Differential Regulation of Adenyl Cyclases by $\text{Ga}_\alpha$*

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Regulation of adenyl cyclase 1, 2, and 6 by $\text{Ga}_\alpha$ was studied. All three mammalian adenyl cyclases were expressed in insect (Sf9 or Hi-5) cells by baculovirus infection. Membranes containing the different adenyl cyclase isoforms displayed distinct signal receiving capabilities from $\text{Ca}^{2+}$ (1–3). Though these adenylyl cyclase isoforms display distinct domain interactions involved in interactions with $\text{G}_\alpha$, the $\text{G}_\alpha$ stimulation of AC1 was best explained by a two-site model with a “high affinity” site at 0.9 nM and a “low affinity” site at 15 nM. Occupancy of the high affinity site appears to be sufficient for $\text{G}_\gamma$ stimulation of AC2. $\text{G}_\alpha$ stimulation of AC6 was also best explained by a two-site model with a high affinity site at 0.6–0.8 nM and a low affinity site at 8–22 nM: however, in contrast to AC2, only a model that assumed interactions between the two sites best fit the AC6 data. With 100 μM forskolin, $\text{G}_\alpha$ stimulation of all three adenyl cyclases showed very similar profiles. $\text{G}_\alpha$ stimulation in the presence of forskolin involved a single site with apparent $K_{\text{act}}$ of 0.1–0.4 nM. These observations indicate a conserved mechanism by which forskolin regulates $\text{G}_\alpha$ coupling to the different adenyl cyclase isoforms. However, there are fundamental differences in the mechanism of $\text{G}_\alpha$ stimulation of the different adenyl cyclase isoforms with AC2 and AC6 having multiple interconvertible sites. These mechanistic differences may provide an explanation for the varied responses by different cells and tissues to hormones that elevate cAMP levels.

Nine adenyl cyclase isoforms have been cloned and characterized (1–3). Though these adenyl cyclase isoforms display distinct signal receiving capabilities from $\text{Ca}^{2+}$ (4, 5), $\text{G}_\gamma$ (6), and protein kinase C (7), they all share the common capability to be stimulated by $\text{G}_\alpha$. Due to its expected nature, regulation by $\text{G}_\alpha$ has received somewhat less attention than other modes of regulation. Little is known about the regions of adenyl cyclase involved in $\text{G}_\alpha$ interactions. Early studies with the purified olfactory adenyl cyclase showed that $\text{G}_\alpha$ could be covalently linked to adenyl cyclase (8). As a prelude to the mapping of adenyl cyclase regions involved in interactions with $\text{G}_\alpha$, we have studied regulation of recombinant AC6, AC2, and AC1 by mutant (Q227L) activated $\text{G}_\alpha$. To our surprise, we find $\text{G}_\alpha$ regulation of the three different adenyl cyclases appears to be mechanistically different. Stimulation of AC1 is the simplest and appears to involve a single site of interaction. Stimulation of AC2 and AC6 appears to be best explained by multiple sites of interaction. This complex regulation is observable only in the absence of forskolin. In the presence of forskolin, $\text{G}_\alpha$ stimulation by all three adenyl cyclase isoforms tested appears to involve a single high affinity site.

EXPERIMENTAL PROCEDURES

Materials—In vitro translation kits and Flexi rabbit reticulocyte lysates and reagents for RNA synthesis were from Promega (Madison, WI), and $[^{32P}]\text{ATP}$ was from ICN. Most biochemicals were from Sigma, and cell culture supplies were from Life Technologies, Inc. All other reagents were the highest grade available.

In Vitro Synthesis of $\text{Ga}_\alpha$ and Q227L-$\text{Ga}_\alpha$—The wild-type $\text{Ga}_\alpha$(short form without the Ser) cloned from a human liver library (8) and the Q227L-$\text{Ga}_\alpha$ ($\text{Ga}_\alpha$*) in pAGA were the gift of Dr. Juan Codina (UT Health Sciences Center, Houston). As described previously by Sanford et al. (9) pAGA is derived from pGEM-SZ (−) and contains the 5′-untranslated sequences of the alfalfa mosaic virus RNA (for sequence, see Ref. 10) upstream of the $\text{G}_\alpha$* sequence. Transcription and translation was according to the method of Sanford et al. (9). The vector containing pAGA-$\text{G}_\alpha$* was linearized with $\text{XhoI}$, and RNA was transcribed using T7 RNA polymerase. The newly transcribed mRNA was purified by phenol/chloroform extraction, precipitated by ethanol, and stored in RNase-free water until use. The $\text{G}_\alpha$* mRNA was translated in rabbit reticulocyte lysates with all 20 amino acids (20 μM) and approximately 3 × 10⁴ cpm of $[^{35}S]\text{Met}$ (~1,200 Ci/mmol). The translation product was washed by 10-fold dilution and concentrated in a Centricon-10 concentrator. After three washes, the proteins in 25 mM Na-Hepes, 1 mM EDTA, and 20 mM-β-mercaptoethanol were frozen in a dry-ice acetone bath and stored at −80 °C until used. There are seven Met in $\text{G}_\alpha$*. Assuming that all seven methionines were labeled, specific activity of the translated $\text{G}_\alpha$* was calculated by measurement of percent incorporation of $[^{35}S]$-labeled t-Met into trichloroacetic acid precipitable material.

Expression of Adenyl Cyclases in Insect Cells—AC2 and AC6 were expressed in Sf9 or Hi-5 cells as described previously (11, 12). Bovine AC1 was tagged with the FLAG epitope at the N terminus using a strategy similar to that used for AC2 (11). The recombinant F-AC1 containing virus was then constructed by use of the shuttle vector pVL1392. Sf9 cells were grown in serum-free insect medium while Hi-5 cells were grown in Grace’s medium supplemented with l-glutamine, yeastolate, and lactalbumin with 10% fetal bovine serum and 10% fetal bovine serum. Infected cells were grown in Grace’s medium supplemented with l-glutamine, yeastolate, and lactalbumin with 10% fetal bovine serum and 10% fetal bovine serum.

Preparation of Adenyl Cyclase-containing Membranes—Infected cells were collected by centrifugation at 4 °C. All further procedures were at 4 °C. Cells were washed in lysis buffer containing 20 mM Na-Hepes, pH 8.0, 5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM dithiothreitol, and a mixture of protease inhibitors including 1 mM phenylmethylsulfonyl fluoride (freshly prepared), 1 mM-β-mercaptoethanol, 3.2 μg/ml each leupeptin and soybean trypsin inhibitor, and 2 μg/ml aprotinin and then resuspended in 10 volumes of lysis buffer. Cells were lysed by nitrogen cavitation in a Parr bomb at 600 p.s.i. for 30 min. Lysates were centrifuged at 1000 × g for 10 min (without break) to pellet the cell debris. The 1000 × g pellet was resuspended in 20 mM Na-Hepes, 1 mM EDTA, pH 8.0, 200 mM sucrose, 2 mM dithiothreitol, and the protease mixture to obtain a final concentration of 3–5 mg/ml protein. Aliquots were frozen in a dry-ice acetone bath and stored at −80 °C.

Immunoblotting—Membranes containing AC2 and AC6 were blotted

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1 The abbreviations used are: $\text{G}_\alpha$*, Q227L-$\text{G}_\alpha$; Gpp(NH)p, guanosine 5′-(β,γ-imidodiposphate); RMS, residual mean squares.
with the AGcom antibody at 1:2000 dilution. The bands were visualized by horseradish peroxidase-coupled second antibody using the ECL system.

Adenylyl Cyclase Assays—Sf9 or Hi-5 cells containing adenylyl cyclase was assayed for activity in the presence of 0.1 mM[^32]P|ATP (~1000 cpm/pmol), 10 mM MgCl₂ and other additives as described previously. Typically, 3–5 mg of crude membrane protein was assayed in a volume of 30–50 μl for 15 min at 32 °C. The concentration of Gα used is described in the individual experiments.

Data Analysis—The Gα concentration effect curves were analyzed using the program PROPHET (Version 4.3) on a Sun Sparc workstation. PROPHET is sponsored by the NIH and distributed by BBN Systems Technology. Initial determinations of the models to be used for fitting were made by visual inspections of the Hofstee plots. AC1 data were fitted to a single-site model using the equation \( V = \frac{V_{\text{max}}^{\alpha} \cdot A}{K_{\text{act}}^{\alpha} + A} \), where \( V \) is the activity of adenylyl cyclase at \( A \), the concentration of the activator Gα, \( V_{\text{max}}^{\alpha} \) is the maximum reaction rate, and \( K_{\text{act}}^{\alpha} \) is the concentration of the activator at half \( V_{\text{max}} \). Initially, the AC2 data were also fitted to a one-site model. However, a significant improvement in the fit was obtained when the data were fit to a noninteractive two-site model using the equation \( V = \frac{V_{\text{max}}^{\alpha} \cdot A}{K_{\text{act}}^{\alpha} + A} + \frac{V_{\text{max}}^{\beta} \cdot A}{K_{\text{act}}^{\beta} + A} \), where \( V \) is the activity of AC2 at concentration \( A \) of Gα, and the parameters for the two sites are labeled with corresponding subscripts. The AC6 data were first fit to a one-site model and then to a noninteractive two-site model. Neither model fit the data adequately. Hence, the AC6 data were then fitted to an interactive two-site model. At low concentrations of Gα, the data are fitted with function, \( V = \frac{V_{\text{max}} \cdot A}{K_{\text{act}} + A} \), since only one (the high affinity) site would be effective at low concentrations of Gα. We then assumed that the low affinity site would become operative only above a certain threshold concentration of Gα. The data points above this threshold concentration were then fitted to a modified two-site model described by the equation \( V = \frac{V_{\text{max}}^{\alpha} \cdot A}{K_{\text{act}}^{\alpha} + A} + \frac{V_{\text{max}}^{\beta} \cdot A}{K_{\text{act}}^{\beta} + A} \), where \( A_{\text{th}} \) represents the threshold concentration for Gα, and \( V_{\text{max}}^{\alpha} = \frac{V_{\text{max}}}{K_{\text{act}}^{\alpha} + A_{\text{th}}} \) and \( V_{\text{max}}^{\beta} = \frac{V_{\text{max}}}{K_{\text{act}}^{\beta} + A_{\text{th}}} \). These threshold concentrations were estimated by initial visual inspection of the Gα concentration-effect curve followed by an iterative fitting process to obtain a threshold value that generated a curve that best fit the data points. Three parameters were used to evaluate the model that best fit the data. The first was the \( p \) values of fitted constants, the second was the residual mean square (RMS) value of the fit, and the third was the F-test statistics to show the significance of the improved fit. Plots of the data points and the fitted curves were generated by Prophet. The printed plots were exported to the Canvas program in a Mac 8100 by use of an optical scanner. The plots were labeled and assembled within Canvas. The final plots as shown were printed as Canvas files.

RESULTS

The wild type and mutant activated Gα, expressed in reticulocytes, was tested for its ability to stimulate S49 cycl- membranes. Wild-type Gα did not stimulate adenylyl cyclase activity in the presence of GTP. However, extensive stimulation was seen with the nonhydrolyzable GTP analog Gpp(NH)p (data not shown). However, with Gα, extensive stimulation was observed with or without added GTP (Fig. 1). In contrast, control lysate did not stimulate the cycl- adenylyl cyclase. These results indicated that we had synthesized an activated Gα, which we used in further studies. When we studied different adenylyl cyclase isoforms expressed in Sf9 cells, we were cognizant of the activity of the endogenous adenylyl cyclases of Sf9 cell membranes. In the case of AC1 and AC2, the endogenous activities represented 3–5% of the total activity under all conditions and hence were not subtracted. In the case of AC6, however, the endogenous activity was between 20–30%. Hence for all AC6 experiments, the difference between control (Sf9 cells infected with baculovirus containing thyroid peroxidase) and AC6-containing membranes for all concentration points was taken as AC6 activity.

The effect of varying concentrations of Gα in the presence and absence of forskolin on AC1 expressed in Sf9 cells is shown in Fig. 2. In the absence of forskolin, Gα stimulated AC1 with an apparent \( K_{\text{act}} \) of 0.9 nM. Stimulation by Gα showed a simple one-site Michaelis-Menten mechanism, and a standard Hofstee transformation yielded a straight line (Fig. 2A, inset). Addition of 100 μM forskolin decreased the apparent \( K_{\text{act}} \) of Gα 6-fold to 0.16 nM; however, the nature of the curve was unaltered (Fig. 2B).

We studied the effect of varying concentrations of Gα in the presence and absence of forskolin on AC2. In the absence of
AC2-containing membranes by immunoblotting with the AC-comm antiserum. A single labeled band of approximately 120 kDa was observed. This profile indicates that the expressed AC2 is not a mixture of differentially modified proteins. We next determined the profile of the Goa response in the presence of forskolin. With 100 μM forskolin, the Goa stimulation of AC2 yielded a simple profile (Fig. 3B); the Hofstee transformation was linear, with a single apparent Kact of 0.39 nM measured (Fig. 3B, inset). In Fig. 3B, the activity due to forskolin alone (313 pmol/min/mg) had been subtracted prior to data analysis and fitting. We next tried to define the conditions of Goa interaction with AC2 that was optimal for Gβγ stimulation. Without Goa, no significant stimulation of AC2 by Gβγ was observable (data not shown). At 2.0 nM, Goa stimulation by Gβγ was seven-fold. In contrast, at 20 nM Goa, Gβγ stimulated only three-fold over Goa (Fig. 3C). These results suggest that occupancy of the high affinity site may be sufficient for Gβγ stimulation.

We next studied Goa stimulation of AC6. Goa stimulation of AC6 was biphasic (Fig. 4A). Hofstee transformation yielded a curvilinear profile (Fig. 4A, inset II). The Goa concentration-response data for AC6 best fit to a two-site model. However, the two-site fitting for AC6 was different from that used for AC2. At low concentrations of Goa, the data showed a single-site interaction profile (Fig. 4A, inset I). The second site was observable only at concentrations of Goa above a certain threshold value. For the experiment shown in Fig. 4A, the threshold value was 3 nM. AC6 activities above 3 nM Goa are the summa-

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**Fig. 3.** Effect of varying concentrations of Goa* on AC2 activity in the absence (A) and presence (B) of 100 μM forskolin. 5 μg of S9 cell membranes containing AC2 was assayed in the presence of indicated concentrations of Goa*. Immunoblot of the AC2 containing membranes with the AC-comm antiserum is shown to the right of the curve in panel A. 5 μg of membrane protein was used for immunoblotting. The Prophet program was used to analyze the data and obtain the fitted curves. Inset shows Hofstee transformations of the data for panels A and B. The apparent Kact and Vmax obtained from the fitting are shown in the boxes. For the data in Fig. 3A, the p values for the constants using the two-site model were p(Vmax1) < 0.006, p(Kact1) < 0.001, p(Vmax2) < 0.001, and p(Kact2) < 0.004. The RMS values of the one-versus the two-site fits were 436 versus 206. The F statistic for the one-site model was 1298 and for the two-site model was 1380. For the experiment shown in Fig. 3B, the F statistic for the one-site model was 1298 and for the two-site model was 1380. The data in panel B, the p values for constants were less than 0.001. C, effect of varying concentrations of Goa* on stimulation of AC2 by 100 nM Gβγ subunits purified from bovine brain. Values are means of triplicate determinations. Coefficient of variance was less than 5%. These experiments are representative of five other experiments except for panel C, which was repeated twice. For other details, see "Experimental Procedures."
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FIG. 4. Effect of varying concentrations of \( \text{Go}_s^* \) on AC6 activity. 5 \( \mu \)g of Hi-5 cell membranes containing AC6 was assayed in the presence of indicated concentrations of \( \text{Go}_s^* \). The Prophet program was used to analyze the data and obtain the fitted curves. Insets (except I in panel A) show Hofstee transformations of the data. The apparent \( K_{\text{act}} \) and \( V_{\text{max}} \) obtained from the fitting are shown in the boxes. A, profile of stimulation by varying concentrations of \( \text{Go}_s \). Inset I is a magnified plot of the data at lower concentrations of \( \text{Go}_s \). Immunoblot of the AC6-containing membranes with the AC6 antisera is shown to the right of the curve in panel A. 5 \( \mu \)g of membrane protein was used for immunoblotting. The \( p \) values for the constants using the interactive two-site model were \( > 0.001, 0.001, K_{\text{act}} < 0.001, V_{\text{max}} < 0.001 \), and \( K_{\text{act}} < 0.001 \). The RMS value of the one-site fit was 208, the non-interactive two-site fit was 17.8, and the interactive two-site model was 5. The \( F \) statistic for the one-site model was 38, for the non-interactive two-site model was 275, and for the interactive two-site model was 1377. \( \text{Go}_s \) stimulation was measured in the presence of forskolin. The \( p \) value in the absence of forskolin. The \( p \) value for the constants using the interactive two-site model was less than 0.001. C, \( \text{Go}_s \) stimulation was measured in the presence of 100 \( \mu \)M forskolin. These data and those in Fig. 4B were part of one experiment. The \( p \) value for constant was less than 0.01. D, stimulation of solubilized AC6 by different concentrations of \( \text{Go}_s \). AC6 was solubilized in 0.8% dodecyl maltoside as described previously (11). The 60,000 \( \times \) g supernatant was used for the assay. Detergent concentration in the assay 0.03%. 5 \( \mu \)g of solubilized membrane protein was used for the assay. The experiments in panels A-C are representative of five other experiments. The experiment in panel D was repeated twice. For other details, see “Experimental Procedures.”

tonic fashion and is the simplest of the three isoforms studied here. Forskolin increases the affinity of \( \text{Go}_s \) for AC1 without altering the profile of the activity curve. It is noteworthy that the apparent \( K_{\text{act}} \) for \( \text{Go}_s \) is similar to the \( K_c \) for \( \beta \gamma_2 \) (13), thus for all practical purposes \( \text{Go}_s \)-coupled receptors would be unable to activate AC1, as has been recently demonstrated by Storm and co-workers (14). Stimulation of AC2 and AC6 by \( \text{Go}_s \) is more complex. In the absence of forskolin, \( \text{Go}_s \) stimulation of AC2 and AC6 is best explained by a two-site model. However, forskolin not only increases the affinity of \( \text{Go}_s \) but also renders stimulation monotonic, indicating that both the low and high affinity sites can be converted to a higher single affinity site in the presence of forskolin. The single \( \text{Go}_s \) site in the presence of forskolin indicates that the two sites displayed by AC2 and AC6 are not due to two populations of enzymes when expressed in Sf9 cells.

For AC2, occupancy of the “high affinity” \( \text{Go}_s \) site appears to be sufficient for \( \beta \gamma_2 \) stimulation, since maximal-fold stimulation by \( \beta \gamma_2 \) is observed at 2 nM \( \text{Go}_s \). This may indicate that some of the determinants for \( \text{Go}_s \) binding are in the cytoplasmic tail. Interaction of \( \text{Go}_s \) with these determinants could open up one of the regions involved in \( \beta \gamma_2 \) binding. Such a model would provide a molecular explanation for why \( \beta \gamma_2 \) only stimulates AC2 activated by \( \text{Go}_s \).

Though \( \text{Go}_s \) stimulation of both AC2 and AC6 best fit a two site model, our kinetic analysis indicates that there may be crucial mechanistic differences in \( \text{Go}_s \) stimulation of the two adenylyl cyclases. The AC2 data can be fit to Michaelis-Menten type of equations, suggesting two noninteracting sites. In contrast, the model used to fit the AC6 data required an iterative two-site fitting procedure where the estimated \( V_{\text{max}} \) of the high affinity site had to be subtracted from the observed activity at higher \( \text{Go}_s \) concentrations to fit the second site so as to obtain an overall best fit. Such a fitting protocol may indicate interactions between the two sites such that the high affinity site needs to be occupied to obtain occupancy of the low affinity site.

The kinetic descriptions of \( \text{Go}_s \) interactions with the various adenylyl cyclases provided here should not be construed as an indication that each molecule of AC2 or AC6 contains two \( \text{Go}_s \) binding sites. This is one possible explanation. In such a case, AC1 would either have only one \( \text{Go}_s \) site or two sites with very similar affinities. Studies by Neer and co-workers on the hydrodynamic properties of the \( \text{Go}_s \)-adenylyl cyclase from bovine caudate which yield molecular weights for \( \text{Go}_s \)-adenylyl cyclase complex in the presence of forskolin of 197–225 kDa (15) may support the stoichiometry of 2 \( \text{Go}_s \)-adenylyl cyclase. This post-
sibility needs to be explored further in detail for the different adenylyl cyclases. There also exist data that do not support 2 $G_{\alpha}$ sites/adenylyl cyclase model. Cross-linking of $G_{\alpha}$ to the olfactory adenylyl cyclases suggest a 1:1 stoichiometry (8). If each adenylyl cyclase molecule contains one $G_{\alpha}$ site, our data may suggest that adenylyl cyclases have a propensity to dimerize in the membrane environment. If different adenylyl cyclases have differing capabilities to form homodimers, then our data would suggest that AC1 had a very low capability to form homodimers while AC2 and AC6 are more likely to dimerize. Future studies analyzing cross-linking $G_{\alpha}$ to adenylyl cyclase as well as accurate molecular size determinations of the $G_{\alpha}$-adenylyl cyclase complex within the membrane are required to determine the stoichiometry of $G_{\alpha}$-adenylyl cyclase interactions. Irrespective of the details that emerge from these studies, it has become abundantly clear that each adenylyl cyclase can respond differently to $G_{\alpha}$ and that such differential responses could be crucial factors in determining why different tissues and organs show specialized capability to regulate cAMP elevation.

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