Distinct Patterns of Bidirectional Regulation of Mammalian Adenylyl Cyclases

The capacities of the α subunits of pertussis toxin-sensitive guanine nucleotide-binding regulatory proteins (G proteins) to inhibit different isoforms of mammalian adenylyl cyclases were assessed. Membranes from Sf9 cells infected with recombinant baculoviruses encoding either type I, II, V, or VI adenylyl cyclase were reconstituted with purified G protein subunits. Types V and VI adenylyl cyclase are most sensitive to inhibition by Gi11, Gβδ, and Gαs; type I adenylyl cyclase can be inhibited by these three Gα proteins and by Gαo, as well. Type II adenylyl cyclase appears to be immune to inhibition by these proteins.

Examination of the effects of native and mutant Gαo proteins, as well as analysis of competition for binding of Gαo to adenylyl cyclase, indicate that at least certain adenylyl cyclases have independent sites for interaction with Gαo (site 1, stimulatory) and Gαo (site 2, inhibitory). High concentrations of Gαo can interact with site 1 on types I and II adenylyl cyclase and activate the enzymes. Types I and II adenylyl cyclase also appear to have independent sites for interaction with G protein βγ subunits. The type I enzyme is strongly inhibited, while type II adenylyl cyclase is activated if Gαo is also present.

Many cellular functions are responsive to changes in concentrations of cyclic AMP and thus to changes in the activities of adenylyl cyclases, the enzymes that catalyze synthesis of the intracellular second messenger from ATP. Classically, adenylyl cyclases respond to receptor-initiated stimulatory and inhibitory regulation, mediated by the homologous heterotrimeric G proteins, Gα, and Gβγ, respectively. The mechanism of stimulation of adenylyl cyclase activity by Gα is relatively well understood.

The binding of an appropriate agonist to a Gα-coupled receptor (e.g., a β-adrenergic receptor) catalyzes the exchange of GDP (bound to Gα) for GTP, with resultant dissociation of GTP-Gαo from βγ; adenylyl cyclase is activated by interaction with GTP-Gαo (1–3). Mechanisms of inhibition of adenylyl cyclase activity have been more difficult to fathom. In most cases hormonal inhibition of adenylyl cyclase is sensitive to disruption by pertussis toxin, implicating one of the Gα proteins (Gα11, Gα2, or Gα5) or Gα in coupling inhibitory receptors (e.g., an α2-adrenergic receptor) to adenylyl cyclase (4, 5). Although activation of the Gα11 and Gα heterotrimer results from an identical process of guanine nucleotide exchange and subunit dissociation, it has been difficult to decide whether GTP-α11, βγ, or both mediate inhibition of adenylyl cyclase.

During the past 4 years cDNAs encoding six distinct isoforms of adenylyl cyclase have been cloned and expressed (6–13). These discoveries and those of additional partial clones (14–16) indicate that the family of adenylyl cyclases is unexpectedly large and diverse. The proteins share the same basic topology and have extensive regions of sequence homology, but they differ in their tissue distribution, relative abundance, and, most interestingly, regulatory properties (17, 18). The latter fact has compounded the difficulties in assessing mechanisms of inhibition of adenylyl cyclase activity.

Although all membrane-bound forms of mammalian adenylyl cyclase are activated by Gαo, they differ dramatically in their responses to other regulatory molecules. For example, types V and VI adenylyl cyclase are inhibited by low micromolar concentrations of Ca2+, while types I and III are activated by Ca2+/calmodulin; types II and IV adenylyl cyclase are insensitive to physiological concentrations of Ca2+ (13, 18–20). The G protein βγ subunit complex inhibits type I adenylyl cyclase, but it greatly potentiates Gα-mediated activation of the type II and IV enzymes; the other forms of adenylyl cyclase are relatively insensitive to βγ (9, 19, 21). Observations of this type have heightened appreciation of the necessity for systematic identification of the particular isoform(s) of adenylyl cyclase present in individual cells and thorough characterization of the regulatory properties of each. Toward this end we have expressed each isoform of adenylyl cyclase in Sf9 cells using the recombinant baculovirus system. We have used membranes derived from these cells to assess the regulatory properties of individual adenylyl cyclases with respect to interactions with forskolin, calmodulin, Gαo, and G protein βγ subunits (9, 19, 21), and we have purified types I and II adenylyl cyclase from these membranes to demonstrate their direct interactions with βγ (22).

Recently, we also demonstrated that recombinant (Escherichia coli-derived) myristoylated Gα11 could inhibit adenylyl cyclase activity in membranes derived from Sf9 cells expressing either the type I or the type V enzyme (23). To characterize such responses more thoroughly, we have now examined the effects of myristoylated Gα11, Gα2, Gα5, and Gαo on adenylyl cyclases types I, II, V, and VI, and we have initiated studies designed to determine the mechanism of inhibition of adenylyl cyclase activity by these proteins.

EXPERIMENTAL PROCEDURES

Sf9 Cell Culture and Recombinant Baculoviruses—Procedures for the culture of Sf9 cells and the production, cloning, and amplification of recombinant baculoviruses have been described by Summers and Smith (24). Baculoviruses encoding types I, II, and V adenylyl cyclase have been described previously (19, 21, 23). A cDNA that encodes casrine type VI adenylyl cyclase (11) was excised from pCDNAamp27–6 with EcoRI and SspI and was cloned into pVL1392 that had been digested with
Membranes were prepared from SF9 cells expressing individual isoforms of the type V adenyl cyclase (22). Briefly, cells (10^6/mL) were infected with the desired baculovirus (1 plaque-forming unit/cell), harvested 48–58 h later, and lysed by nitrogen cavitation. After removal of nuclei by centrifugation, membranes were collected, washed, and re suspended. Protein concentrations were determined by the method of Bradford (25).

Generation and Purification of Truncated Gαα Subunits—All G protein α subunits were synthesized in E. coli as described by Lee et al. (26). For synthesis of myristoylated α subunits (Gαi1, Gαs, Gαa, and Gαo), proteins were coexpressed with yeast protein N-myristoyltransferase (26, 27). Purification of recombinant α subunits was achieved by modifications of the methods of Linder et al. (26), as described by Lee et al. (26). Protein concentrations were estimated by staining with Amido Black (28).

Purified α subunits were activated by incubation with 50 mM Na-HEPES (pH 8.0), 5 mM MgSO4, 1 mM EDTA, 1 mM dithiothreitol, and 400 μM GTPγS at 30 °C for 30 min (Gαi1 and Gαas) or 2 h (Gαs and the Gα12i13mutant) (28, 30). Free GTPγS was removed by gel filtration.

G protein βγ subunits were purified from bovine brain as described by Sternweiss and Riboehsh (31).

Adenylyl Cyclase Assays—Adenylyl cyclase activity was measured as described by Smigol (32). All assays were performed for 5–7 min at 30 °C in a final volume of 100 μl. The concentration of MgCl2 was 4 mM. Membranes containing individual G protein subunits were incubated for 5 min at 30 °C in a total volume of 40 μl prior to initiation of the assay; GDPβS (25 μM) was included during this incubation, as was recombinant Gαi1 (30) or calcimodulin (33), where indicated. When used, forskolin was added at the start of the assay. When the effects of G protein βγ subunits were assessed, the final concentration of detergent (Lubrol PX) in the assay was maintained at 0.2%.

Binding of Gαi1 to SF9 Cell Membranes Containing Adenylyl Cyclase—[3H]GTPγS-Gαi1 was prepared by incubating 0.6 μM Gαi1 (short form) with 1.2 μM [3H]GTPγS/5 for 1 h at 30 °C in 20 mM Na-HEPES (pH 8.0), 5 mM MgSO4, 1 mM dithiothreitol, and 1 mg/ml of bovine serum albumin. The reaction mixture was then filtered through Sephadex G-25 to remove free nucleotide. The Gαi1-binding assay was performed by mixing 20 μg of SF9 cell membranes expressing the indicated type of adenylyl cyclase, 80 fmol of [3H]GTPγS-Gαi1 (final concentration 4 nM), and variable amounts of the indicated GTPγS-activated G protein α subunit in 20 μl of buffer containing 20 mM Na-HEPES (pH 8.0), 4 mM MgCl2, 1 mM dithiothreitol, 100 μM forskolin, and 100 μM GDPβS. The reaction mixtures were incubated at 30 °C for 10 min (2–3 min are required to reach equilibrium). [3H]GTPγS-Gαi1, that was not associated with membranes was removed by filtration through 0.22-μm Millipore durepore membranes, followed by washing with 6 ml of 20 mM Na-HEPES (pH 8.0), 4 mM MgCl2, 1 mM dithiothreitol, and 10 μM forskolin. The amount of labeled Gαi1 retained on the filters was determined by scintillation counting. Specific binding was calculated by subtracting binding to SF9 cell membranes prepared from cells expressing β-galactosidase from that observed to membranes from cells expressing adenylyl cyclase. Further details of this assay will be presented elsewhere.2

Generation and Purification of Truncated Gαα—Methods for the expression in E. coli and purification of Gαα subunits containing hexahistidine tags at the amino terminus have been described by Lee et al. (26). Hexa-histidine Gα1 (16 mg) was purified to near homogeneity from a 5-liter culture of E. coli by Ni2+-NTA affinity chromatography (Qia gen). Gαa lacking the amino-terminal 34 amino acid residues (and the hexahistidine tag, designated NC-Gαa) was synthesized by limited trypic digestion. Purified hexa-histidine Gα1i11 (in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol) was activated at 25 °C for 10 min with either 10 μM GTPγS and 10 mM MgSO4 or with 30 μM AICl3, 10 mM MgCl2, 10 mM NaF, and 0.1 μM GDP (AMFG) and then diluted to a final concentration of 1.7 mg/ml in the same buffer. Trypsin was added (0.02 mg/ml final concentration) and the sample was incubated at 4 °C for 30 min. Digestion was terminated by addition of soy bean trypsin inhibitor (0.08 mg/ml final concentration). More than 95% of the Gαa was truncated and existed as a single species with an apparent molecular mass of 40 kDa. Immunoblotting with site-specific antibodies indicated that the amino terminus was missing and that the carboxyl terminus was intact.

The sample was diluted 5-fold and loaded directly to a 10-ml MonoQ anion-exchange column for fast protein liquid chromatography equilibrated with buffer A (50 mM Tris-HCl (pH 8.0), 20 mM 2-mercaptoethanol, 5 mM MgCl2, 10% glycerol, and 0.02 mg/ml phenylmethylsulfonyl fluoride) containing the appropriate activator (either GTPγS or AMFG). Protein was eluted from the column with a 60-ml linear gradient of NaCl (0–300 mM) in the same buffer. Fractions were analyzed by electrophoresis. To remove residual undigested hexa-histidine Gαa, the sample was loaded to a 2-ml Ni2+-NTA column equilibrated with buffer A containing the appropriate activator, and the flow-through and washes were analyzed for NC-Gαa by electrophoresis. Fractions containing NC-Gαa were stored in 50 mM Na-HEPES (pH 8.0), 1 mM EDTA, and 0.1 mM GDP. Sequence analysis indicated that both GTPγS- and AMFG-activated NC-Gαa were homogeneous preparations that lacked the first 34 amino acid residues of the native protein. GTPγS- and AMFG-activated NC-Gαa displayed identical capacities to activate adenylyl cyclase. GDP-bound NC-Gαa was generated by incubating the AMFG-activated protein with excess EDTA for 1 h at 22 °C, and AMFG and EDTA were removed by gel filtration.

The affinity of NC-Gαa for βγ was assessed by examining the capacity of βγ to inhibit the steady-state GTPase activity of the protein. GTPase assays were performed as described (34), except the concentration of Mg2+ was 0.2 mM.

RESULTS

Inhibition of Isoforms of Adenyl Cyclase by Gαα Subunits: Types V and VI—Our initial observation that purified myristoylated Gα1i11 could inhibit type V adenyl cyclase (23) prompted us to examine the generality of this response with additional isoforms of the enzyme. Results with type VI adenyl cyclase, which shares many regulatory properties with the type V enzyme, are shown in Fig. 1. The activity of type VI adenyl cyclase (activated with GTPγS-Gαα) is also inhibited by recombinant myristoylated GTPγS-Gαα. The apparent affinity of type VI adenyl cyclase for the inhibitory protein is roughly 50 nm, and more than 80% of enzymatic activity can be inhibited (at a 50 nm concentration of activated Rαα). Boiled protein is without effect, ruling out inhibition due to components of the buffer or to unbound GTPγS. The significance of the concentrations of Gαα proteins required to inhibit adenylyl cyclase activity has been discussed previously (23). As anticipated, substantially higher concentrations of the GDP-bound form of myristoylated Gα1i11 were required to inhibit type VI adenyl cyclase than are needed for the GTPγS-bound form of the protein, presumably reflecting the relative affinities.
of the two forms of the α subunit for the enzyme. (Some of the activity observed with the GDP-bound form of myristoylated Gi₃⁵ could be due to exchange of GDP for an activating nucleotide (e.g. GTPαS).) In addition, as described for type V adenylyl cyclase, myristoylation of Gi₃⁵ at the amino terminus is required to observe inhibition; micromolar concentrations of the unmodified protein are without effect. Myristoylated, activated Gα₅ is also ineffective.

Similar patterns of effects were observed when activated, myristoylated Gi₁₁, Gi₃₂, and Gi₃₃ were compared for their capacities to inhibit types V and VI adenylyl cyclase in Sf9 cell membranes (Fig. 2). Neither adenylyl cyclase distinguishes among the three related Gi₅ proteins. Inhibition of type VI adenylyl cyclase appeared to occur at slightly lower concentrations of the Gi₅ proteins, and the extent of inhibition is somewhat greater than with type V adenylyl cyclase. In addition, the Gi₅ proteins are somewhat more efficacious inhibitors of Gi₅-activated adenylyl cyclase activity than of forskolin-stimulated activity with both the types V and VI enzyme. The effects of the activated Gi₅ proteins on Gα₅-stimulated adenylyl cyclase activity in Sf9 cell cyclase membranes are very similar to those seen with the type VI enzyme in Sf9 cell membranes, consistent with the observation that Sf9 cells express predominantly type VI (35).

Type II—We were next interested in determining if activated Gi₅ could inhibit forms of adenylyl cyclase (e.g. type I and type II) that have distinctly different regulatory properties. Fig. 3 shows results obtained with membranes from Sf9 cells expressing the type II enzyme. In the presence of GTPγS-Gα₅₃, myristoylated GTPγS-Gα₅₃ inhibits enzymatic activity only weakly, while micromolar concentrations of GTPγS-Gα₅ are without effect. The effects of GTPγS-activated Gi₅₃ and Gi₅₃ are similar to those of Gi₅₃ (not shown). Since G protein βγ subunits activate type II adenylyl cyclase (conditionally, in the presence of Gα₅), we suspected that GTPγS-Gα₅ does not itself inhibit type II adenylyl cyclase but rather the observed inhibition might be due to interaction between small amounts of GDP-Gi₅ in the preparation and endogenous βγ in the Sf9 cell membranes, thereby preventing βγ from activating adenylyl cyclase. Consistent with this hypothesis is the finding that the GDP-bound forms of both Gi₅ and Gα₅ are more potent inhibitors of type II adenylyl cyclase than is myristoylated GTPγS-Gα₅₃. Also consistent with the hypothesis is the fact that GTPγS-activated Gi₅₃ could not inhibit forskolin-activated type II adenylyl cy-

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**Fig. 2.** Inhibition of type V, VI, and cyc- adenylyl cyclases by Gi₁₁, Gi₃₂, and Gi₃₃. Membranes were incubated with G protein α subunits, and adenylyl cyclase activity was assayed as described in the legend to Fig. 1, except that forskolin, when used, was added at the start of the assay. Membranes prepared from Sf9 cells expressing type V adenylyl cyclase (10 µg A and D), Sf9 cells expressing type VI adenylyl cyclase (20 µg B and E), or cyc- Sf9 cells (50 µg, C and F) were assayed in the presence of 50 nM GTPγS-Gα₅₃ (A-C) or 50 µM forskolin (D-F) and either recombinant myristoylated GTPγS-Gα₅₃ (B), GTPγS-Gα₅ (C), or GTPγS-Gα₅ (D). All activities are expressed as percentages of control values measured in the absence of Gα₅, protein: 2.5, 1.5, 0.7, 1.9, 0.5, and 0.2 nmol·min⁻¹·mg⁻¹ for A-F, respectively. All determinations were performed in duplicate and are representative of at least two experiments.

**Fig. 3.** Effects of Gα₅ subunits on type II adenylyl cyclase. Adenylyl cyclase activities of membranes prepared from Sf9 cells expressing type II adenylyl cyclase were assayed in the presence of 50 nM GTPγS-Gα₅₃ (A) or 50 µM forskolin (B). Membranes (10 µg) were incubated with GDP- (open symbols) or GTPγS- (filled symbols) bound myristoylated Gα₅₃ (circles) or Gα₅ (squares). Control values were 5.9 and 0.7 nmol·min⁻¹·mg⁻¹ for A and B, respectively. Determinations were performed in duplicate and are representative of two experiments.
enzyme at concentrations in the range (Fig. 4B); Gia2 and Gia3 behaved similarly (not shown). In this case the GTPyS-bound form of Gia1 was more active than the GDP-bound form of the protein, and GTPyS-Gia was nearly devoid of activity. Because forskolin and Gia activate type II adenylyl cyclase synergistically (7), these data suggest that high concentrations of activated Gia may be mimicking Gia in these experiments. Additional data that substantiate this notion are presented.

Type I—We have shown previously that activated myristoylated Gia can inhibit type I adenylyl cyclase partially when the enzyme is stimulated by Ca2+/calmodulin and less extensively in the presence of forskolin; inhibition is very modest when Gia1, Gia2, and Gia3 are compared (Fig. 4). Maximal inhibition is achieved at 100–300 nM concentrations of the proteins; the extent of inhibition is 50% with Ca2+/calmodulin and only 20% with forskolin.

The inhibitory effect of the G protein βγ subunit complex on type I adenylyl cyclase, described previously (19), is both more prominent and is exerted at somewhat lower concentrations than the effect obtained with Gia. The effects of βγ and myristoylated Gia1 are compared in Fig. 5 for both the Gia1- and the Ca2+/calmodulin-activated type I enzyme. The inhibitory effects of Gia and βγ are additive, at least at certain concentrations (Fig. 6A); the percentage inhibition that can be obtained with Gia is larger in the presence of βγ (Fig. 6B). The experiments in Fig. 6 were performed in the presence of forskolin. A similar pattern was seen with Ca2+/calmodulin-activated type I adenylyl cyclase.

Surprisingly, myristoylated GTPyS-Gia is also capable of inhibiting Ca2+/calmodulin- or forskolin-activated type I adenylyl cyclase (Fig. 7). Although the extent of inhibition of enzymatic activity by Gia and Gia is comparable, Gia is approximately 10 times less potent. Nevertheless, this effect of Gia may be physiologically relevant, since the concentrations of Gia in brain are very high and exceed those of Gia by a considerable amount (31). Activated Gia (300 nM) did not inhibit type I adenylyl cyclase (not shown).
We observed a consistent capacity of activated $G_{\text{ia}}$ proteins to cause a paradoxical increase in type I adenylyl cyclase activity at concentrations higher than those necessary to observe maximal inhibition. This effect was obvious in the presence of $\text{Ca}^{2+}$/calmodulin (Figs. 4, 5, and 7) or forskolin (Figs. 4, 6, and 7), but not $G_{\text{ia}}$ (Fig. 5). While less dramatic, this behavior is reminiscent of the effects of $G_{\text{ia}}$ on forskolin-stimulated type II adenylyl cyclase described above. The fact that this phenomenon is not observed in the presence of $G_{\text{ia}}$ again suggests that it is due to interaction of high concentrations of $G_{\text{ia}}$ with a binding site for $G_{\text{ia}}$ on adenylyl cyclase (see below).

**Mechanism of Inhibition of Adenylyl Cyclase by $G_{\text{ia}}$**—The observation that $G_{\text{ia}}$ can inhibit adenylyl cyclase activity in the absence of $G_{\text{ia}}$ (i.e., in the presence of forskolin or calmodulin) indicates that inhibition need not be due to competition between the two homologous G protein α subunits for a common binding site on adenylyl cyclase. Although kinetic analysis of the effects of $G_{\text{ia}}$ on $G_{\text{ia}}$-activated type V adenylyl cyclase indicates a largely competitive relationship between the two proteins over a relatively narrow range of concentrations of $G_{\text{ia}}$ (2–20 nm; not shown), this is not true when a broader range of concentrations is examined (Fig. 8). As the concentration of $G_{\text{ia}}$ is raised, the maximal extent of inhibition that can be obtained with $G_{\text{ia}}$ is clearly reduced.

These data are thus consistent with a model in which $G_{\text{ia}}$ and $G_{\text{ia}}$ bind to distinct sites on adenylyl cyclase. Activation results from binding of $G_{\text{ia}}$ to site 1, while binding of $G_{\text{ia}}$ to site 2 causes inhibition. We propose that $G_{\text{ia}}$ may also have a modest affinity for site 1 and make contacts appropriate to activate adenylyl cyclase under unusual circumstances. Thus, in the presence of forskolin or $\text{Ca}^{2+}$/calmodulin, $G_{\text{ia}}$ first inhibits type I adenylyl cyclase (site 2) but then activates at high concentrations (site 1). Binding of $G_{\text{ia}}$ to site 1 of type II adenylyl cyclase leads to substantial activation of the enzyme in the presence of forskolin, presumably because site 2 is absent or inapparent and because of synergistic activation of the enzyme by forskolin and $G_{\text{ia}}$ (or $G_{\text{ia}}$ in site 1).

To test this model, we expressed and purified the myristoylated form of a mutant of $G_{\text{ia}}$ predicted to have an increased affinity for site 1. Mutational analysis has identified several regions of $G_{\text{ia}}$ that are necessary for activation of adenylyl cyclase (34, 36, 37). For example, replacement of residues between positions 263 and 269 of $G_{\text{ia}}$ with the corresponding residues of $G_{\text{ia}}$ (254–260) results in $G_{\text{ia}}$ mutants with little affinity for adenylyl cyclase. We reasoned that reciprocal mutations in $G_{\text{ia}}$ (replacement with $G_{\text{ia}}$ sequence) should increase the affinity of the mutant $G_{\text{ia}}$ for site 1.

Analysis of the effects of a mutant designated myristoylated $G_{\text{ia}}$ (residues 258–261 (Phe-Trp-Asp-Trp) changed to Leu-Arg-Tyr-Ile) is shown in Fig. 9. In the absence of other activators, $G_{\text{ia}}$ has a weak stimulatory effect on types I and II adenylyl cyclase and little effect on the type VI enzyme (Fig. 9, A–C). However, in the presence of forskolin (which activates adenylyl cyclase synergistically with $G_{\text{ia}}$ (particularly types II and VI)), $G_{\text{ia}}$ is a more potent activator of type I or type II adenylyl cyclase than is the wild-type $G_{\text{ia}}$ protein; it is obviously not as potent as $G_{\text{ia}}$ (Fig. 9, D–E). A more dramatic effect of the mutation is observed on forskolin-activated type VI adenylyl cyclase (Fig. 9F). The mutant $G_{\text{ia}}$ is not an inhibitor of the enzyme; it is an activator, presumably because of preferential affinity for site 1 under these conditions.

**Binding of $G_{\text{ia}}$—**A binding assay was developed to detect interactions of activated $G_{\text{ia}}$ with membranes from SF9 cells expressing different isoforms of adenylyl cyclase. Binding of $[^{35}S]GTPyS-G_{\text{ia}}$ to membranes from cells expressing either type I or type II adenylyl cyclase is 3–6-fold higher than binding of the labeled protein to membranes from cells infected with a baculovirus encoding β-galactosidase. The amount of binding observed is consistent with the amount of adenylyl cyclase present in these membranes, based on their specific enzymatic activity. Although the extent of binding of $G_{\text{ia}}$ to membranes containing type I or type II adenylyl cyclase is similar in the presence or absence of forskolin, it is necessary to include forskolin to obtain an adequate signal when using membranes containing type V or type VI adenylyl cyclase (data not shown). Because the signal-to-noise ratio in these assays is not high, we interpret the results semiquantitatively.
As expected, addition of unlabeled GTPγS-Gsa inhibits binding of the labeled complex by competition (Fig. 10). Half-maximal competition is observed at roughly 10–30 nM concentrations of GTPγS-Gsa, in reasonable agreement with concentrations of GTPγS-Gsa necessary to activate the adenylyl cyclases (Fig. 9). Activated myristoylated Gia1(–10) is also an effective competitor for Gsa-binding sites with all three types of adenylyl cyclase. Required concentrations (μM) are close to those required to observe activation of adenylyl cyclase by this mutant protein. High concentrations of myristoylated GTPγS-Gia1 compete for Gsa-binding sites on type I and type II adenylyl cyclases (compare curves with those for nonmyristoylated Gia1, which we take as an ineffective control); these are the two adenylyl cyclases where activation is seen at high concentrations of myristoylated Gia1. There is no significant competition by myristoylated Gia1 for Gsa-binding sites on type VI adenylyl cyclase. Under the conditions utilized, this protein inhibits and does not activate the enzyme. These data are entirely consistent with the model discussed above, wherein Gsa and Gia1 interact with distinct sites on adenylyl cyclase to activate and inhibit the enzyme, respectively.

**Interactions Between Gsa and βγ on Types I and II Adenylyl Cyclase**—As discussed above, βγ appears to be the most potent and efficacious inhibitor of type I adenylyl cyclase, and the protein is also an effective conditional activator of the type II enzyme, stimulating activity dramatically in the presence of Gia1 (but not forskolin). Despite the fact that βγ inhibits both Gia1- and Ca2+/calmodulin-activated type I adenylyl cyclase, the question has arisen about the nature of the interaction between Gia1, βγ, and types I and II adenylyl cyclase. Do Gsa and βγ interact independently with these enzymes or might they interact as the heterotrimer (despite the low affinity of GTPγS-Gia1 for βγ)?

In the absence of a workable binding assay for βγ, we have addressed this issue by examining the effects of βγ on types I and II adenylyl cyclase in conjunction with an altered Gsa that has been truncated at the amino terminus by digestion of the GTPγS-activated protein with trypsin (Fig. 11A). This treatment, which removes the amino-terminal 34 amino acid residues, leaves the guanine nucleotide binding properties of the protein intact, as well as its interactions with adenylyl cyclase. However, it greatly reduces the affinity of the protein for βγ (38, 39). The reduced affinity of truncated Gsa for βγ is documented in Fig. 11B, wherein interactions between Gsa and βγ were assayed by examination of the capacity of βγ to inhibit the steady-state GTPase activity of Gsa. The basis of this effect lies in βγ-induced slowing of the rate of dissociation of GDP from Gsa. Interaction between βγ and Gsa could not be detected at the highest concentrations of βγ tested (100 nM). Truncated Gsa activates types I and II adenylyl cyclase normally (Fig. 12, A and C). Of interest, there was no change in the capacity of βγ to inhibit Gsa-activated type I adenylyl cyclase or to activate Gsa-stimulated type II adenylyl cyclase when these assays were performed in the presence of wild-type Gia1 or truncated Gia1 (Fig. 12, B and D). These experiments support the hypothesis that these adenylyl cyclases have independent binding sites for Gsa and βγ.

**DISCUSSION**

The discoveries of several isoforms of membrane-bound adenylyl cyclases in mammals and the elucidation of pathways for type-specific regulation of their activities permit synthesis of this information in the form of distinct schemes for regulation of intracellular concentrations of cyclic AMP (Fig. 13). Three patterns have emerged to date: those represented by adenylyl cyclases types V and VI, by types II and IV (although information is incomplete about type IV; it is poorly expressed in Sf9 cells), and by type I. (Type III is similar to type I in that it is activated by Ca2+/calmodulin, but other information is incomplete.) The complexity of these schemes is remarkable, as is the plasticity among them. They will certainly become more complex and interesting as additional isoforms of adenylyl cyclase are studied and as additional layers of regulation are incorporated (e.g. covalent modifications and allosteric regulation by small molecules).

There are a few constant features. All types of adenylyl cyclase studied to date are activated by Gsa. All types studied to date are regulated, directly or indirectly, by members of three of

**Fig. 9. Effect of Gia1(–10) on types I, II, and VI adenylyl cyclase.** Membranes prepared from Sf9 cells expressing type I (A and D), type II (B and E), or type VI (C and F) adenylyl cyclase were incubated with GTPγS-Gia1 (○), myristoylated GTPγS-Gia1 (□), or myristoylated GTPγS-Gia1(–10) (Δ) and assayed in the absence (A–C) or presence (D–F) of 50 μM forskolin. Activities are expressed in nmol-min⁻¹·mg⁻¹. Assays were performed in duplicate, and values are representative of results from at least two separate experiments.
the four major classes of G proteins (G₁α, G₂α, and G₃α; the functions of G₁₂/₁₃ are unknown). Of less obvious regulatory significance are the facts that all types are activated by forskolin and inhibited by P-site analogs. Beyond this, evolution has endowed the different isoforms with impressive diversity, often involving different mechanisms: direct inhibition of types V and VI by Ca²⁺, or indirectly in all cases, and three different Ca²⁺-related mechanisms: and Gi₁δ, Go₁δ, Ca²⁺/calmodulin, and, indirectly, G_i, and G_i₂α, and G_i₃δ, and G_i₄δ can apparently collaborate as activators. G_i, and G_i₂α can collaborate as inhibitors. The effects of G_i, are indirect in all cases, and three different Ca²⁺-related mechanisms are involved: direct inhibition of types V and VI by Ca²⁺ (13, 18, 20), activation of type II by protein kinase C (mechanism unknown) (40–42), and activation of type I by Ca²⁺/calmodulin (19). Given the symmetrical structural relationships of the adenylyl cyclases, we speculate that duplication of the gene provided the opportunity to acquire independent binding sites for the two homologous G protein α subunits, G₁α, and G₁₂/₁₃.

Types V and VI adenylyl cyclase represent the simplest pattern of regulation. They are activated by G₁α, and they are inhibited strongly by G₁β. Under the relatively simple conditions explored to date, the inhibitory effects of Ca²⁺ appear to be more modest than are those of G₁β. It can be anticipated that additional forms of adenylyl cyclase will be discovered that are simply activated by G₁α and inhibited by G₁β without being subject to regulation by Ca²⁺; this is the classical picture.

Type II is noteworthy for strong, conditional activation by βγ. The apparent function of this adenylyl cyclase as a detector for coincidental activation of G₄α and G₁α₂δ, or G₄α₂δ-linked receptors has been discussed previously (17, 40). Interaction between G₄α₂δ and G₁α₂δ-controlled pathways appears to be based on different affinity of the cyclase for G₁β and βγ. Thus, the concentra-

Fig. 10. Binding of [³²S]GTPγS-G₁α to Sf9 cell membranes containing adenylyl cyclase. Unlabeled GTPγS-bound forms of G₁α (C), myristoylated G₁α-myristoyl (M), myristoylated G₁α (V), or nonmyristoylated G₁α (W) were included at the indicated concentrations as competitors. Membranes were from Sf9 cells expressing type I (A), type II (B), or type VI (C) adenylyl cyclase. The amount of labeled G₁α bound to 20 μg of Sf9 cell membranes in the absence of competitors was 19, 27, and 14 fmol for A, B, and C, respectively.

Fig. 11. Effect of βγ on the steady-state GTPase activity of truncated G₁α and wild-type G₁α. A. Purified G protein subunits. Approximately 2.5 μg each of purified wild-type G₁α (WTα), truncated G₁α (NCα), or bovine brain βγ was treated with N-ethylmaleimide and then resolved on an 11% polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue. B. GTP hydrolysis by G₁α, WTα (C) or NCα (O) or NCα (O) (15 nM each) was incubated in 50 μl of buffer containing 0.5 μM 1,25[P]GTP (16,500 counts/min/pmol), 1 mM EDTA, and 2 mM MgSO₄ in the presence of increasing amounts of bovine brain βγ at 30°C. Release of 32P, was determined and expressed as percent of P, released in the absence of βγ (100%), 0.088 pmol and 0.070 pmol for WTα and NCα, respectively. P, release associated with βγ alone was subtracted from each value. Results are the mean of duplicate determinations and are representative of three similar experiments.

Fig. 12. Stimulation of type I and type II adenylyl cyclase by wild-type G₁α and truncated G₁α and modulation of these responses by βγ. A. Stimulation of type I adenylyl cyclase by either wild-type G₁α (WTα) or truncated G₁α (NCα). B, type I adenylyl cyclase activity in the presence of the indicated amounts of βγ alone (C), βγ and 100 nM WTα (B), or βγ and 100 nM NCα (A). C, Stimulation of type II adenylyl cyclase by either WTα (C) or NCα (O). D, type II adenylyl cyclase activity in the presence of the indicated amounts of βγ alone (C), βγ and 100 nM WTα (B), or βγ and 100 nM NCα (A). All assays were performed for 5 min at 30°C in the presence of 10 μM GTPγS. Adenylyl cyclases were provided as Sf9 cell membranes. WTα and NCα were each activated with GTPγS prior to assay.

3 W.-J. Tang, unpublished observations.
inhibited strongly by $G_{i/}$, This result appears to maintain the condition not tested in the present experiments.

Type I adenyl cyclase appears to have independent sites for regulation by the following four types of proteins: $G_{i/}$, $G_{o/}$, $\beta$-arrestin, and Ca$^{2+}$/calmodulin. $G_{i/}$ and Ca$^{2+}$/calmodulin activate the enzyme to similar extents, and they can interact synergistically in doing so (19). Under the conditions studied, $\beta$-arrestin is the more efficacious inhibitor of the enzyme. $G_{i/}$ and $G_{o/}$ can also inhibit, but this effect is largely limited to the Ca$^{2+}$/calmodulin stimulated activity. This is the only form of adenyl cyclase studied that can be inhibited by $G_{o/}$.

Although the apparent affinity of $G_{o/}$ for type I adenyl cyclase is lower than that of $G_{i/}$, $G_{o/}$ is present in higher concentrations. We assume that $G_{i/}$ also acts at site 2 on type I adenyl cyclase. $G_{o/}$ has no obvious effect on types V or VI adenyl cyclase, which are inhibited strongly by $G_{i/}$. This result appears to maintain harmony among the multitude of regulatory interactions, since types V and VI are inhibited by Ca$^{2+}$ and the inhibitory effect of $G_{o/}$ on Ca$^{2+}$ influx would oppose this mechanism. By contrast, $G_{o/}$ can apparently inhibit type I adenyl cyclase both directly (at site 2) and by opposing $G_{i/}$-mediated increments in intracellular Ca$^{2+}$ concentrations.

We detected no differences in the capacity of $G_{i/}$, $G_{o/}$, and $G_{o/}$ to inhibit types V, VI, and I adenyl cyclase. The lack of specificity has been observed with other effectors that are controlled by G protein subunits. Different isoforms of phospholipase Cβ fail to discriminate among $G_{i/}$, $G_{o/}$, and $G_{o/}$, and all three $G_{o/}$s activate cardiac K$^+$ channels with similar potencies and efficacies (45). Although information is less complete, the same is largely true for different isoforms of G protein $\beta$-arrestin subunit complexes (with the exception of the retinal complex, $\beta_{1Y}$). Several different species of $\beta$-arrestin have indistinguishable interactions with adenyl cyclases (46) and phospholipases. The significance of heterogeneity within subgroups of G protein subunits may lie with interactions between G protein oligomers and their receptors (48-50) or with patterns of cellular and subcellular distribution.

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REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615-649
2. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) Nature 346, 125-132
3. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Science 252, 802-808
4. Iwakura, O., Krupinski, J., Chen, L., Halnon, N., Horwitz, J., and Downes, J. (1992) J. Biol. Chem. 267, 3739-3746
5. Kataoka, T., Amano, T., and Usui, M. (1982) J. Biol. Chem. 257, 3739-3746
6. Krupinski, J., Coussen, F., Bakalyar, H. A., Tung, W.-J., Feinstein, P. G., Ort, K., Slaughter, C., Reed, R., and Gilman, A. G. (1989) Science 244, 1558-1564
7. Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W.-J., Krupinski, J., Gilman, A. G., and Reed, R. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10173-10177
8. Bakalyar, H. A., and Reed, R. R. (1990) Science 250, 1403-1406
9. Gao, B., and Gilman, A. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10178-10182
10. Ishikawa, Y., Katsushika, S., Chen, L., Halnon, N. J., Yamada, K., and Horwitz, J. (1992) J. Biol. Chem. 267, 13553-13557
11. Premont, R. T., Chen, J.-Q., Ma, H. W., Ponnapalli, M., and Iyengar, R. (1992) J. Biol. Chem. 267, 8968-8983
12. Yoshino, M., and Cooper, D. M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6716-6720
13. Krupinski, J., Lehman, T. C., Frankenfield, C. D., Zwaagstra, J. C., and Wat-