Distinct Patterns of Bidirectional Regulation of Mammalian Adenylyl Cyclases

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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 Giα1, Gβγ, and Gαs; type I adenylyl cyclase can be inhibited by these three Gα proteins and by Gαi, as well. Type II adenylyl cyclase appears to be immune to inhibition by these proteins.

Examination of the effects of native and mutant Gα proteins, as well as analysis of competition for binding of Gαi to adenylyl cyclases, indicate that at least certain adenylyl cyclases have independent sites for interaction with Gαi (site 1, stimulatory) and Gαo (site 2, inhibitory). High concentrations of Gαi 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 Gαi) for GTP, with resultant dissociation of GTP-Gαi from βγ; adenylyl cyclase is activated by interaction with GTP-Gαi (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 (G1i1, G1i2, or G1i3) or Gα in coupling inhibitory receptors (e.g., an α2-adrenergic receptor) to adenylyl cyclase (4, 5). Although activation of the G1i

and Gβ heterotrimer results from an identical process of guanine nucleotide exchange and subunit dissociation, it has been difficult to decide whether GTP-α, 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 complicated the difficulties in assessing mechanisms of inhibition of adenylyl cyclase activity.

Although all membrane-bound forms of mammalian adenylyl cyclase are activated by Gαs, 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 Gαo-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 assay the regulatory properties of individual adenylyl cyclases with respect to interactions with forskolin, calmodulin, Gαs, 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 Gαs1 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 examined the effects of myristoylated Gαs1, Gαi2, Gαi3, 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 canine type VI adenylyl cyclase (11) was excised from pcDNAamp27–4 with EcoRI and SspI and was cloned into pVL1392 that had been digested with

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EcoRI and Smal. The resultant plasmid was used to construct recombinant virus B-RAC-V1 as described (21).

Membranes were prepared from Sf9 cells expressing individual isoforms of Gi (adenylyl cyclase) (22). Briefly, cells (10%/mL) were infected with the desired baculovirus (1 plaque-forming unit/mL), harvested 48–58 hours later, and lysed by nitrogen cavitation. After removal of nuclei by centrifugation, membranes were collected, washed, and resuspended. Protein concentrations were determined by the method of Bradford (25).

Membrane-bound G protein subunits were synthesized in E. coli as described by Lee et al. (26). For synthesis of myristoylated α subunits (Gαi1, Gαi2, Gαs, and Gαot), 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. (28), as described by Lee et al. (26). Protein concentrations were estimated by staining with Amido Black (29).

Purified α subunits were activated by incubation with 50 mM NaHEPES (pH 8.0), 5 mM MgSO4, 1 mM EDTA, 1 mM dithiothreitol, and 400 μM GTPyS at 30 °C for 30 min (Gαs and Gαot) or 2 h (Gαt and the Gαot trm) (28, 30). Free GTPyS was removed by gel filtration.

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

Adenylyl Cyclase Assays—Adenylyl cyclase activity was measured as described by Smigiel (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 and 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 Gαt (30) or calmodulin (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 0.1%.

Binding of Gαt to Sf9 Cell Membranes Containing Adenylyl Cyclases—[35S]GTPγS-Gαt was prepared by incubating 0.6 μM Gαt (short form) with 1.2 μM [35S]GTPγS for 1 h at 30 °C in 20 mM NaHEPES (pH 8.0), 5 mM MgSO4, 1 mM dithiothreitol, and 1 μg/mL of bovine serum albumin. The reaction mixture was then filtered through Sephadex G-25 to remove free nucleotide. The Gαt binding assay was performed by mixing 20 μM of Sf9 cell membranes expressing the indicated type of adenylyl cyclase, 80 fmol of [35S]GTPγS-Gαt (final concentration 4 nM), and variable amounts of the indicated GTPγS-activated G protein α subunit in 20 μL of buffer containing 20 mM NaHEPES (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). [35S]GTPγS-Gαt, 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 NaHEPES (pH 8.0), 4 mM MgCl2, 1 mM dithiothreitol, and 10 μM forskolin. The amount of labeled Gαt 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 Gαt—Methods for the expression in E. coli and purification of Gαt subunits containing hexahistidine tags at the amino terminus have been described by Lee et al. (26). Hexa-histidine Gαt (16 mg) was purified to near homogeneity from a 5-liter culture of E. coli by Ni2+-NTA affinity chromatography (Qia-gen). Gαt lacking the amino-terminal 34 amino acid residues (and the hexahistidine tags at the amino terminus have been described by Lee et al. (26)). Gαt lacking the amino-terminal 34 amino acid residues (and the hexahistidine tags at the amino terminus have been described by Lee et al. (26)). Gαt lacking the amino-terminal 34 amino acid residues (and the hexahistidine tags at the amino terminus have been described by Lee et al. (26)).

The sample was diluted 5-fold and loaded directly to a 10-μL MonoQ anion-exchange column for fast protein liquid chromatography equilibrated with buffer A (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2, 10% glycerol, and 0.02 mM phenylmethylsulfonyl fluoride) containing the appropriate activator (either GTPγS or AMFG). Protein was eluted from the column with a 60-μL linear gradient of buffer containing 20 mM NaHEPES (pH 8.0), 5 mM MgSO4, 1 mM dithiothreitol, and 1 μg/mL of bovine serum albumin. The reaction mixture was then filtered through Sephadex G-25 to remove free nucleotide. The Gαt binding assay was performed by mixing 20 μM of Sf9 cell membranes expressing the indicated type of adenylyl cyclase, 80 fmol of [35S]GTPγS-Gαt (final concentration 4 nM), and variable amounts of the indicated GTPγS-activated G protein α subunit in 20 μL of buffer containing 20 mM NaHEPES (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). [35S]GTPγS-Gαt, 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 NaHEPES (pH 8.0), 4 mM MgCl2, 1 mM dithiothreitol, and 10 μM forskolin. The amount of labeled Gαt 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

RESULTS

Inhibition of Isoforms of Adenylyl Cyclase by Gαt Subunits: Types V and VI—Our initial observation that purified myristoylated Gαi1 could inhibit type V adenylyl cyclase (23) prompted us to examine the generality of this response with additional isoforms of the enzyme. Results with type VI adenylyl cyclase, which shares many regulatory properties with the type V enzyme, are shown in Fig. 1. The activity of type VI adenylyl cyclase (activated with GTPγS-Gαt) is also inhibited by recombinant myristoylated GTPγS-Gαot. The apparent affinity of type VI adenylyl 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 Gαot). Boiled protein is without effect, ruling out inhibition due to components of the buffer or to unbound GTPγS. The significance of the concentrations of Gαt proteins required to inhibit adenylyl cyclase activity has been discussed previously (23).

As anticipated, substantially higher concentrations of the GDP-bound form of myristoylated Gαi1 were required to inhibit type VI adenylyl 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 Gia1 may be due to exchange of GDP for an activating nucleotide (e.g. GTPβS).) In addition, as described for type V adenylyl cyclase, myristoylation of Gia1 at the amino terminus is required to observe inhibition; micromolar concentrations of the unmodified protein are without effect. Myristoylated, activated Gia1 is also ineffective.

Similar patterns of effects were observed when activated, myristoylated Gial, Gia2, and Gia3 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 Gia proteins. Inhibition of type VI adenylyl cyclase appeared to occur at slightly lower concentrations of the Gia proteins, and the extent of inhibition is somewhat greater than with type V adenylyl cyclase. In addition, the Gia proteins are somewhat more efficacious inhibitors of Gia-activated adenylyl cyclase activity than of forskolin-stimulated activity with both the types V and VI enzyme. The effects of the activated Gia proteins on Gia-stimulated adenylyl cyclase activity in Sf9 cell cyclic-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 Gia 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 GTPβS-Gia-stimulated GTPβS-Gia inhibits enzymatic activity only weakly, while micromolar concentrations of GTPβS-Gia are without effect. The effects of GTPβS-activated Gia2 and Gia3 are similar to those of Gia-stimulated (not shown). Since G protein βγ subunits activate type II adenylyl cyclase (conditionally, in the presence of Gia), we suspected that GTPβS-Gia does not itself inhibit type II adenylyl cyclase but rather the observed inhibition might be due to interaction between small amounts of GDP-Gia1 in the

![Fig. 2. Inhibition of type V, VI, and cyc- adenylyl cyclases by Gia1, Gia2, and Gia3. 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 GTPβS-Gia1 (A-D) or 50 nM forskolin (E-F) and either recombinant myristoylated GTPβS-Gia1 (solid symbols) or GTPβS-Gia3 (open symbols). All activities are expressed as percentages of control values measured in the absence of Gia 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.](image)

![Fig. 3. Effects of Gia 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 GTPβS-Gia-stimulated Gia, which is shown in A with Gia, filled symbols, and in B with Gia3, open symbols. 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.](image)
enzyme at concentrations in the additional data that substantiate this notion are presented synergistically of activated Gi, may be mimicking G,, in these experiments. Inhibition is achieved at the enzyme is stimulated by Ca2+/calmodulin and less extensively in the presence of forskolin; inhibition is very modest when Gia1, Gia2, and Giu3 are compared (Fig. 4). Type I adenylyl cyclase activities in the absence of G,, were performed in duplicate and are representative of three experiments.

Type I—We have shown previously that activated myristoylated G,, can inhibit type I adenylyl cyclase partially when Gia1, Gia2, and Giu3 behaved similarly (not shown). In this case the GTPyS-bound form of the protein, and GTPyS-G,,was nearly devoid of activity. Because forskolin and G,, activate type I adenylyl cyclase synergistically (7), these data suggest that high concentrations of activated G,, may be mimicking G, in these experiments. Additional data that substantiate this notion are presented below.

Fig. 4. Inhibition of type I adenylyl cyclase by G,, subunits. Membranes (10 µg) prepared from Sf9 cells expressing type I adenylyl cyclase were incubated with varying concentrations of GTPyS-bound myristoylated G,, or G,, and adenylyl cyclase activity was assayed. A, calmodulin, CaCl2, and EDTA were included during the 3-min incubation prior to assay to achieve final concentrations of 50 nM, 1 mM, and 1 mM, respectively. B, forskolin (50 µM) was added at the start of the assay. Type I adenylyl cyclase activities in the absence of G,, subunits were 2.2 (A) and 2.3 (B) nmol·min⁻¹·mg⁻¹. Determinations were performed in duplicate and are representative of three experiments.

Fig. 5. Inhibition of type I adenylyl cyclase by G,, or βγ subunits. Sf9 cell membranes (10 µg) containing type I adenylyl cyclase were incubated with 50 nM GTPyS-G,, (A) or 100 nM calmodulin, 1 mM CaCl2, and 1 mM EDTA (B) and varying concentrations of GTPyS-bound myristoylated G,, or brain G protein βγ subunits (C). Adenylyl cyclase activity was assayed in duplicate, and data are representative of at least two experiments.

Fig. 6. Effects of GTPyS-G,, and βγ on type I adenylyl cyclase. Adenylyl cyclase activity of membranes (10 µg) prepared from Sf9 cells expressing type I adenylyl cyclase was assayed following incubation with myristoylated recombinant GTPyS-G,, and βγ subunits. Activities were measured in the presence of the indicated concentrations of GTPyS-G,, and 50 µM forskolin and in the absence (■) or presence of 3 nM (○) or 100 nM (▲). βγ A, activities are expressed in nmol·min⁻¹·mg⁻¹. B, values are plotted as a percentage of control (absence of GTPyS-G,,).

Surprisingly, myristoylated GTPyS-G,, 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 G,, and G,, is comparable, G,, is approximately 10 times less potent. Nevertheless, this effect of G,, may be physiologically relevant, since the concentrations of G,, in brain are very high and exceed those of G,, by a considerable amount (31). Activated G,, (300 nM) did not inhibit type I adenylyl cyclase (not shown).
We observed a consistent capacity of activated $G_{\alpha}$ 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 Ca²⁺/calmodulin (Figs. 4, 5, and 7) or forskolin (Figs. 4, 6, and 7), but not $G_{\beta\gamma}$ (Fig. 5). While less dramatic, this behavior is reminiscent of the effects of $G_{\alpha}$ on forskolin-stimulated type II adenylyl cyclase described above. The fact that this phenomenon is not observed in the presence of $G_{\beta\gamma}$ again suggests that it is due to interaction of high concentrations of $G_{\alpha}$ with a binding site for $G_{\alpha}$ on adenylyl cyclase (see below).

**Mechanism of Inhibition of Adenylyl Cyclase by $G_{\alpha}$**—The observation that $G_{\alpha}$ can inhibit adenylyl cyclase activity in the absence of $G_{\beta\gamma}$ (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 $G_{\alpha}$ on $G_{\alpha}$-activated type V adenylyl cyclase indicates a largely competitive relationship between the two proteins over a relatively narrow range of concentrations of $G_{\alpha}$ (2–20 nm; not shown), this is not true when a broader range of concentrations is examined (Fig. 8). As the concentration of $G_{\alpha}$ is raised, the maximal extent of inhibition that can be obtained with $G_{\beta\gamma}$ is clearly reduced.

These data are thus consistent with a model in which $G_{\alpha}$ and $G_{\beta\gamma}$ bind to distinct sites on adenylyl cyclase. Activation results from binding of $G_{\alpha}$ to site 1, while binding of $G_{\beta\gamma}$ to site 2 causes inhibition. We propose that $G_{\alpha}$ 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 Ca²⁺/calmodulin, $G_{\alpha}$ first inhibits type I adenylyl cyclase (site 2) but then activates at high concentrations (site 1). Binding of $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$ (or $G_{\alpha}$ in site 1).

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

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

**Binding of $G_{\alpha}$**—A binding assay was developed to detect interactions of activated $G_{\alpha}$ with membranes from S9 cells expressing different isoforms of adenylyl cyclase.² Binding of [³²S]GTPyS-γ-GST to membranes from cells expressing either type I or type II adenylyl cyclase is 3–6-fold higher than is the 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 $G_{\alpha}$ 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.

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**Fig. 7. Inhibition of type I adenylyl cyclase by $G_{\alpha}$.** Membranes prepared from S9 cells expressing type I adenylyl cyclase were incubated with varying concentrations of myristoylated GTPyS-γ-GST (C) or myristoylated GTPyS-γ-GST (C) and assayed as described. A, calmodulin, CaCl₂, and EDTA were included to yield final concentrations of 50 nm, 1 mm, and 1 mm, respectively. B, forskolin (50 μM) was included in the assay. Assays were performed in duplicate, and results are representative of two experiments. Activities are expressed as a percentage of control values measured in the absence of $G_{\alpha}$ or $G_{\beta\gamma}$ (A, 1.3 and 1.5, 2.0 nmol/min/mg).

**Fig. 8. GTPyS-γ-GST-mediated inhibition of type V adenylyl cyclase at different concentrations of GTPyS-γ-GST.** Membranes prepared from S9 cells expressing type V adenylyl cyclase were incubated with varying concentrations of myristoylated GTPyS-γ-GST, and activities were assayed. GTPyS-γ-GST was included in the incubations to give final concentrations of 15 (C), 50 (C), 500 (C), or 450 (C) nm. Activities are expressed in nmol/min/mg, and numbers in parentheses indicate the percent of control activity at the highest concentration of $G_{\alpha}$ tested. All determinations were performed in duplicate, and results are representative of at least three experiments.
As expected, addition of unlabeled GTPγS-Gsα inhibits binding of the labeled complex by competition (Fig. 10). Half-maximal competition is observed at roughly 10–30 nM concentrations of GTPγS-Gsα, in reasonable agreement with concentrations of GTPγS-Gsα necessary to activate the adenylyl cyclases (Fig. 9). Activated myristoylated Gsα is also an effective competitor for Gsα-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-Gsα compete for Gsα-binding sites on type I and type II adenylyl cyclases (compare curves with those for nonmyristoylated Gsα, which we take as an ineffective control); these are the two adenylyl cyclases where activation is seen at high concentrations of myristoylated Gsα. There is no significant competition by myristoylated Gsα for Gsα-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 Gsα and Gsα interact with distinct sites on adenylyl cyclase to activate and inhibit the enzyme, respectively.

**Interactions Between Gsα 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 Ca2+/calmodulin. Despite the fact that βγ inhibits both Gsα- and Ca2+/calmodulin-activated type I adenylyl cyclase, the question has arisen about the nature of the interaction between Gsα, βγ, and types I and II adenylyl cyclase. Do Gsα and βγ interact independently with these enzymes or might they interact as the heterotrimer (given the low affinity of GTPγS-Gsα 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 Gsα 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 Gsα for βγ is documented in Fig. 11B, wherein interactions between Gsα and βγ were assessed by examination of the capacity of βγ to inhibit the steady-state GTPase activity of Gsα. The basis of this effect lies in βγ-induced slowing of the rate of dissociation of GDP from Gsα. Interaction between βγ and Gsα could not be detected at the highest concentrations of βγ tested (100 nM). Truncated Gsα 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 Gsα-activated type I adenylyl cyclase or to activate Gsα-stimulated type II adenylyl cyclase when these assays were performed in the presence of wild-type Gsα or truncated Gsα (Fig. 12, B and D). These experiments support the hypothesis that these adenylyl cyclases have independent binding sites for Gsα 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 Gsα. All types studied to date are regulated, directly or indirectly, by members of three of
the four major classes of G proteins (Gαs, Gαi, and Gαq; 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 by using the same or related molecular players to accomplish different responses. Direct inhibition of types V and VI by Ca2+ is now clearly understood (13, 18, 19). Given the symmetrical structures 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, Gαs and Gαi.

Types V and VI adenylyl cyclase represent the simplest pattern of regulation. They are activated by Gαs and they are inhibited strongly by Gαi. Under the relatively simple conditions explored to date, the inhibitory effects of Ca2+ appear to be more modest than are those of Gαi. It can be anticipated that additional forms of adenylyl cyclase will be discovered that are simply activated by Gαs and inhibited by Gαi 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 Gαi and Gαo or Gαi-linked receptors has been discussed previously (17, 40). Interaction between Gαi and Gαo-controlled pathways appears to be based on differential affinity of the cyclase for Gαs and βγ. Thus, the concentra-
tions of βγ required to coactivate the enzyme are hypothesized to be generated only by activation of Gβ or Gγ, not by activation of Gi, which is present in much lower abundance (17, 21). We are unable to detect substantial inhibition of type II adenylyl cyclase by Gi or Go. This makes sense in view of the stimulatory effect of βγ. It would be difficult to rationalize release of both an activator and an inhibitor as a result of dissociation of the subunits of the Gi or Go oligomer. However, it is conceivable that Gαs might inhibit type II adenylyl cyclase under some condition not tested in the present experiments.

Type I adenylyl cyclase appears to have independent sites for regulation by the following four types of proteins: Gαs, Gαi/Go, βγ, and Ca2+/calmodulin. Gαs and Ca2+/calmodulin activate the enzyme to similar extents, and they can interact synergistically in doing so (19). Under the conditions studied, βγ is the more efficacious inhibitor of the enzyme. Gαi and Gαo can also inhibit, but this effect is largely limited to the Ca2+/calmodulin stimulated activity. This is the only form of adenylyl cyclase studied to date that can be inhibited by Gαo. Although the apparent affinity of Gαo for type I adenylyl cyclase is lower than that of Gαi, Gαo is present in higher concentrations. We assume that Gαo also acts at site 2 on type I adenylyl cyclase. Gαo has no obvious effect on types V or VI adenylyl cyclase, which are inhibited strongly by Gαi. This result appears to maintain harmony among the multitude of regulatory interactions, since types V and VI are inhibited by Ca2+ and the inhibitory effect of Gαi on Ca2+ influx would oppose this mechanism. By contrast, Gαi can apparently inhibit type I adenylyl cyclase both directly (at site 2) and by opposing Gαs-mediated increments in intracellular Ca2+ concentrations.

We detected no differences in the capacity of Gαi, Gαo, and Gαs to inhibit V, VI, and I adenylyl 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 Gαi, Gαo, and Gαs (43, 44), and all three Gαs 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 βγ subunit complexes (with the exception of the retinal complex, βγRII). Several different species of βγ have indistinguishable interactions with adenylyl 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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