Inhibition of adenylyl cyclase isoforms V and VI by various $G_{\beta\gamma}$ subunits

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

Key Words: $G_{\beta\gamma}$ · signal transduction · inhibition · plasmid

ADENYLYL CYCLASE (AC)[2] is a major target enzyme whose activity is modulated by receptor-coupled G-proteins. AC activity can be stimulated by $G_{\alpha s}$ and is inhibited by $G_{\alpha i/z}$. It has recently been shown that the wide array of AC isoforms (nine are currently known) display differential sensitivity to free $G_{\beta\gamma}$ subunits. Previous results have demonstrated that AC I is significantly inhibited whereas AC II, IV, and presumably VII are activated by $G_{\beta\gamma}$ (1–9).

Six G_{β} and 12 G_{γ} isoforms have been cloned to date (6). Most previous experiments investigating the role of free $G_{\beta\gamma}$ on AC activity were performed in cell-free systems with either baculovirus/Sf9 recombinant G_{β} and G_{γ} preparations (10, 11) or purified brain $G_{\beta\gamma}$ preparations that consist of a mixture of various $G_{\beta\gamma}$ heterodimers (3, 12, 13). There is little information about the possible variations between the effects of the various G_{β} and G_{γ} combinations in intact cells. This is important because the various G_{β} and G_{γ} subunits do not necessarily have the same regulatory activities. Indeed, we recently showed that activation of PLC-β_2 by $G_{\beta\gamma}$ is G_{β} isoform independent ($G_{\beta 1}$ being equally effective as $G_{\beta 5}$), whereas activation of MAPK/ERK and JNK/SAPK appears to be G_{β} isoform dependent (14).

AC V and VI represent a subfamily within the group of AC isozymes. These two isozymes share high sequence homology and are both inhibited by activated $G_{\alpha i}$, but were not found to be sensitive to free $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$ combinations, using cotransfection of G_{β} and G_{γ} together with these AC isoforms. Due to the divergence in sequence homology between $G_{\beta 1}$ and $G_{\beta 5}$, we have concentrated our studies on these two G_{β} subunits and their modulation of activity of the AC isoforms in question. We observed that the activities of AC V and VI are inhibited by $G_{\beta\gamma}$ and that $G_{\beta 1}$ is more efficient than $G_{\beta 5}$ 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 μg/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.

Plasmids

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 $G_{\beta 1}$, $G_{\beta 5}$, $G_{\gamma 2}$, $G_{\beta 1}$E10K, and $G_{\gamma 2}$C68S 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 [^{3}H]adenine. Total [^{3}H]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 [^{3}H]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 $G_{\beta 1}$), or SGS polyclonal antibody (against $G_{\beta 5}$) (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 $G_{\beta 1}$, $G_{\beta 1/\gamma 2}$ and $G_{\beta 5/\gamma 2}$

In these experiments, AC V and, where indicated, G_β and G_γ subunits, were transiently transfected into COS cells and the amounts of cAMP in the cells were determined after 10 min activation with FS (**Fig. 1*A***) or TSH (**Fig. 2**). The activity of AC V was markedly inhibited by $G_{\beta 1/\gamma 2}$ (ca. 60%) and by $G_{\beta 5/\gamma 2}$ (ca. 40%). In addition, a significant inhibition could be observed with $G_{\beta 1}$ but not with $G_{\beta 5}$ transfected alone. The addition of $G_{\gamma 2}$, which by itself had no activity (**Fig. 3**), only slightly increased the $G_{\beta 1}$-mediated inhibition, but significantly increased the inhibition of AC V by $G_{\beta 5}$. Western blot analysis (Fig. 1*B*) of the cotransfected cells revealed that AC V expression was

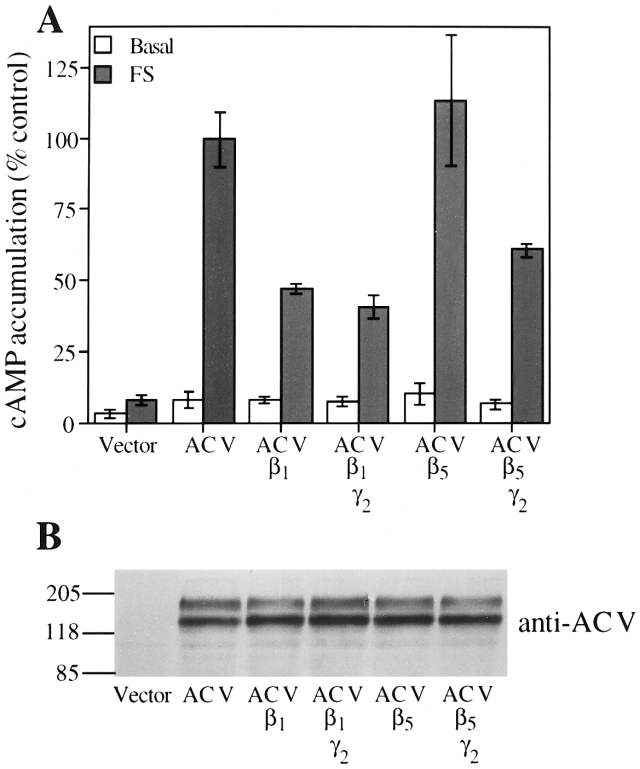

Figure 1. AC V activity is inhibited by $G_{\beta 1/\gamma 2}$ and $G_{\beta 5/\gamma 2}$. *A)* Effects of transfected $G_{\beta/\gamma}$ combinations on basal or FS-stimulated AC V activity. Transfections in 10 cm dishes contained 2 μg AC V, 2 μg $G_{\beta 1}$, 2 μg $G_{\beta 5}$, and 1 μg $G_{\gamma 2}$ cDNAs. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE 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).

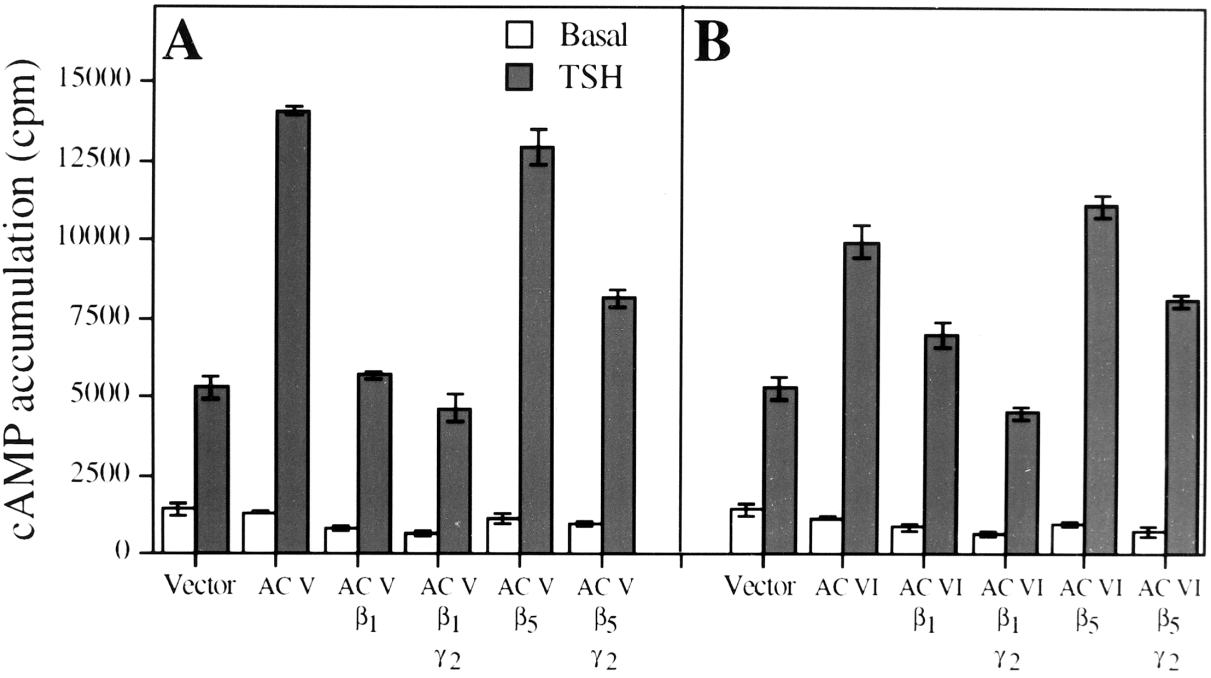

Figure 2. Inhibition of AC V and AC VI by $G_{\beta 1}$ and $G_{\beta 5}$ is independent of mode of activation. Effect of $G_{\beta\gamma}$ combinations on the activities of *A)* AC V and *B)* AC VI under basal conditions or after TSH stimulation. COS cells were transfected with 1 μg TSH receptor cDNA and, where indicated, with 2 μg AC V or AC VI, 2 μg $G_{\beta 1}$ or $G_{\beta 5}$, and 1 μg $G_{\gamma 2}$ 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 $G_{\beta 1}$, $G_{\beta 5}$, and $G_{\gamma 2}$. These results show that in COS cells, $G_{\beta 1}$ is more effective in modulating AC V than $G_{\beta 5}$ and that the addition of $G_{\gamma 2}$ strengthens the efficacy of $G_{\beta 5}$.

The experiment shown in Fig. 1 was performed with relatively large amounts of G_{β} and G_{γ} cDNAs. It was therefore repeated with $G_{\beta 1}$, $G_{\beta 5}$, and $G_{\gamma 2}$ cDNAs at various concentrations. Figure 3*A* demonstrates that the inhibition of AC V reached maximal values when the concentration of cDNA of $G_{\beta 1}$ was above 1 μg (per 10 cm culture plate). A half-maximal effect was observed with ca. 250 ng of transfected $G_{\beta 1}$ cDNA. The addition of $G_{\gamma 2}$ cDNA (2 μg/plate) did not have a marked effect on the $G_{\beta 1}$-mediated inhibition of AC V at any of the $G_{\beta 1}$ cDNA concentrations. Expression of $G_{\beta 5}$ 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 $G_{\beta 5}$ cDNA, together with increasing levels of $G_{\gamma 2}$, was inhibition of AC V observed, demonstrating the dependency of $G_{\beta 5}$ activity on the presence of $G_{\gamma 2}$. Moreover, the inhibition of AC V by $G_{\beta 1}$ or by $G_{\beta 1/\gamma 2}$ was much more pronounced than that observed with $G_{\beta 5}$ together with $G_{\gamma 2}$.

Western blot analysis using selective antibodies to $G_{\beta 1}$ and $G_{\beta 5}$ (Fig. 3*B*, see also **Fig. 4*B***) demonstrated that in agreement with our previous results (14), untransfected COS cells have endogenous $G_{\beta 1}$ but are devoid of $G_{\beta 5}$. Maximal expression of $G_{\beta 1}$ was achieved at ≥ 500 ng of $G_{\beta 1}$ cDNA, in agreement with the effect of $G_{\beta 1}$ on AC activity. Western blot analysis also showed that the lack of $G_{\beta 5}$ effect on AC V activity is not due to a low level of $G_{\beta 5}$ 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 $G_{\beta 1}$, $G_{\beta 1/\gamma 2}$, and $G_{\beta 5/\gamma 2}$ 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 $G_{\beta 1}$, $G_{\beta 1/\gamma 2}$, and $G_{\beta 5/\gamma 2}$ 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 $G_{\alpha s}$ 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 G_{β} and G_{γ} subunits. AC VI activity was inhibited upon cotransfection with the $G_{\beta 1}$ subunit alone while remaining insensitive to $G_{\beta 5}$. The addition of $G_{\gamma 2}$ in both cases contributed to further inhibition observed with $G_{\beta 1}$ as well as with $G_{\beta 5}$. The same pattern of inhibition by $G_{\beta 1}$ and $G_{\beta 5}$ (in the presence of $G_{\gamma 2}$) 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-

tivity (stimulated by TSH or FS) was not inhibited by transfected $G_{\beta 1}$ or $G_{\beta 5}$ (data not shown). Thus, the inhibitions observed result from modulations in the activity of the transfected AC.

Diminished inhibition of AC V by $G_{\beta 1}$E10K mutant

To determine whether $G_{\beta 1}$-mediated inhibition is dependent on endogenous G_{γ} subunits, we cotransfected AC V with the previously described point mutant, $G_{\beta 1}$E10K, shown to be defective in its capacity to associate with $G_{\gamma 2}$ subunits (19). Indeed, $G_{\beta 1}$E10K was much weaker than $G_{\beta 1}$ in inhibiting AC V activity (Fig. 4*A*). Using antibodies against $G_{\beta 1}$ (recognizing both $G_{\beta 1}$ and $G_{\beta 1}$E10K), Western blot analysis of total cellular protein shows that similar expression was achieved for both $G_{\beta 1}$ and $G_{\beta 1}$E10K (Fig. 4*B*). In contrast, immunoblot analysis of the cholate-soluble cell membrane fraction revealed lower expression of $G_{\beta 1}$E10K compared to the wild-type $G_{\beta 1}$, consistent with the mutant's impaired ability to interact with G_{γ}. This result indicates that proper localization of the $G_{\beta 1}$ protein to the membrane (probably via endogenous G_{γ}) is important for the inhibitory modulation of AC V and suggests that the active inhibitory mediator is the $G_{\beta\gamma}$ dimer itself.

Sequestration of $G_{\beta\gamma}$ activates AC V

The above-described result suggests that $G_{\beta\gamma}$ dimers are involved in the inhibition of AC V activity. Therefore, it would follow that molecules that sequester free $G_{\beta\gamma}$ should relieve AC V of such tonic inhibitory activity. A number of protein molecules have been shown to interfere with $G_{\beta\gamma}$ activities. A mutant form of $G_{\gamma 2}$ that lacks the prenylation site ($G_{\gamma 2}$C68S) and therefore cannot anchor to the membrane has been shown to redirect G_{β} subunits into the cellular cytosol, thus reducing G_{β} content at the cell membrane (28, 29). In addition, G_{α} subunits such as αT will combine with $G_{\beta\gamma}$ and interfere with $G_{\beta\gamma}$-mediated signaling (5, 30). We have checked the effect of cotransfection of cDNAs of αT or $G_{\gamma 2}$C68S on AC V activity. As shown in **Fig. 5**, both approaches increased the level of activation of AC V by FS. αT increased AC V activity by threefold and $G_{\gamma 2}$C68S by 1.6-fold. These results suggest there is a tonic inhibition of AC V activity that is mediated by endogenous $G_{\beta\gamma}$.

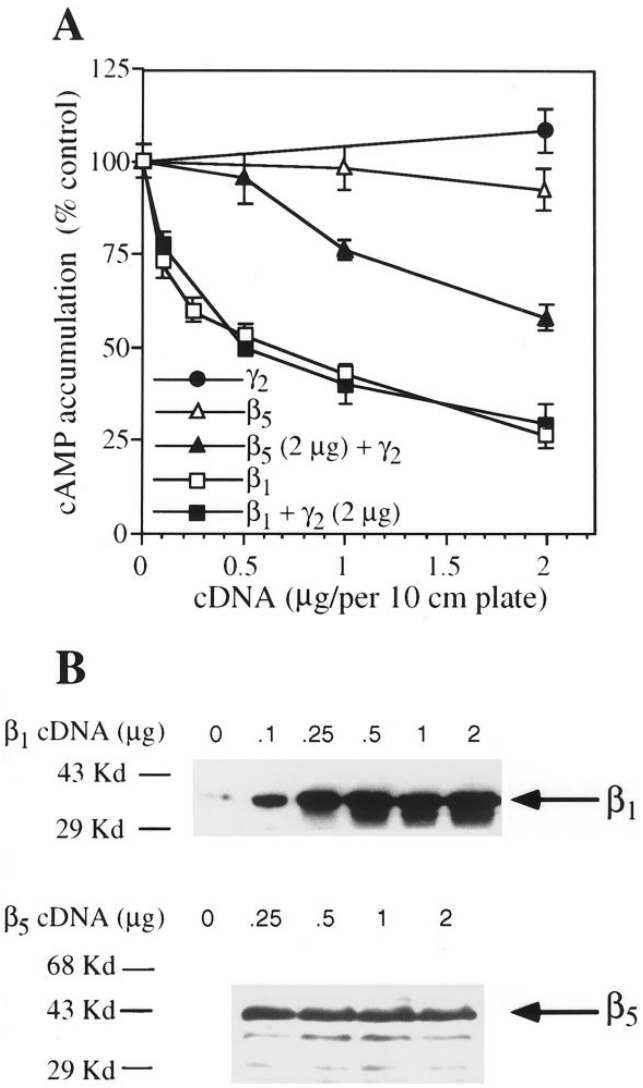

Figure 3. $G_{\beta/\gamma}$ modulates AC V in a dose-dependent manner. *A)* Effect of increasing amounts of G_{β} and G_{γ} cDNA on AC V activity. COS cells were transfected with AC V cDNA (2 μg) together with the indicated amounts of $G_{\beta 1}$, $G_{\beta 5}$, and $G_{\gamma 2}$ cDNAs. FS-stimulated cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE of three experiments. *B)* Expression of $G_{\beta 1}$ and $G_{\beta 5}$. COS cells were transfected with 2 μg of AC V cDNA and the indicated amounts of $G_{\beta 1}$ and $G_{\beta 5}$ cDNAs. Aliquots containing 15 μg total cellular protein were separated by SDS-PAGE (12% acrylamide) and analyzed by Western blotting using RA polyclonal antibody (against $G_{\beta 1}$) and SGS polyclonal antibody (against $G_{\beta 5}$). Arrows represent positions of $G_{\beta 1}$ and $G_{\beta 5}$. The molecular mass of $G_{\beta 5}$ (39 kDa) is higher than that of $G_{\beta 1}$ (36 kDa), in agreement with the difference in amino acid number between the two proteins (340 vs. 353 for $G_{\beta 1}$ and $G_{\beta 5}$, respectively).

DISCUSSION

$G_{\beta\gamma}$ dimers are involved in the regulation of various effector systems including PLC-β2, MAPK/ERK, JNK/SAPK, PI3-kinase, and several AC isozymes (6, 14, 30–33). AC isozymes were shown to differ in their response to $G_{\beta\gamma}$. AC types II, IV, and presumably VII are stimulated by free $G_{\beta\gamma}$, whereas AC type I was shown to be inhibited (1, 2, 5–7, 9, 32, 34). Here we report that $G_{\beta\gamma}$ inhibits the activation of AC V and AC VI.

$G_{\beta 1}$ and $G_{\beta 5}$ represent two distinct forms of G_{β} as demonstrated by their sequence, expression pattern ($G_{\beta 5}$ being predominantly found in brain), and ability to differentially affect downstream signaling pro-

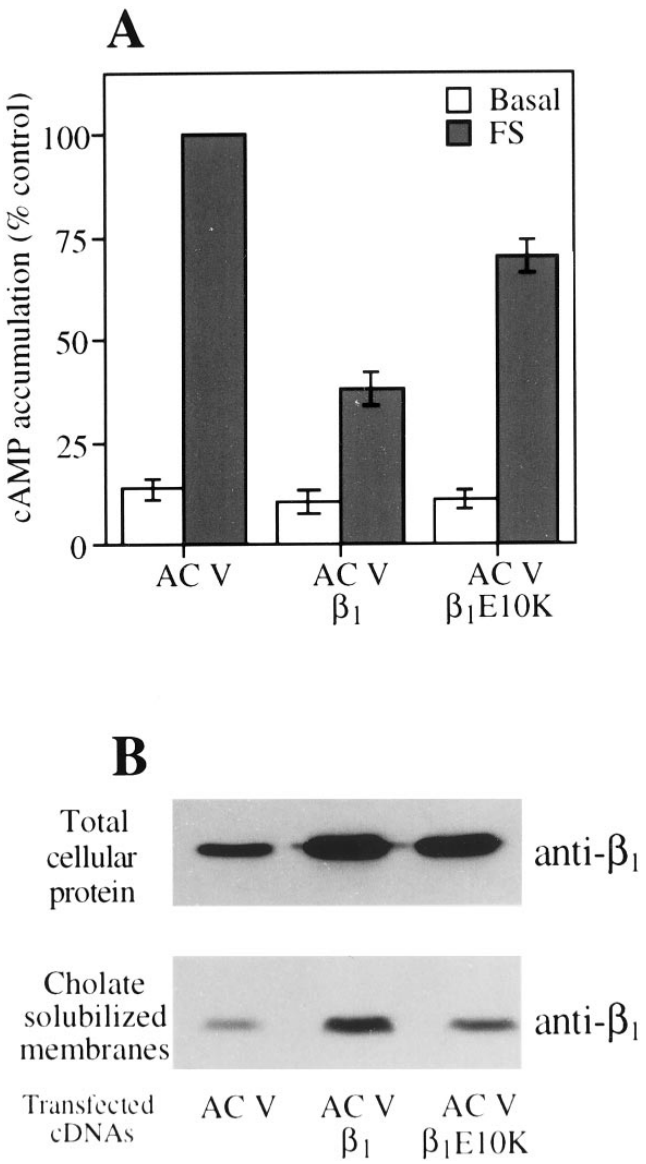

Figure 4. Efficacy of the $G_{\beta 1}$E10K mutant in inhibiting AC V activity. *A)* Effect of cotransfection of $G_{\beta 1}$E10K mutant on basal or 1 μM FS-stimulated AC V activity. COS cells were transfected with 2 μg AC V and either 2 μg $G_{\beta 1}$ or 2 μg $G_{\beta 1}$E10K cDNAs. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE of three experiments. *B)* Expression of $G_{\beta 1}$ vs. $G_{\beta 1}$E10K. 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 G_{β} subunits detected by the RA polyclonal antibody.

teins such as ERK and JNK (14, 35). We have recently shown that $G_{\beta 1/\gamma 2}$ and $G_{\beta 5/\gamma 2}$ 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 $G_{\beta 1}$ subunit efficiently inhibits AC V when it is transfected alone, whereas $G_{\beta 5}$ was functional only upon the addition of $G_{\gamma 2}$. Moreover, the $G_{\beta 1}$E10K mutant, which was shown to be defective in its ability to couple to the $G_{\gamma 2}$ subunit and possibly to other G_{γ} subunits (19, 29), was much less efficient in inhibiting AC V activity. It follows that $G_{\beta 1}$ is more efficient than $G_{\beta 5}$ in recruiting and coupling with endogenous G_{γ} subunits present in COS cells to form $G_{\beta\gamma}$ dimers. It has been shown that $G_{\beta 1}$ can couple with a number of G_{γ} subunits equally well (37, 38), whereas $G_{\beta 5}$ appears to have a more restrictive selectivity to $G_{\gamma 2}$ (14, 35). Accordingly, we have shown that sequestration of endogenous $G_{\beta\gamma}$ by a $G_{\beta\gamma}$ scavenger molecule such as αT, or the removal of $G_{\beta\gamma}$ from the membrane by interfering with G_{γ} anchoring (using the prenylation-deficient mutant), enhances AC V activity. These data led us to suggest that a pool of $G_{\beta\gamma}$ 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 Ca^{2+} channels can be modulated by $G_{\beta\gamma}$ subunits (39). In agreement with our results, it was shown that transfection of $Gl_{\beta 2}$ alone into tsA-201 cells was almost as effective as $G_{\beta 2/\gamma 3}$ in regulating Ca^{2+} channel activation in these cells. It has been speculated that the overexpression of $G_{\beta 1}$ and $G_{\beta 2}$ could lead to increased levels of $G_{\beta\gamma}$ dimers or that the G_{β} has an intrinsic activity on its own (39).

AC V and AC VI were reported to be insensitive to $G_{\beta\gamma}$ in membrane reconstitution assays (2, 16). Here we report, using COS cells transfected with AC V and AC VI, that $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$

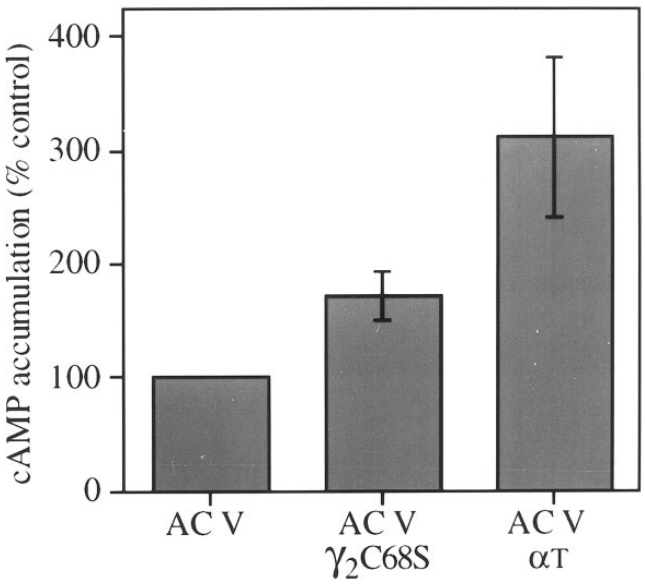

Figure 5. Effect of $G_{\beta\gamma}$ sequestration on AC V activity. COS cells were transfected with 2 μg AC V and either 2 μg $G_{\gamma 2}$C68S or 2 μg αT. cAMP accumulation is expressed as percent of control (AC V transfected alone) and is the mean ± SE of three experiments.

interaction with AC II as well as with voltage-dependent N-type calcium channels (8, 40). In addition, AC VI contains a similar sequence (RXXER) at the homologous position. Additional experiments are needed to show whether the above-described sequences are involved in AC V-VI/$G_{\beta\gamma}$ interactions and why such interaction leads to inhibition of AC V and VI activity, whereas in AC II it allows for $G_{\beta\gamma}$-mediated stimulation. We cannot rule out the possibility that the effect of $G_{\beta\gamma}$ on AC V and AC VI may be indirect. Such indirect effects of $G_{\beta\gamma}$ have been observed for the activation of MAPK/ERK, where it has been shown that free $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$ heterodimers, demonstrating that after agonist-mediated activation of $G_{i/o}$-coupled receptors, the activities of AC V and VI can be inhibited via activated $G_{\alpha i/o}$ and $G_{\beta\gamma}$ dimers released from G-protein heterotrimers. FJ

This work was supported by the National Institute of Drug Abuse (DA-06265), the German-Israeli Foundation for Scientific Research and Development, the Forschheimer Center for Molecular Genetics, and the Israeli Ministries of Science and Arts and of Absorption (fellowship to M.B.). Z.V. is the incumbent of the Ruth and Leonard Simon Chair for Cancer Research.

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Received for publication December 5, 1997.
Revised for publication March 9, 1998.