombinant $G_{s\alpha}$ 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 γ S at 30 °C for 30 min. Free GTP γ S was removed by gel filtration. Recombinant $\beta_1\gamma_2$ and nonprenylated $\beta_1\gamma_2$ Cys⁶⁸ → 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 H6pTrc(271)I₁I₂L₃; 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 -D-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

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¹ The abbreviations used are: 2'-d3'-AMP, 2'-deoxyadenosine 3'-monophosphate; GTP γ S, guanosine 5'-3'-O-(thio)triphosphate; NTA, nitrilotriacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

frozen in liquid N₂. The mixed protease inhibitors used were 22 mg/liter each L-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-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 mg/liter aprotinin, and mixed protease inhibitors), followed by the 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 membranes and cell debris were pelleted by centrifugation for 30 min at 100,000 $\times g$. The supernatant was supplemented with NaCl (250 mM final concentration) and loaded onto a 10-ml Ni²⁺-NTA column equilibrated with lysis buffer. The column was washed with 25 column volumes of buffer A (50 mM Tris-HCl (pH 8.0), 10 mM β -mercaptoethanol, 2 mM MgCl₂, 400 mM NaCl, 5 mM imidazole, and mixed protease inhibitors), followed by 15 column volumes of buffer A containing 15 mM imidazole and 10 column volumes of buffer B (buffer A, except with 10 mM NaCl and 15 mM imidazole). The hexahistidine-tagged protein was eluted with 50 ml of buffer B containing 150 mM imidazole. 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 dithiothreitol) and then eluted with a gradient of 0–400 mM NaCl. Fractions containing adenylyl cyclase activity were pooled, and (NH₄)₂SO₄ was added to a final concentration of 0.4 M. This solution was loaded onto two phenyl-Superose HR 5/5 columns run in tandem, and protein was eluted with two sequential gradients (using buffer C): 0.4 to 0 M (NH₄)₂SO₄ over 30 ml, followed by 0–15 mM CHAPS over 30 ml. Fractions were pooled based on adenylyl cyclase activity and purity (silver-stained SDS-polyacrylamide gels). The protein was stored in small aliquots at –70 °C in buffer C containing 11 mM CHAPS.

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 μ l 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 μ l. Experiments involving the kinetics of P-site inhibition and determinations of K_m were initiated by the addition of enzyme. Assays that contained G protein $\beta\gamma$ 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 charcoal method (14) in which charcoal was equilibrated with 50 mM 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 (16) to assess their purity and to quantitate contaminants in 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 [³H]cAMP, 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 generate recombinant adenovirus, 1 μ g of the shuttle vector pH₆(271)I₁II₂L₃ACCMV and 4 μ g of the plasmid pJM17 (17) were cotransfected into 293 cells using the modified bovine serum transfection kit (Stratagene) according to the manufacturer's protocol. After transfection, the culture medium was changed every 2–3 days until cytopathic effects were observed. Primary virus was identified by polymerase chain reaction (18) using primers within the cytomegalovirus early gene promoter and the coding region of the adenylyl cyclase gene. The virus was plaque-purified (19), and the appropriate viral plaques were amplified and stored in cell culture medium. Viral titers were estimated by plaque assay and ranged from 0.5 to 1.5 $\times 10^5$ plaque-forming units/ml. The appropriate ratios for infection of HC-1 cells were determined by immunoblotting and by assay of adenylyl cyclase or β -galactosidase activity in cytosolic fractions of infected cells.

Cyclic AMP Accumulation in HC-1 Cells—Changes in intracellular concentrations of cyclic AMP were measured by determining the ratio of radioactivity in cyclic AMP to that in the total ATP and ADP pools in [³H]adenine-labeled cells (20). HC-1 cells growing in 12-well plates (0.4 $\times 10^6$ cells/well) were infected with 100–150 μ l of adenovirus encoding β -galactosidase or the soluble adenylyl cyclase. Medium was

removed after 30 h and replaced with 1.5 ml of medium containing 2 μ Ci/ml [³H]adenine. After an additional 18 h, the cells were washed once with Dulbecco's minimal essential medium containing 20 mM NaHepes (pH 7.4) and then treated at 37 °C with 1.0 mM 1-methyl-3-isobutylxanthine (a cyclic-nucleotide phosphodiesterase inhibitor) and various effectors as indicated. Incubations were terminated by aspiration of medium and addition of 0.9 ml of ice-cold 5% trichloroacetic acid containing 0.1 mM cyclic AMP. The cells remained on ice for 45 min, and [³²P]cAMP (5000 cpm) was added to each well to quantify recoveries from columns. Acid-soluble nucleotides were separated on Dowex and alumina columns according to the method of Salomon *et al.* (15). Assays were performed in duplicate, and the values shown are representative of three experiments.

RESULTS AND DISCUSSION

Expression and Purification of the Soluble Adenylyl Cyclase—Expression of the hexahistidine-tagged soluble adenylyl cyclase (H₆(271)I₁II₂L₃) 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 chaperonins GroEL and GroES 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 IIC₂ domain (detected by antibody IIC₂-870) also accumulated (Fig. 1A), but fragments containing the IC₁ 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 migrated on sodium dodecyl sulfate-containing polyacrylamide gels with an apparent M_r of 55,000, as judged by silver staining and immunoblotting, and appeared to be >90% pure (Fig. 1A); the predicted M_r of the protein is 56,900.

Our functional criterion for adequate purification was the absence of interfering enzymatic activities (ATPases, phosphodiesterases, pyrophosphatases, etc.). For each molecule of ATP consumed by the preparation, one molecule of cyclic AMP and PP_i were produced (Fig. 2). There is no detectable additional consumption of ATP even in the absence of any stimulator of adenylyl cyclase (data not shown). The enzymatic reaction approaches the equilibrium for the interconversion of ATP, cyclic AMP, and PP_i as measured by Hayaishi *et al.* (21) with the adenylyl cyclase from *Brevibacterium liquefaciens* (K_{eq} = 0.065 M). Under conditions where ATP is not limiting, enzymatic activity is linear for >90 min (data not shown). Since mammalian adenylyl cyclases have two similar domains that could be involved in nucleotide binding, it is significant that only one molecule of ATP is utilized for each molecule of cyclic AMP that is synthesized. Any conformational changes that occur upon activation of the enzyme are not mediated by hydrolysis of ATP at a regulatory site.

Activation of the Soluble Adenylyl Cyclase—Although the soluble adenylyl cyclase is a chimera and lacks its integral

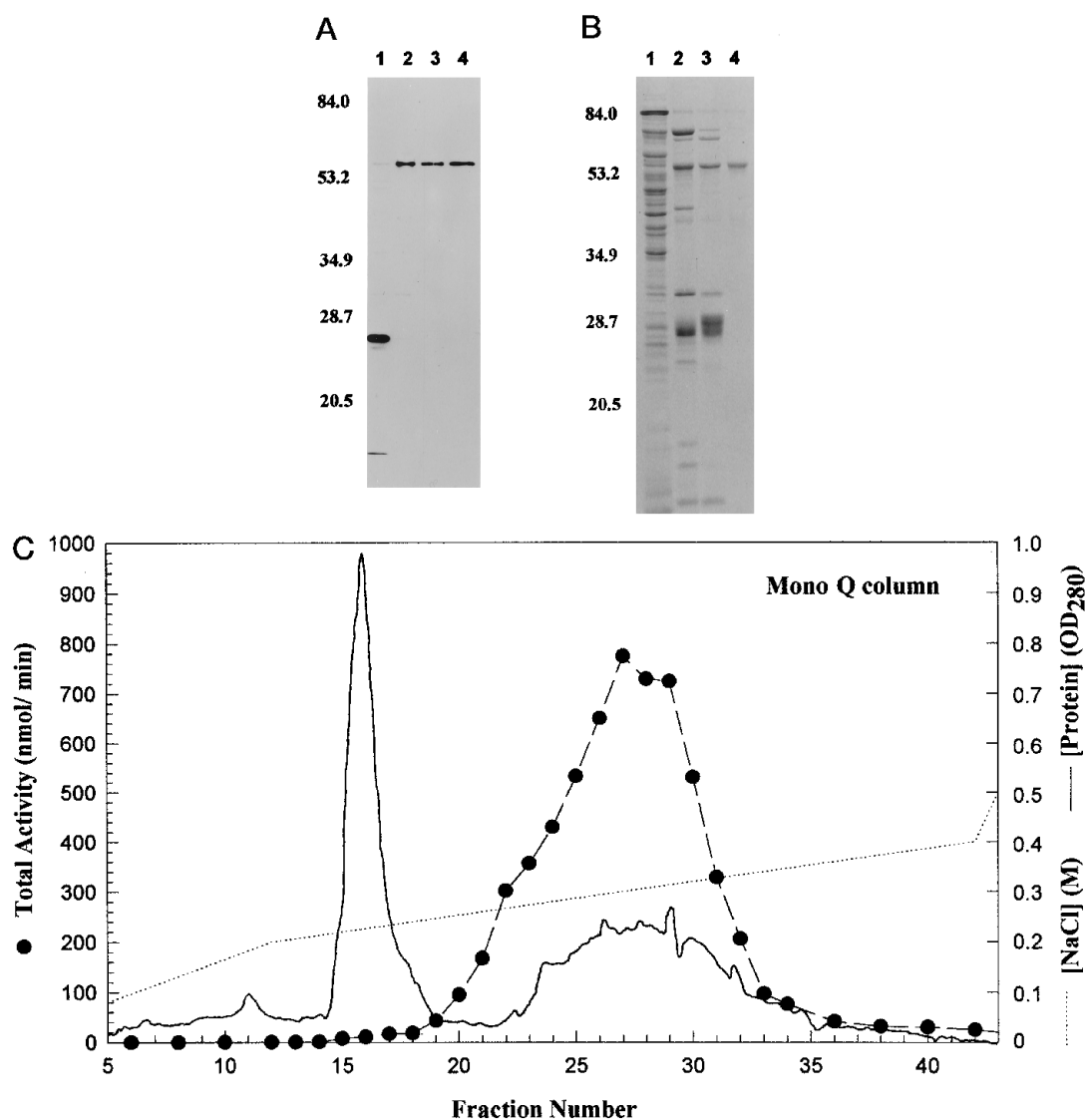


FIG. 1. **Purification of the soluble adenylyl cyclase.** A, Western blot analysis of the purification of the soluble adenylyl cyclase from 18 liters of *E. coli* BL21(DE3) harboring plasmid H₆pTrc(271)I₁II₂L₃. Proteins were treated with *N*-ethylmaleimide, resolved by SDS-polyacrylamide gel electrophoresis on an 11% polyacrylamide gel, and stained using affinity-purified rabbit IIC₂-870 antiserum. Lane 1, 10 μ g of clarified lysate; lane 2, 0.9 μ g of the Ni²⁺-NTA pool; lane 3, 0.3 μ g of the Mono Q pool; lane 4, 50 ng of the phenyl-Superose pool. B, Coomassie Blue stain of SDS gels. Lane 1, 20 μ g of clarified lysate; lane 2, 10 μ g of the Ni²⁺-NTA pool; lane 3, 4 μ g of the Mono Q pool; lane 4, 1 μ g of the phenyl-Superose pool. C, chromatographic profile of the Ni²⁺-NTA eluate on a Mono Q HR 10/10 column. Adenylyl cyclase activity (●) was assayed in the presence of 100 μ M forskolin, 0.5 mM ATP, and 10 mM MgCl₂.

TABLE I
Purification of the soluble adenylyl cyclase from *E. coli*

Assays were performed with 10 mM MgCl₂, 0.5 mM ATP, and 100 μ M forskolin.

Purification step	Volume	Protein	Specific activity	Total activity	Recovery	Purification
	ml	mg	nmol/min/mg	μ mol/min	%	-fold
Lysate	1500	13,200	2.7	35.6	100	1
Ni ²⁺ pool	50	71	137	9.7	27	50
Mono Q pool	81	21	405	8.5	24	150
Phenyl-Superose pool	3.8	2.0	1900	3.7	10	700

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 γ S-G_{s α} or forskolin. The K_m for Mn²⁺-ATP is $\sim$ 50 μ M and is little affected by the presence of enzyme activators. However, the K_m for Mg²⁺-ATP remains relatively low when the enzyme is stimulated by GTP γ S-G_{s α} (65 μ M), but increases to 620 μ M in the presence of forskolin (Fig. 3). When both GTP γ S-G_{s α} and forskolin are present, the

V_{max} is 12.8 μ mol/min/mg, and the K_m remains elevated (300 μ 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 μ M with Mn²⁺ and forskolin and a K_m of 440 μ M with Mg²⁺ 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.

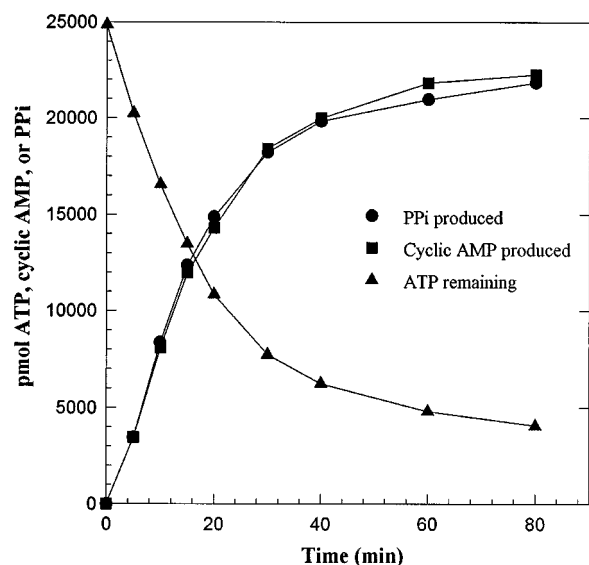


FIG. 2. **Stoichiometry of the reaction catalyzed by the soluble adenylyl cyclase.** Assays (0.1 μ g of protein) were performed in the absence of any ATP-regenerating system and in the presence of 10 mM MgCl_2 , 50 μ M forskolin, and 200 nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$. ATP ($\blacktriangle$), cyclic AMP ($\blacksquare$), and PP_i ($\bullet$) were quantified as described under "Experimental Procedures." All determinations were performed in duplicate. The experiment shown is representative of several.

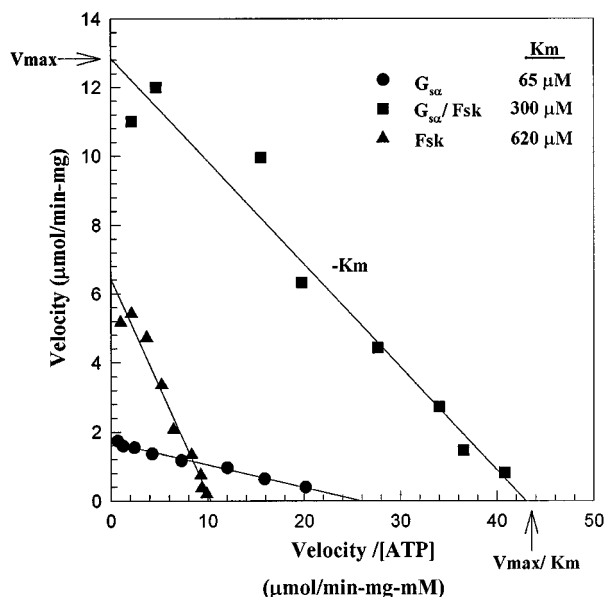


FIG. 3. **Determination of V_{max} and K_i values for ATP.** Assays (7.6 nM protein, 15 min) were performed in the presence of 10 mM MgCl_2 and 500 nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ ($\bullet$), 50 μ M forskolin ($\blacktriangle$), or 100 nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ plus 50 μ M forskolin ($\blacksquare$). All determinations were performed in duplicate and are representative of at least two experiments.

The turnover number is 360 min^{-1} in the presence of forskolin and increases to 720 min^{-1} with both forskolin and $\text{GTP}\gamma\text{S-G}_{\text{sa}}$. 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 Mn^{2+} (24). The low V_{max} for the $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ -stimulated soluble adenylyl cyclase (Fig. 3) is due only to the amount of $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ used in the assay compared with the EC_{50} for activation by G_{sa} (Fig. 4A). With maximal amounts of $\text{GTP}\gamma\text{S-G}_{\text{sa}}$, the soluble adenylyl cyclase displays a V_{max} approaching that observed in the presence of both $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ and forskolin (Fig. 4A).

The affinity of the soluble adenylyl cyclase for activated *E. coli*-derived G_{sa} is ~ 100 -fold lower than that of type I adenylyl

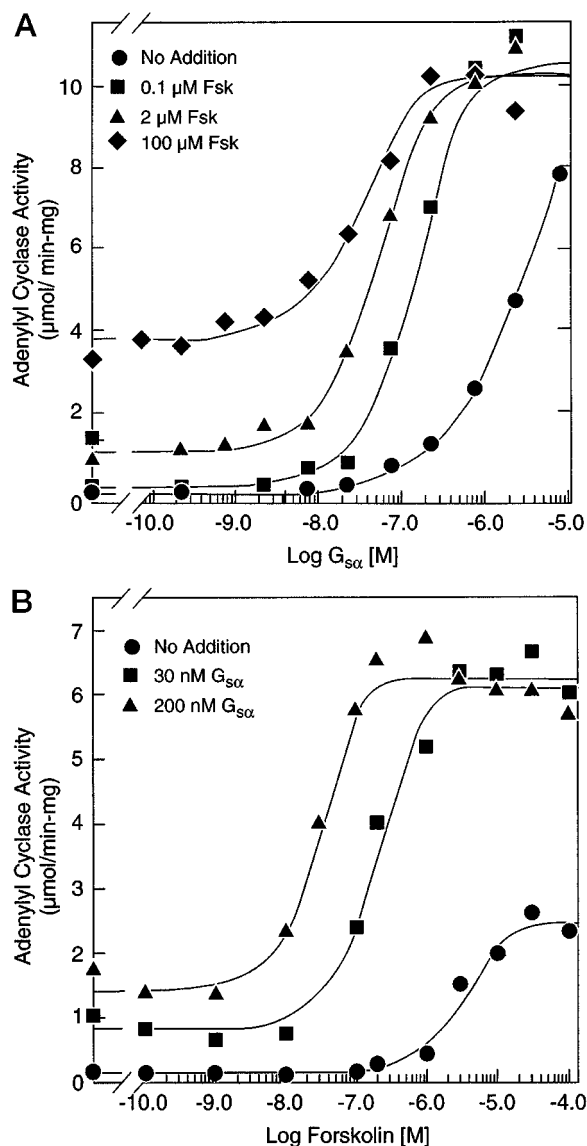


FIG. 4. **Synergistic activation of adenylyl cyclase by $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ and forskolin.** Assays (0.9 nM protein, 20 min) were performed in the presence of 10 mM MgCl_2 and 1 mM ATP. A, $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ plus 0 ($\bullet$), 0.1 ($\blacksquare$), 2 ($\blacktriangle$), and 100 ($\blacklozenge$) μ M forskolin (*Fsk*); B, forskolin plus 0 ($\bullet$), 30 ($\blacksquare$), and 200 ($\blacktriangle$) nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$. Variations in the maximal activity of the enzyme are due to losses caused by freezing and thawing. All determinations were performed in duplicate and are representative of at least two experiments.

cyclase (Fig. 4A) (25). However, forskolin potentiates the effect of low concentrations of $\text{GTP}\gamma\text{S-G}_{\text{sa}}$, shifting the apparent affinity of the soluble adenylyl cyclase for $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ by 2 orders of magnitude. For example, adenylyl cyclase activity was 9.2 $\mu\text{mol/min/mg}$ in the presence of 225 nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ and 2 μ M forskolin, while the activities in the presence of these activators alone were 1.2 and 0.8 $\mu\text{mol/min/mg}$, 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 μ M forskolin (Fig. 4B), the same concentration required for type I and II adenylyl cyclases (22). Low concentrations of $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ shift the apparent affinity of forskolin by 2 orders of magnitude. Unlike the situation with $\text{GTP}\gamma\text{S-G}_{\text{sa}}$, maximal activity in the presence of forskolin is significantly less than that observed with $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ or $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ plus forskolin (Fig. 4B).

Ca^{2+} -calmodulin had no stimulatory effect on the soluble adenylyl cyclase (data not shown); calmodulin activates type I

adenylyl cyclase. However, the C_{1b} 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 $G_{i\alpha}$ proteins and the G protein $\beta\gamma$ subunit complex. $G_{i\alpha}$, $G_{o\alpha}$, and $\beta\gamma$ all inhibit type I adenylyl cyclase (1, 30). The type II enzyme is not affected by $G_{i\alpha}$ and is greatly stimulated by $\beta\gamma$ in the presence of $G_{s\alpha}$ (1, 30). The chimeric type IC₁/type IIC₂ soluble enzyme is unresponsive to $G_{i\alpha}$, even at 10 μ M concentrations (data not shown), but it can be inhibited almost completely by $\beta\gamma$ (Fig. 5). This is surprising since the extent of inhibition of purified type I adenylyl cyclase by $\beta\gamma$ 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 $\beta\gamma$ with $G_{s\alpha}$ since $\beta\gamma$ inhibits forskolin- and $G_{s\alpha}$ -activated enzymatic activity equally. The apparent affinity of the soluble adenylyl cyclase for $\beta\gamma$ 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 $\beta_1\gamma_2$ Cys⁶⁸ $\rightarrow$ 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 $\beta\gamma$ to the carboxyl-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 $\beta\gamma$ 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 $\beta\gamma$ 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 $\beta\gamma$ and these domains can be examined definitively.

It is not clear why $G_{s\alpha}$ and $\beta\gamma$ have reduced affinities for the soluble adenylyl cyclase relative to its membrane-bound counterparts or what role the membrane spans play in signal transduction. $\beta\gamma$ 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 $\beta\gamma$ 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 $\beta\gamma$ 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 $G_{s\alpha}$. However, the recombinant (*E. coli*-derived) $G_{s\alpha}$ used here does not contain lipid modifications and already has a lower affinity for membrane-bound adenylyl cyclases than does tissue-derived $G_{s\alpha}$ (33). All mammalian adenylyl cyclases are activated by $G_{s\alpha}$ *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, C_{1b} , etc.) or conformational constraints imposed by linkage of C₁ and C₂ are responsible for the loss of affinity.

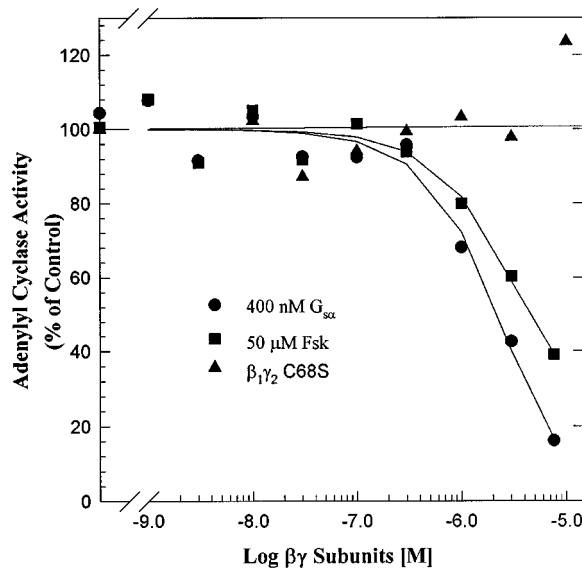


FIG. 5. **Inhibition of adenylyl cyclase by recombinant G protein $\beta_1\gamma_2$.** The Soluble adenylyl cyclase (3.6 nM) was assayed for 20 min with 10 mM MgCl₂, 1 mM ATP, and the indicated concentrations of $\beta\gamma$ in the presence of 400 nM GTP γ S- $G_{s\alpha}$ (●) or 50 μ M forskolin (Fsk; ■). The effects of $\beta_1\gamma_2$ Cys⁶⁸ $\rightarrow$ Ser (▲) were tested in the presence of GTP γ S- $G_{s\alpha}$. Activities are expressed as percentages of control values measured in the absence of $\beta\gamma$: 0.4 and 1.0 μ mol/min/mg for GTP γ S- $G_{s\alpha}$ and forskolin, respectively. All determinations were performed in duplicate and are representative of three experiments.

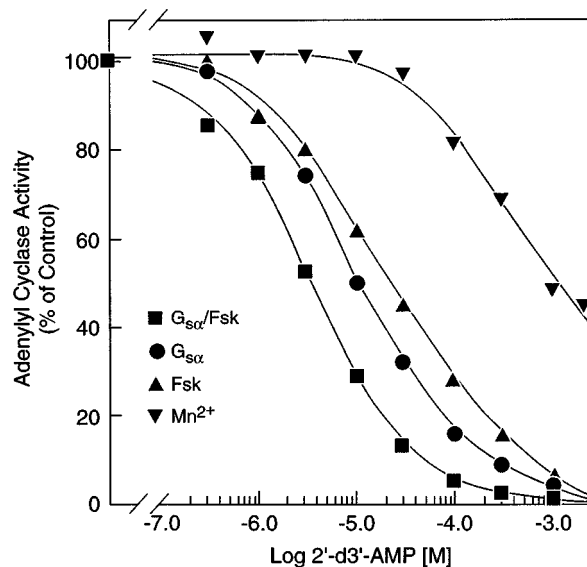


FIG. 6. **Inhibition by the P-site inhibitor 2'-d3'-AMP.** Assays were performed with 0.5 mM ATP for 15 min plus the following: 400 nM GTP γ S- $G_{s\alpha}$ (●), 50 μ M forskolin (Fsk; ▲), 100 nM GTP γ S- $G_{s\alpha}$ plus 50 μ M forskolin (■), or 2 mM MnCl₂ (▼). Control activities for these conditions were 1.1, 1.4, 4.5, and 0.05 μ mol/min/mg of protein, respectively. All determinations were performed in duplicate and are representative of two experiments.

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 are 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

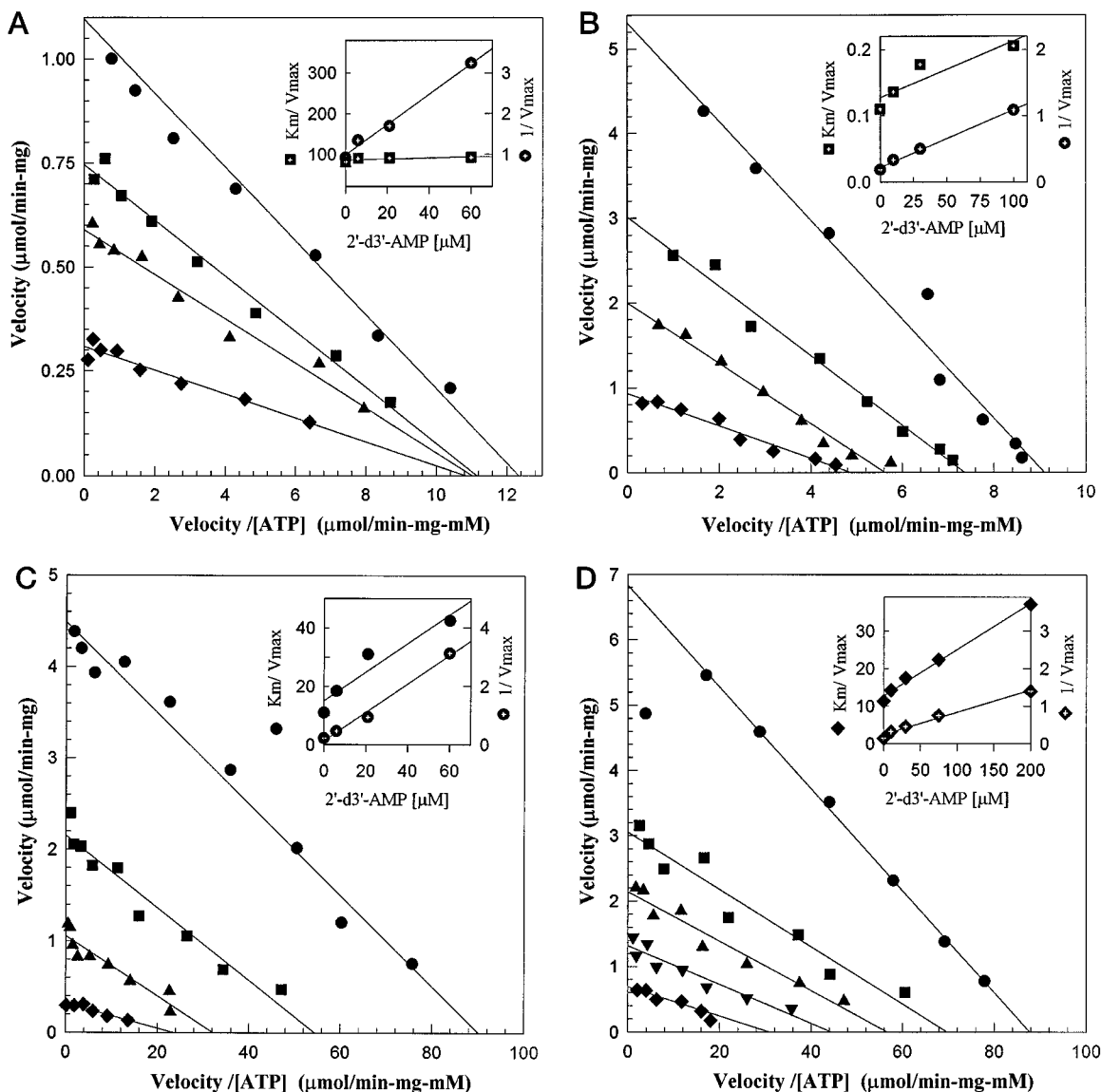


FIG. 7. **Kinetic analysis of inhibition by 2'-d3'-AMP.** Shown are Eadie-Hofstee analysis and replots (*inset*) of $1/V_{\max}$ and $K_m/V_{\max}$ from plots of inhibition by 2'-d3'-AMP. A, assays (2.7 nm protein, 15 min) were performed in the presence of 10 mM MgCl_2 , 400 nM $\text{GTP}\gamma\text{S-G}_{\text{sa}}$, and no inhibitor ($\bullet$) or 6 μM ($\blacksquare$), 21 μM ($\blacktriangle$), or 60 μM ($\blacklozenge$) 2'-d3'-AMP. B, assays were performed in the presence of 10 mM MgCl_2 , 50 μM forskolin, and no inhibitor ($\bullet$) or 10 μM ($\blacksquare$), 30 μM ($\blacktriangle$), or 100 μM ($\blacklozenge$) 2'-d3'-AMP. C, assays were performed as described for A except with 5 mM MnCl_2 . D, assays were performed as described for B except with 2.5 mM MnCl_2 . All determinations were performed in duplicate and are representative of at least two experiments.

G_{sa} -activated enzyme is noncompetitive with respect to Mg^{2+} -ATP (38). All of these features are preserved in the soluble adenylyl cyclase. This enzyme is significantly more sensitive to 2'-d3'-AMP when activated by forskolin, $\text{GTP}\gamma\text{S-G}_{\text{sa}}$, or the combination of the two regulators than when assayed with Mn^{2+} alone (Fig. 6). Thus, the $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ - and forskolin-stimulated enzyme, which is 100-fold more active than the Mn^{2+} -stimulated enzyme, is >300 times more sensitive to P-site inhibition ($\text{IC}_{50} = 3 \mu\text{M}$ versus $\sim 1 \text{ mM}$). The IC_{50} of bovine brain adenylyl cyclase for 2'-d3'-AMP is 2–3 μM in the presence of forskolin or $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ (35).

P-site inhibition of the $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ -stimulated soluble adenylyl cyclase is noncompetitive with respect to Mg^{2+} -ATP. This is also true of detergent-solubilized adenylyl cyclase activity in rat brain (38). Noncompetitive inhibition is rare in unreactant systems and is characterized by a lower $V_{\max}$, a decreased apparent K_m , and an unchanged $K_m/V_{\max}$. The interpretation is that the inhibitor binds only to the enzyme-substrate complex, yielding an inactive enzyme. All other activated forms of the

soluble adenylyl cyclase show a mixed noncompetitive pattern of inhibition with respect to metal-ATP, where both $1/V_{\max}$ and $K_m/V_{\max}$ 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 K_m remains constant. $V_{\max}$, K_m , and $V_{\max}/K_m$ all decrease with increasing concentrations of inhibitor when the soluble adenylyl cyclase is stimulated with forskolin (Mg^{2+} or Mn^{2+}) or $\text{GTP}\gamma\text{S-G}_{\text{sa}}$ in the presence of Mn^{2+} .

The x intercepts of replots of $1/V_{\max}$ or $K_m/V_{\max}$ versus the concentration of 2'-d3'-AMP represent the values of αK_i and K_i , respectively, where K_i is the dissociation constant of 2'-d3'-AMP from the enzyme-inhibitor complex and αK_i is the dissociation constant of 2'-d3'-AMP from the enzyme-substrate-inhibitor complex (Table II) (39). The calculated αK_i values correspond to the observed IC_{50} values for P-site inhibition and correlate well with those shown in Fig. 6. The factor α is equal

TABLE II
Effects of activators and P-site inhibitors on K_m , V_{max} , and K_i values for the soluble adenylyl cyclase

The K_m for ATP and V_{max} were determined by Eadie-Hofstee analysis. Reactions were performed as described in the legend to Fig. 7.

Activator	K_m	V_{max}	K_i	αK_i	α factor	Inhibition pattern
	μM	$\mu mol/min/mg$	μM	μM		
Mn ²⁺	163	0.04		1000 ^a		Mixed
GTP γ S-G _{sα} /Mg ²⁺	89	1.1	22			Noncompetitive
Fsk ^b /Mg ²⁺	585	5.3	102	25	0.25	Mixed
GTP γ S-G _{sα} /Mn ²⁺	50	4.5	31	3	0.1	Mixed
Fsk/Mn ²⁺	78	6.8	100	41	0.41	Mixed

^a The αK_i for activation by Mn²⁺ is an approximation based on the IC₅₀ from Fig. 6 and Eadie-Hofstee analysis (data not shown).

^b Fsk, forskolin.

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 α determined here are <1 in all cases, showing a clear preference of the inhibitor for the adenylyl cyclase-ATP complex compared with adenylyl 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_{max} , and the inhibition patterns is complex. Mn²⁺ (compared with Mg²⁺) 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 γ S-G_{sα} is the activator, the type of metal determines if 2'-d3'-AMP binds solely to the adenylyl cyclase-ATP complex or to both this complex and free adenylyl cyclase. Mn²⁺ can also influence the V_{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_{sα} and binding activity characteristic of the β -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 β -galactosidase was utilized as a control.

There was no detectable accumulation of cyclic AMP in non-infected HC-1 cells or in those expressing β -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 adenylyl cyclase can be activated by a G protein-coupled receptor when its apparent affinity for G_{sα} is increased by forskolin.

The failure to detect stimulation of cyclic AMP synthesis in the presence of isoproterenol alone was not unexpected. The apparent affinities of the soluble adenylyl cyclase for the stimulator G_{sα} and the inhibitor $\beta\gamma$ are similar. However, in the presence of forskolin, the apparent affinity of the enzyme for G_{sα} is increased dramatically, while that for $\beta\gamma$ is not altered. Furthermore, the soluble adenylyl cyclase studied here is not unique in its failure to respond to G_{sα} *in vivo* in the absence of

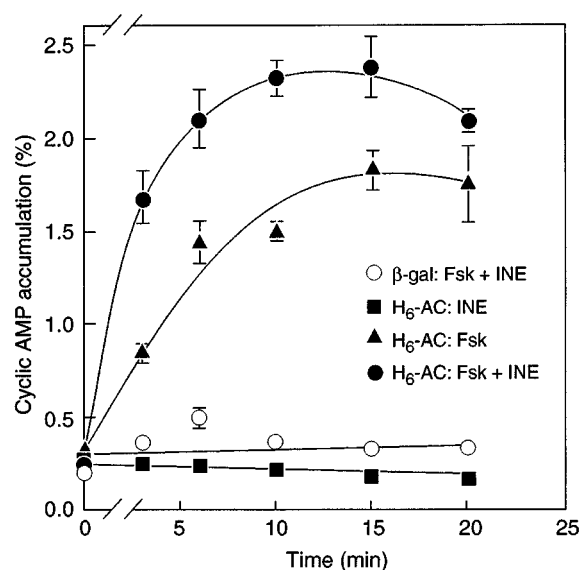


FIG. 8. Synergistic stimulation of soluble adenylyl cyclase activity by forskolin and isoproterenol in HC-1 cells. HC-1 cells infected with β -galactosidase adenovirus (β -gal; $\circ$) or H₆(271)I₁I₂L₃ adenovirus (H₆-AC; $\bullet$, $\blacktriangle$, $\blacksquare$) were exposed to 10 μM isoproterenol (INE; $\blacksquare$), 50 μM forskolin (Fsk; $\blacktriangle$), or 10 μM isoproterenol + 50 μM forskolin ($\bullet$, $\circ$) as described under "Experimental Procedures." Relative accumulation of cyclic AMP was determined as described under "Experimental Procedures." Each time point was performed in duplicate, and the experiment shown is representative of three experiments.

forskolin. When expressed by transfection, several membrane-bound adenylyl 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_{iα}, 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

protein and related constructs will permit detailed appreciation of mechanisms of regulation of cyclic AMP synthesis and their structural counterparts.

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Additions and Corrections

Vol. 271 (1996) 16967–16974

Purification and characterization of a soluble form of mammalian adenylyl cyclase.

Carmen W. Dessauer and Alfred G. Gilman

Pages 16972 and 16973: The word “noncompetitive” was substituted incorrectly during the publication process. The correct sentences are shown below.

Page 16972, line 1: “...G_{sα}-activated enzyme is uncompetitive with respect to Mg²⁺-ATP (38).”

Page 16972, line 12: “P-site inhibition of GTPγS-G_{sα}-stimulated soluble adenylyl cyclase is uncompetitive with respect to Mg²⁺-ATP.”

Page 16972, line 15: “Uncompetitive inhibition is rare in unireactant systems and is characterized by a lower $V_{\max}$, a decreased apparent K_m , and an unchanged $K_m/V_{\max}$.”

Page 16973, Table II:

TABLE II						
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^a The αK_i for activation by Mn²⁺ is an approximation based on the IC₅₀ from Fig. 6 and Eadie-Hofstee analysis (data not shown).

^b Fsk, forskolin.

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Purification and Characterization of a Soluble Form of Mammalian Adenylyl Cyclase

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