Inhibition of adenylyl cyclase isoforms V and VI by various G\textsubscript{\(\beta\gamma\)} subunits

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ABSTRACT An intriguing development in the G-protein signaling field has been the finding that not only the G\textsubscript{\(\alpha\)} subunit, but also G\textsubscript{\(\beta\gamma\)} subunits, affect a number of downstream target molecules. One of the downstream targets of G\textsubscript{\(\beta\gamma\)} is adenylyl cyclase, and it has been demonstrated that a number of isoforms of adenylyl cyclase can be either inhibited or stimulated by G\textsubscript{\(\beta\gamma\)} subunits. Until now, adenylyl cyclase type I has been the only isoform reported to be inhibited by free G\textsubscript{\(\beta\gamma\)}. Here we show by transient cotransfection into COS-7 cells of either adenylyl cyclase V or VI, together with G\textsubscript{\(\gamma\)2} and various G\textsubscript{\(\beta\)} subunits, that these two adenylyl cyclase isoforms are markedly inhibited by G\textsubscript{\(\beta\gamma\)}. In addition, we show that G\textsubscript{\(\beta\)1} and G\textsubscript{\(\beta\)5} subunits differ in their activity. G\textsubscript{\(\beta\)1} transfected alone markedly inhibited adenylyl cyclase V and VI (probably by recruiting endogenous G\textsubscript{\(\gamma\)} subunits). On the other hand, G\textsubscript{\(\beta\)5} produced less inhibition of these isoforms, and its activity was enhanced by the addition of G\textsubscript{\(\gamma\)2}. These results demonstrate that adenylyl cyclase types V and VI are inhibited by G\textsubscript{\(\beta\)1} dimers and that G\textsubscript{\(\beta\)1} and G\textsubscript{\(\beta\)5} subunits differ in their capacity to regulate these adenylyl cyclase isoforms.—Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F., Vogel, Z. Inhibition of adenylyl cyclase isoforms V and VI by various G\textsubscript{\(\beta\gamma\)} subunits. FASEB J. 12, 1019–1025 (1998)

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ADENYLYL CYCLASE (AC)\textsuperscript{2} is a major target enzyme whose activity is modulated by receptor-coupled G-proteins. AC activity can be stimulated by G\textsubscript{\(\alpha\)} and is inhibited by G\textsubscript{\(\alpha\)/2}. It has recently been shown that the wide array of AC isoforms (nine are currently known) display differential sensitivity to free G\textsubscript{\(\beta\gamma\)} subunits. Previous results have demonstrated that AC I is significantly inhibited whereas AC II, IV, and presumably VII are activated by G\textsubscript{\(\beta\gamma\); 1–9). Six G\textsubscript{\(\beta\)} and 12 G\textsubscript{\(\gamma\)} isoforms have been cloned to date (6). Most previous experiments investigating the role of free G\textsubscript{\(\beta\gamma\)} on AC activity were performed in cell-free systems with either baculovirus/Sf9 recombinant G\textsubscript{\(\beta\)} and G\textsubscript{\(\gamma\)} preparations (10, 11) or purified brain G\textsubscript{\(\beta\gamma\)} preparations that consist of a mixture of various G\textsubscript{\(\beta\gamma\)} heterodimers (3, 12, 13). There is little information about the possible variations between the effects of the various G\textsubscript{\(\beta\)} and G\textsubscript{\(\gamma\)} combinations in intact cells. This is important because the various G\textsubscript{\(\beta\)} and G\textsubscript{\(\gamma\)} subunits do not necessarily have the same regulatory activities. Indeed, we recently showed that activation of PLC-\(\beta\)2 by G\textsubscript{\(\beta\gamma\)} is G\textsubscript{\(\beta\)} isoform independent (G\textsubscript{\(\beta\)1} being equally effective as G\textsubscript{\(\beta\)5}), whereas activation of MAPK/ERK and JNK/SAPK appears to be G\textsubscript{\(\beta\)5} isoform dependent (14).

AC V and VI represent a subfamily within the group of AC isoymes. These two isoymes share high sequence homology and are both inhibited by activated G\textsubscript{\(\alpha\)1s, but were not found to be sensitive to free G\textsubscript{\(\beta\gamma\)} subunits when using in vitro reconstitution assays (4, 6, 7, 15, 16). In this study, we have investigated the modulation of AC V and VI by specific G\textsubscript{\(\beta\gamma\)} combinations, using cotransfection of G\textsubscript{\(\beta\)} and G\textsubscript{\(\gamma\)}, together with these AC isoymes. Due to the divergence in sequence homology between G\textsubscript{\(\beta\)1} and G\textsubscript{\(\beta\)5}, we have concentrated our studies on these two G\textsubscript{\(\beta\)} subunits and their modulation of activity of the AC isoymes in question. We observed that the activities of AC V and VI are inhibited by G\textsubscript{\(\beta\)5} and that G\textsubscript{\(\beta\)1} is more efficient than G\textsubscript{\(\beta\)5} in conferring this inhibition.

MATERIALS AND METHODS

Cell cultures

COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin.

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² Abbreviations: AC, adenylyl cyclase; FS, forskolin; HRP, horseradish peroxidase; TSH, thyroid-stimulating hormone; IBMX, 3-isobutyl-1-methylxanthine; αT, α-transducin; PBS, phosphate-buffered saline.
cDNAs of AC V and VI were used in the pXMD1 vector under control of the adenovirus-2 major late promoter (17). The preparation of these plasmids as well as plasmids containing Gp1, Gp5, Gp2, GpE10K, and GpC68S cDNAs were described previously (9, 14, 18, 19). The cDNA for α-transducin (αT) and thyroid-stimulating hormone (TSH) receptor cDNA were described (5, 20).

**Transfection of COS cells**

COS-7 cells in 10 cm dishes were transfected transiently by the DEAE-dextran chloroquine method (21, 22) with the indicated cDNA. The total amount of cDNA per transfection was maintained at a constant level using vector cDNA. Twenty-four hours later, the cells were trypsinized and cultured for an additional 24 h in 24-well plates for AC activity assay or in 10 cm dishes for determination of protein expression by Western blots. Transfection efficiency was optimized and determined by staining for β-galactosidase (23) after transfection with plasmid expressing the enzyme. Efficiency of transfections was always in the range of 60–80% as determined by microscopic visualization of stained cells.

**Adenylyl cyclase assay**

Cells in 24-well plates were incubated for 2 h with 0.25 ml/well of growth medium containing 5 μCi/ml of [3H]adenine. Total [3H]adenine incorporation was not affected by the transfection of various plasmid DNAs. The medium was replaced with Dulbecco’s modified Eagle’s medium containing 20 mM Hepes (pH 7.4), 1 mg/ml bovine serum albumin, and the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) and RO-20-1724 (0.5 mM). The AC stimulants forskolin (FS) or TSH were added immediately at 1 μM concentration and the cells incubated at 37°C for 10 min. Reaction was terminated with 1 ml of 2.5% perchloric acid, neutralized, and applied to a two-step column separation procedure (21, 24). The [3H]cAMP was eluted into scintillation vials and counted.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots**

Transfected COS-7 cells were washed with cold phosphate-buffered saline (PBS), scraped in PBS, spun down at 5000 RPM (at 4°C for 5 min), and cell pellets (unless otherwise specified) were mixed with 100 μl of Laemmli sample buffer (25), sonicated, and frozen. Prior to application on the gel, dithiothreitol (0.1 M final) was added and the samples incubated for 5 min at 100°C (for analysis of G-protein subunits) or incubated for 2 h at 37°C (for analysis of AC isoforms). Proteins were separated on polyacrylamide gel at the concentrations indicated and blotted onto nitrocellulose. The blot was blocked in PBS containing 5% fat-free milk and 0.5% Tween-20, followed by 1.5 h incubation with either BBC-4 monoclonal antibody (against AC V) (26), RA polyclonal antibody (against Gp1), or SGS polyclonal antibody (against Gp5) (14), all diluted 1:1,000 in 5% fat-free milk and 0.5% Tween-20. Blots were washed three times with PBS containing 0.3% Tween-20 and secondary antibody (horseradish peroxidase (HRP) -coupled rat anti-mouse or HRP-coupled goat anti-rabbit; Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) diluted 1:20,000 in 5% fat-free milk plus 0.5% Tween-20, incubated with the blot for 1 h, and the blot was washed extensively (>2 h) with PBS containing 0.3% Tween-20. Peroxidase activity was observed by the ECL chemiluminescence technique (Amersham, Arlington Heights, Ill.).

**RESULTS**

**AC V activity is inhibited by Gp1, Gp1/γ2 and Gp5/γ2**

In these experiments, AC V and, where indicated, Gp and Gγ subunits, were transiently transfected into COS cells and the amounts of cAMP in the cells were determined after 10 min activation with FS (Fig. 1A) or TSH (Fig. 2). The activity of AC V was markedly inhibited by Gp1/γ2 (ca. 60%) and by Gp5/γ2 (ca. 40%). In addition, a significant inhibition could be observed with Gp1 but not with Gp5 transfected alone. The addition of Gγ2, which by itself had no activity (Fig. 3), only slightly increased the Gp1-mediated inhibition, but significantly increased the inhibition of AC V by Gp5. Western blot analysis (Fig. 1B) of the cotransfected cells revealed that AC V expression was...

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**Figure 1.** AC V activity is inhibited by Gp1, Gp1/γ2 and Gp5/γ2. A) Effects of transfected Gp/γ combinations on basal or FS-stimulated AC V activity. Transfections in 10 cm dishes contained 2 μg AC V, 2 μg Gp1, 2 μg Gp5, and 1 μg Gγ2 cDNAs. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± s.e. of three experiments. B) Aliquots containing 15 μg of total cellular protein from COS cells transfected as described above were analyzed by SDS-PAGE (8% acrylamide) using the anti-AC V antibody BBC-4. The two bands of 160 and 150 kDa are equivalent to the previously reported antigen recognition pattern of BBC-4 and may represent alternate glycosylated forms of AC V (41).
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Figure 2. Inhibition of AC V and AC VI by Gbg1 and Gbg5, is independent of mode of activation. Effect of Gbg combinations on the activities of A) AC V and B) AC VI under basal conditions or after TSH stimulation. COS cells were transfected with 1 µg TSH receptor cDNA and, where indicated, with 2 µg AC V or AC VI, 2 µg Gbg1 or Gbg5, and 1 µg Gg2 cDNA. cAMP accumulation is expressed in cpm and is the average of triplicate determinations of a representative experiment repeated three times with similar results.

not affected by the coexpression of Gbg1, Gbg5, and Gg2. These results show that in COS cells, Gbg1 is more effective in modulating AC V than Gbg5 and that the addition of Gg2 strengthens the efficacy of Gbg5.

The experiment shown in Fig. 1 was performed with relatively large amounts of Gbg and Gg cDNAs. It was therefore repeated with Gbg1, Gbg5, and Gg2 cDNAs at various concentrations. Figure 3A demonstrates that the inhibition of AC V reached maximal values when the concentration of cDNA of Gbg1 was above 1 µg (per 10 cm culture plate). A half-maximal effect was observed with ca. 250 ng of transfected Gbg1 cDNA. The addition of Gg2 cDNA (2 µg/plate) did not have a marked effect on the Gbg1-mediated inhibition of AC V at any of the Gbg1 cDNA concentrations. Expression of Gbg3 at all cDNA concentrations failed to mediate any significant inhibition of AC V activity. Only when the COS cells were transfected with 2 µg of Gbg3 cDNA, together with increasing levels of Gg2, was inhibition of AC V observed, demonstrating the dependency of Gbg3 activity on the presence of Gg2. Moreover, the inhibition of AC V by Gbg1 or by Gbg1/g2 was much more pronounced than that observed with Gbg3 together with Gg2.

Western blot analysis using selective antibodies to Gbg1 and Gbg5 (Fig. 3B, see also Fig. 4B) demonstrated that in agreement with our previous results (14), untransfected COS cells have endogenous Gbg1 but are devoid of Gbg5. Maximal expression of Gbg1 was achieved at ≥ 500 ng of Gbg1 cDNA, in agreement with the effect of Gbg1 on AC activity. Western blot analysis also showed that the lack of Gbg5 effect on AC V activity is not due to a low level of Gbg5 protein expression and that expression of this protein was already maximal when 250 ng cDNA was used in the transfection.

Inhibition of AC V and VI by Gbg1, Gbg1/g2, and Gbg5/g2 is independent of mode of AC stimulation

In the previous experiments, FS was used to stimulate AC V. We have found that the inhibition of AC V by Gbg1, Gbg1/g2, and Gbg5/g2 is not dependent on the method used for AC stimulation. COS-7 cells cotransfected with the TSH receptor and stimulated with TSH to activate AC through Gag revealed (Fig. 2) the same inhibitory pattern as established for AC V activated by FS.

AC VI is a close homologue of AC V (4, 15, 16). Indeed, as shown in Fig. 2, AC VI displayed a similar pattern of activity as AC V when cotransfected with Gbg and Gg subunits. AC VI activity was inhibited upon cotransfection with the Gbg1 subunit alone while remaining insensitive to Gbg5. The addition of Gg2 in both cases contributed to further inhibition observed with Gbg1 as well as with Gbg5. The same pattern of inhibition by Gbg1 and Gbg5 (in the presence of Gg2) was observed on basal activity of AC V and VI (e.g., in the absence of TSH stimulation). In addition, COS-7 cells have been shown to contain at least the AC-VII and IX isozymes (27), and the COS endogenous AC ac-
activity (stimulated by TSH or FS) was not inhibited by transfected $G_{b1}$ or $G_{b5}$ (data not shown). Thus, the inhibitions observed result from modulations in the activity of the transfected AC.

**Diminished inhibition of AC V by $G_{b1}E10K$ mutant**

To determine whether $G_{b1}$-mediated inhibition is dependent on endogenous $G_b$ subunits, we cotransfected AC V with the previously described point mutant, $G_{b1}E10K$, shown to be defective in its capacity to associate with $G_{b2}$ subunits (19). Indeed, $G_{b1}E10K$ was much weaker than $G_{b1}$ in inhibiting AC V activity (Fig. 4A). Using antibodies against $G_b$ (recognizing both $G_{b1}$ and $G_{b3}E10K$), Western blot analysis of total cellular protein shows that similar expression was achieved for both $G_{b1}$ and $G_{b1}E10K$ (Fig. 4B). In contrast, immunoblot analysis of the cholate-soluble cell membrane fraction revealed lower expression of $G_{b1}E10K$ compared to the wild-type $G_{b1}$, consistent with the mutant’s impaired ability to interact with $G_b$. This result indicates that proper localization of the $G_{b1}$ protein to the membrane (probably via endogenous $G_eta$) is important for the inhibitory modulation of AC V and suggests that the active inhibitory mediator is the $G_{b1}$ dimer itself.

**Sequestration of $G_{b2}$ activates AC V**

The above-described result suggests that $G_{b2}$ dimers are involved in the inhibition of AC V activity. Therefore, it would follow that molecules that sequester free $G_{b2}$ should relieve AC V of such tonic inhibitory activity. A number of protein molecules have been shown to interfere with $G_{b2}$ activities. A mutant form of $G_{b2}$ that lacks the prenylation site ($G_{b2}C68S$) and therefore cannot anchor to the membrane has been shown to redirect $G_b$ subunits into the cellular cytosol, thus reducing $G_b$ content at the cell membrane (28, 29). In addition, $G_b$ subunits such as $G_b$ itself will combine with $G_{b2}$ and interfere with $G_{b2}$-mediated signaling (5, 30). We have checked the effect of cotransfection of cDNAs of $G_b$ or $G_{b2}C68S$ on AC V activity. As shown in Fig. 5, both approaches increased the level of activation of AC V by FS. $G_b$ increased AC V activity threefold and $G_{b2}C68S$ by 1.6-fold. These results suggest there is a tonic inhibition of AC V activity that is mediated by endogenous $G_{b2}$.

**DISCUSSION**

$G_{b2}$ dimers are involved in the regulation of various effector systems including PLC-$eta_2$, MAPK/ERK, JNK/SAPK, PI3-kinase, and several AC isozymes (6, 14, 30–33). AC isozymes were shown to differ in their response to $G_{b2}$. AC types II, IV, and presumably VII are stimulated by free $G_{b2}$ whereas AC type I was shown to be inhibited (1, 2, 5–7, 9, 32, 34). Here we report that $G_{b2}$ inhibits the activation of AC V and AC VI. $G_{b1}$ and $G_{b5}$ represent two distinct forms of $G_b$ as demonstrated by their sequence, expression pattern ($G_{b5}$ being predominantly found in brain), and ability to differentially affect downstream signaling pro-
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Figure 4. Efficacy of the Gβ1E10K mutant in inhibiting AC V activity. A) Effect of cotransfection of Gβ1E10K mutant on basal or 1 mM FS-stimulated AC V activity. COS cells were transfected with 2 μg AC V and either 2 μg Gβ1 or 2 μg Gβ1E10K cDNAs. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE of three experiments. B) Expression of Gβ1 vs. Gβ1E10K. COS cells were transfected as described above and aliquots of 5 μg of total cellular protein or 5 μg of the cholate-soluble membrane fraction prepared by solubilizing the membranes of transfected COS cells in 1% cholate (14) were separated by SDS-PAGE (12% acrylamide), and the Gβ subunits detected by the RA polyclonal antibody.

Figure 5. Effect of Gγ sequestration on AC V activity. COS cells were transfected with 2 μg AC V and either 2 μg Gγ2C68S or 2 μg αT. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE of three experiments.

teins such as ERK and JNK (14, 35). We have recently shown that Gβ1/γ2 and Gβ5/γ2 have distinct effects on the modulation of AC II activity (36). Our current study shows that they also differ in their potency to modulate the activity of AC V and VI in COS cells. We have observed that the Gβ1 subunit efficiently inhibits AC V when it is transfected alone, whereas Gβ5 was functional only upon the addition of Gγ2. Moreover, the Gβ1E10K mutant, which was shown to be defective in its ability to couple to the Gγ2 subunit and possibly to other Gγ subunits (19, 29), was much less efficient in inhibiting AC V activity. It follows that Gβ1 is more efficient than Gβ5 in recruiting and coupling with endogenous Gγ subunits present in COS cells to form Gγ2 dimers. It has been shown that Gβ1 can couple with a number of Gγ subunits equally well (37, 38), whereas Gβ5 appears to have a more restrictive selectivity to Gγ2 (14, 35). Accordingly, we have shown that sequestration of endogenous Gγ2 by a Gγ2 scavenger molecule such as αT, or the removal of Gγ2 from the membrane by interfering with Gγ anchoring (using the prenylation-deficient mutant), enhances AC V activity. These data led us to suggest that a pool of Gγ2 exists in COS cells that can exert a tonic inhibition on AC V activity.

It has recently been shown that the activity of P/Q- and N-type, voltage-dependent Ca2+ channels can be modulated by Gγ2 subunits (39). In agreement with our results, it was shown that transfection of Gγ2 alone into tsA-201 cells was almost as effective as Gγ2/Gγ2 in regulating Ca2+ channel activation in these cells. It has been speculated that the overexpression of Gγ1 and Gγ2 could lead to increased levels of Gγ2 dimers or that the Gγ1 has an intrinsic activity on its own (39).

AC V and AC VI were reported to be insensitive to Gγ in membrane reconstitution assays (2, 16). Here we report, using COS cells transfected with AC V and AC VI, that Gγ2 dimers can inhibit the activity of these AC isozymes. A search of AC V amino acid sequence reveals that AC V contains the sequence QXXER in the C1 cytoplasmic loop at positions 429–433 (18). This sequence has been shown to be involved in Gγ2

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interaction with AC II as well as with voltage-dependent N-type calcium channels (8, 40). In addition, AC VI contains a similar sequence (RXRER) at the homologous position. Additional experiments are needed to show whether the above-described sequences are involved in ACV-VI/Grbeta interactions and why such interaction leads to inhibition of AC V and VI activity, whereas in AC II it allows for Grbeta-mediated stimulation. We cannot rule out the possibility that the effect of Grbeta on AC V and AC VI may be indirect. Such indirect effects of Grbeta have been observed for the activation of MAPK/ERK, where it has been shown that free Grbeta recruits PI3-kinase to the plasma membrane, which in turn leads to a cascade of events leading to increased MAPK activity (33).

In summary, we have shown that AC V and AC VI are inhibited by Grbeta heterodimers, demonstrating that after agonist-mediated activation of Gralpha/o-coupled receptors, the activities of AC V and VI can be inhibited via activated Gralpha/o and Grbeta dimers released from G-protein heterotrimers.

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