Conditional Stimulation of Type V and VI Adenylyl Cyclases by G Protein βγ Subunits*§

Xianlong Gao‡, Rachna Sadana†, Carmen W. Dessauer‡, and Tarun B. Patel‡†

From the ‡Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois 60153 and †Department of Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, Texas 77030

In a yeast two-hybrid screen of mouse brain cDNA library, using the N-terminal region of human type V adenylyl cyclase (hACV) as bait, we identified G protein β2 subunit as an interacting partner. Additional yeast two-hybrid assays showed that the Gβ1 subunit also interacts with the N-terminal segments of hACV and human type VI adenylyl cyclase (hACVI). In vitro adenylyl cyclase (AC) activity assays using membranes of SF9 cells expressing hACV or hACVI showed that Gβγ subunits enhance the activity of these enzymes provided either Gaq or forskolin is present. Deletion of residues 77–151, but not 1–76, in the N-terminal region of hACVI obliterated the ability of Gβγ subunits to conditionally stimulate the enzyme. Likewise, activities of the recombinant, engineered, soluble forms of ACV and ACVI, which lack the N termini, were not enhanced by Gβγ subunits. Transfection of the C terminus of G protein receptor kinase 2 to sequester endogenous Gβγ subunits attenuated the ability of isoproterenol to increase cAMP accumulation in COS-7 cells overexpressing hACVI even when Gi was inactivated by pertussis toxin. Therefore, we conclude that the N termini of human hACV and hACVI are necessary for interactions with, and regulation by, Gβγ subunits both in vitro and in intact cells. Moreover, Gβγ subunits derived from a source(s) other than Gi are necessary for the full activation of hACVI by isoproterenol in intact cells.

Adenylyl cyclase (AC)* catalyzes the conversion of ATP into cAMP. To date at least nine isoforms of membrane-bound mammalian adenylyl cyclases have been cloned and characterized (for review, see Refs. 1–3). All are stimulated by Gaq and, with the exception of type IX, also by forskolin. The different AC isoforms also share similar structural features consisting of a cytosolic N-terminal region, two sets of six transmembrane spans separated by a cytosolic loop (C1), and a cytosolic tail (C2) (1–4). The N termini of the two cytosolic domains (C1a and C2a) are highly conserved among nine types of AC and form the catalytic core (5–9). These two domains can be separately expressed and reconstituted to restore catalytic activity that is responsive to both forskolin and Gaq (6, 9).

Although nine isoforms of AC share certain common features described above, they vary greatly in their regulatory profiles. For example, ACI and ACVIII are stimulated by Ca2+/calmodulin as is ACIII in vitro (3, 10, 11); ACI, ACV, and ACVI are inhibited by Gaq (9, 12, 13). The function of the mammalian ACs can also be modulated by protein kinases such as cAMP-dependent protein kinase A and protein kinase C. ACV and ACVI activities are inhibited by protein kinase A (14, 15), whereas ACII and ACVII are activated by protein kinase C (16, 17). Differences in regulation between isoforms are, in part, related to the high degree of sequence variation associated with regions outside of the catalytic core. For instance, the C-terminal region of the C1 domain, C1b, is highly variable among the nine AC isoforms and has been found to be involved in some of the type-specific characteristics. In ACII, ACIV, and ACVII, the C1b region is required for stimulatory actions of Gβγ subunits (18, 19). The C1b region in ACI is also necessary for the binding of Ca2+/calmodulin and consequent stimulation of enzyme activity (20, 21) as well as Gβγ-mediated inhibition of activity (9). The N-terminal segment of the different AC isoforms is also highly variable, and this region may play an important role in type-specific regulation of these enzymes. In ACVI, the N-terminal region is critical for both protein kinase C-mediated and Gaq-mediated inhibition of the enzyme (22, 23) as well as binding to Snapin (24), although the functional significance of this latter interaction remains unknown. Moreover, the N-terminal segment of ACVIII has been shown to play a critical role in regulating its activity by capacitative calcium entry in intact cells (25).

The stimulation of G protein-coupled receptors results in the activation of G protein α subunit and the release of Gβγ subunits from the heterotrimeric complex (26, 27). Both Gaq subunit and Gβγ subunits can then interact with their effectors and mediate signal transduction. The known Gβγ effectors include phospholipases (28), ion channels (29), G protein-coupled receptor kinases (30), phosphoinositide 3-kinases (31), and some isoforms of AC. Among the AC isoforms, ACII, ACIV, and ACVII are stimulated by Gβγ subunits provided that some Gaq is present (13), whereas ACI and ACVIII are inhibited by Gβγ (13, 32).

* This research was supported by National Institutes of Health Grants HL59679 and GM073181 (to T. B. P.) and GM060419 (to C. W. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

‡ To whom correspondence should be addressed: Dept. of Pharmacology and Experimental Therapeutics, Loyola University Chicago, Stritch School of Medicine, 2160 S. First Ave., Maywood, IL 60153. Tel.: 708-216-5773; Fax: 708-216-6888; E-mail: tpatel7@lumc.edu.

§ The abbreviations used are: AC, adenylyl cyclase; hACV, human AC type V; GRK2, G protein-coupled receptor kinase 2; CT, C terminus; aa, amino acids; GST, glutathione S-transferase; DTT, dithiothreitol; GTPγS, guanosine 5’-3-O-(thio)triphosphate.
To date, either nothing (ACV) or very little (ACVI) is known about the role of the N-terminal segments of these enzymes in regulating their activity. Therefore, we performed yeast two-hybrid screens using the N-terminal 241 residues of human ACV (hACV) as bait and identified GB\_2 subunit as its potential interacting partner. In vitro AC activity assays showed that in the presence of G\_0, or forskolin GB\_y subunits enhanced the activity of hACV and human ACVI (hACVI). The conditional stimulation (i.e. in presence of G\_0 or forskolin) by GB\_y was also observed with rat ACV and rat ACVI but not canine ACV, which is distinctly dissimilar from its human and rat isoforms. The deletion of residues 77–151 in the N terminus in hACVI abolished the ability of GB\_y to conditionally stimulate activity. Consistent with this, the recombinant soluble forms of ACV and ACVI lacking the N-terminal segments were unaffected by GB\_y subunits. Furthermore, the C terminus of G protein-coupled receptor kinase 2 (GRK2-CT) by sequestering GB\_y subunits in vivo decreased isoproterenol-elevated increase in cAMP accumulation in cells expressing hACVI. Pertussis toxin treatment did not affect the ability of GRK2-CT to attenuate isoproterenol-stimulated cAMP accumulation in these cells. Together these results show that the GB\_y subunits derived from sources other than G\_i participate in mediating the full activation of hACVI by the \( \beta \)-adrenergic receptor agonist, isoproterenol.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—The Clontech MatchMaker Gal4-two-hybrid System 3 was used for the screening of a mouse brain cDNA library. The nucleotide sequence encoding the N terminus of hACV (amino acid residues 1–241) was amplified by PCR from plasmid pCMVSPORT6 that contains the N-terminal region of hACV (Invitrogen). The PCR fragment was cut with BamHI and XhoI and ligated to the binding domain vector pGBK7-7digested with BamHI and SalI. The resulting pGBK7-hACV-N was transformed into yeast strain AH109 together with empty activation domain vector pGADT7 to check for the self-activation. A mouse brain cDNA library was screened with the bait plasmid pGBK7-hACV-N following the manufacturer’s instructions. Transformed yeast cells were grown on medium devoid of Leu, Trp, His, and adenine at 30 °C for 7 days. Colonies growing on the above medium were tested for the \( \beta \)-galactosidase activity. Plasmids from clones showing positive \( \beta \)-galactosidase assay were isolated and sequenced.

To test the interaction between two known proteins, AH109 yeast cells were transformed with plasmids to express hACV-N, hACVI-N (aa 1–151), or the C1 and C2 domains of hACVI as fusion proteins with GAL4 binding domain and GB\_y/GAL4 activation domain. Controls were transformed with one of the fusion constructs and the corresponding empty vector. The transformants were grown on media devoid of Trp and Leu. \( \beta \)-Galactosidase expression was determined using colony-lift filter assay. Cells transformed with pGADT7-T and pGBK7-53 served as a positive control.

Binding of the N-terminal Region of hACV with GB\_y—The N-terminal region of hACV was fused with a GST tag (GST-hACV-N). Protein was expressed in Escherichia coli and purified under non-denaturing conditions using glutathione affinity resin essentially as described (33), except that NaCl concentration was increased to 250 mM in the lysis buffer. The protein was dialyzed overnight in 20 mM Hepes, 1 mM EDTA, 2 mM DTT, 100 mM NaCl, and 5% glycerol. Binding assays were performed with purified recombinant protein. 15 μg of GST-hACV-N or GST was incubated with 5 μg of GB\_y\_1, for 30 min at 4 °C. The glutathione beads equilibrated with buffer (20 mM Hepes, pH 8.0, 2 mM MgCl\_2, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, and 0.2% lube1) were added to the mixture and incubated for 2 h at 4 °C on a rotating mixer. After washing 4 times with wash buffer (20 mM Hepes, pH 8.0, 2 mM MgCl\_2, 150 mM NaCl, 1 mM DTT, and 0.05% lube1), beads were boiled in Laemmli buffer and subjected to SDS-PAGE and analyzed by immunoblotting.

Adenylyl Cylase Constructs and Expression—Human ACV and ACVI expressing baculovirus was generated as described before (34). To construct deletion mutants of hACVI, hACVI\_ΔN (aa 1–151 deleted), hACVI\_Δ1–76, and hACVI\_Δ77–151, fragments encompassing nucleotides 1–1250 of hACVI containing deletions of nucleotides 1–453, 1–228, or 229–453, respectively, were PCR-amplified with an EcoRI site and ATG at the 5’ end. The fragments were cut with EcoRI and SstI and cloned into the cognate sites in the plasmid pFastbactual-hACVI described before (34). The hACVI mutants expressing baculovirus was generated following the manufacturer’s instructions (Invitrogen).

Baculovirus to express rat ACV was obtained from Drs. Yibang Chen and Ravi Iyengar (Mt. Sinai School of Medicine). The baculovirus to express canine ACV was obtained from Dr. Yoshihiro Ishikawa (University of Medicine and Dentistry of New Jersey). Both of these enzymes were expressed in SF9 cells. Rat ACV was expressed in COS-7 cells transfected with pcDNA1-rACVI from Dr. Richard Premont (Duke University). Membranes from SF9 or COS-7 cells expressing ACV and ACVI from different species or hACVI mutants were isolated as described previously (35, 36). Essentially, cells were harvested and washed with phosphate-buffered saline, and pellets were resuspended in hypotonic lysis buffer containing 2 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.2 mM DTT, 1 mM benzamidine, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. After homogenization with a Dounce homogenizer, cells were centrifuged at 290 \( \times \) g for 5 min. The supernatant was centrifuged at 20,000 \( \times \) g for 30 min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose containing the aforementioned protease inhibitors.

Purification of G Protein Subunits—The constitutively active Q213L mutant form of G\_α\_s was used. G\_α was expressed in E. coli and purified as described previously (7). GB\_1\_y\_2 subunits were expressed in SF9 cells and purified as described previously (35).

AC Activity Assays—G\_α was preincubated with 20 μM GTP\_S and 10 mM MgCl\_2 at 30 °C for 30 min for activation in medium containing 20 mM Tris-HCl, pH 8.0, 1 mM DTT, and 1 mM EDTA. SF9 membranes containing the desired AC forms were preincubated at room temperature for 2 min with GB\_1\_y\_2 or GB\_1\_y\_2C68S at the indicated concentrations before initiating
the reactions. The AC assays were performed as described before (38, 39) in a total volume of 100 μl in the presence of 5 mM Mg\textsuperscript{2+}. The reactions were initiated by the addition of Go\textsubscript{α} or forskolin at the indicated concentrations and continued at room temperature for 15 min.

Wherever GST-phosducin was present, 200 nm GST-phosducin (gift from Dr. Heidi Hamm, Vanderbilt University) or GST alone was preincubated with 50 nm GB\textsubscript{1γ2} on ice for 20 min. In parallel, 40 nm Go\textsubscript{α} was preincubated with 4 μg of membranes from Sf9 cells expressing hACV or hACVI at room temperature for 10 min. Thereafter, the two preincubations were mixed together, and AC activity reaction mix was added to initiate the assays.

**Cyclic AMP Accumulation Assay**—Cyclic AMP accumulation in intact cells was monitored as described previously (36). The day before transfection, COS-7 cells (1.2 × 10\textsuperscript{6}) were seeded in 100-mm dishes and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were transfected with plasmids expressing hACV, GRK2-CT, Go\textsubscript{α}Q213L, or M2 muscarinic receptor separately or together using FuGene 6 (Roche Diagnostics) as indicated in the figure legends. Empty vector was added to keep the total amount of plasmid the same for each transfection. One day later cells were trypsinized and plated in 24-well plates. The next day, cells were washed and labeled with \[^{3}H\]adenine (1 μCi/well) for 4 h in Dulbecco’s modified Eagle’s medium without serum. Pertussis toxin (25 nm), when present, was incubated with cells for 6 h before treatments. Cells were then washed twice with Dulbecco’s modified Eagle’s medium containing 20 mM Na-Hepes, pH 7.4, and preincubated for 15 min with Dulbecco’s modified Eagle’s medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.2 mM) before the addition of agonist, isoproterenol, forskolin, or carbacol for 10 min at the indicated concentrations. Incubations were terminated by the addition of 10% ice-cold trichloroacetic acid containing \[^{14}C\]cAMP as an internal standard to correct for recovery. Cyclic AMP in the trichloroacetic acid extracts was isolated as described above. The expression of hACV and GRK2-CT was monitored by Western blotting using anti-ACV/ACVI antibody SC-590 (Santa Cruz) and anti-GRK2 antibody 3A10, provided by Dr. Adriano Marchese (Loyola University Chicago).

**RESULTS**

Although the N-terminal region of ACV1 may interact with Snapin (24), the functional consequences of this interaction remain unknown. Moreover, the function(s) of the relatively large N-terminal region of hACV (241-aa residues) and hACV1 (151-aa residues) has not been defined. We postulated that the N-terminal region of hACV may regulate the activity of hACV by interacting with other proteins. Therefore, to identify the proteins that interact with the N-terminal domain of hACV, we performed a yeast two-hybrid screen of a mouse brain cDNA library using a construct that expressed amino acids 1–241 of hACV as bait. Of 1.1 million clones screened, we identified 11 independent clones that showed positive interactions with the N-terminal domain of hACV. Among them, GB\textsubscript{2} subunit was the most interesting candidate (the other 10 clones are presented in supplemental Table S1). The interaction with GB\textsubscript{2} was confirmed by re-transforming the AH109 cells with the isolated pACT2-GB\textsubscript{2} and bait plasmids and monitoring β-galactosidase activity as well as the ability of the transformants to grow on medium devoid of Leu, Trp, His, and adenine (not shown). Because GB\textsubscript{2} is highly homologous to GB\textsubscript{α}, we transformed cells with constructs to express the N-terminal region of hACV and GB\textsubscript{2}. Similar to the results with GB\textsubscript{α}, the N terminus of hACV also interacted with GB\textsubscript{2} (Fig. 1A). Because hACVI and its close homologue hACV are similarly regulated (2, 3), the N terminus (aa 1–151) of hACV1 interacted with the GB\textsubscript{2} subunit. Like its counterpart in hACV, the N-terminal segment of hACV1 also showed interaction with GB\textsubscript{1} (Fig. 1A). As a control, the binding domain vector pGBK7 together with pGADT7-β1 or the activating vector pGADT7 together with pGBK7-N-terminal region of hACV or hACVI were co-transformed into AH109. The β-galactosidase activity in these controls was similar to background (Fig. 1A). Moreover, specificity of the interactions between N termini of hACV and hACVI with GB\textsubscript{1} and GB\textsubscript{2} was also demonstrated by the inability of the C1 (aa 306–673) and C2 (aa 861–1115) domains of hACV to interact with GB\textsubscript{1} (Fig. 1A).

GB\textsubscript{2} subunits are tightly bound to G\textsubscript{γ} subunits, and activation of heterotrimeric G proteins permits the Go\textsubscript{α} and GB\textsubscript{γ} subunits to regulate their respective effectors (26, 37). Therefore, using purified GB\textsubscript{1γ2}, we investigated whether GB\textsubscript{1γ2} bound the N-terminal region of hACV. As shown in Fig. 1B, the N-terminal region of hACV, which was expressed in and purified from bacteria as a GST fusion (GST-hACV-N), interacted with GB\textsubscript{1γ2}, confirming our data from the yeast two-hybrid assay. Next, we tested the ability of GB\textsubscript{1γ2} to modulate hACV and hACVI activity. Membranes from Sf9 cells expressing hACV or hACVI were preincubated with GB\textsubscript{1γ2} and then stimulated with forskolin or Go\textsubscript{α}. As shown in Fig. 1C, and GB\textsubscript{1γ2} did not affect the basal activity of hACV or hACVI. However, GB\textsubscript{1γ2} increased the activation of hACV and hACVI by forskolin or Go\textsubscript{α} (Fig. 1C and D). GB\textsubscript{1γ2} was more effective at augmenting hACV activity than hACVI (Figs. 1C and D). Thus, 100 nm GB\textsubscript{1γ2} increased the activity of hACV stimulated by forskolin or Go\textsubscript{α} by 57 and 106%, respectively, whereas the corresponding values for hACVI were 133 and 194%, respectively (Fig. 1C and D). Because the isoprenylation of G\textsubscript{γ} subunit is required for GB\textsubscript{γ} to modulate their effectors (38), controls were also performed with mutant of G\textsubscript{γ2}(C68S) that cannot be isoprenylated. As shown in Figs. 1C and D, the non-isoprenylated GB\textsubscript{1γ2}(C68S) did not further stimulate Go\textsubscript{α} or forskolin-stimulated hACV or hACVI activity. Moreover, when phosducin, which binds and sequesters GB\textsubscript{γ} (39), was added, the ability of GB\textsubscript{1γ2} to stimulate hACV or hACVI activities was obliterated (Fig. 1E). Overall, the data in Fig. 1 demonstrate that the N termini of ACV and ACVI specifically interact with GB\textsubscript{1γ2} and that isoprenylation is required for GB\textsubscript{1γ2}-mediated enhancement of forskolin- or Go\textsubscript{α}-stimulated hACV and hACVI activity. Moreover, the sequestration of GB\textsubscript{1γ2} by phosducin abolishes the ability of GB\textsubscript{1γ2} to stimulate hACV and hACVI activities.
Regulation of ACV and ACVI by Gβγ Subunits

We also determined if the Gβ1γ2 subunits altered the ability of purified, myristoylated Gαs to inhibit hACVI. Essentially, the ability of Gαs to inhibit Gαs-stimulated activities of hACVI was monitored. As shown for hACVI in supplemental Fig. S1, Gβ1γ2 did not alter the ability of Gαs to inhibit the activities of hACVI. These data show that Gβ1γ2 specifically alters the ability of Gαs to stimulate hACV and hACVI.

Gβ1γ2 enhanced Gαs-stimulated hACV and hACVI activity in a dose-dependent manner with an EC50 of ~10 nM (Fig. 2A). This value is similar to the EC50 value for the conditional stimulation of ACII by Gβγ subunits (18, 40). To determine whether Gβ1γ2 altered the efficacy or potency of forskolin and Gαs to stimulate hACVI, enzyme activity was monitored in the presence of increasing concentrations of forskolin or Gαs with and without Gβ1γ2. As shown in Figs. 2, B and C, Gβ1γ2 did not alter the EC50 values of forskolin or Gαs to stimulate hACVI. However, the maximal stimulation by both forskolin and Gαs was markedly increased in the presence of Gβ1γ2 (Figs. 2, B and C). These data clearly show that Gβ1γ2 increases the efficacy of both forskolin and Gαs. The ability of Gβγ to augment hACV and hACVI activity in the presence of Gαs is reminiscent of the conditional stimulation of the activities of ACII and ACIV by Gβγ subunits (40, 41). Thus, here onward the term “conditional stimulation” is used to describe the effects of Gβγ subunits on ACV and ACVI. Because the Gβγ binding sites on ACII have been reported to reside in the C2 domain and the C1b region (18, 19, 42), we investigated whether Gβ1γ2 regulated the activity of the recombinant, engineered, soluble forms of canine ACV and hACVI that can be activated by Gαs and forskolin (5, 7). The canine ACV was used because, except for one residue in each of its C1 and C2 domains, the sequences of these regions are identical to those of the corresponding regions in hACV. Although both forskolin and Gαs activated the soluble forms of ACV and ACVI comprising their C1 and C2 domains (supplemental Fig. S2), Gβ1γ2 did not alter the ability of either of these stimulatory agonists to regulate AC activity. These findings suggest that the C1 and C2 domains of ACV and ACVI, which form the catalytic core, are not sufficient for Gβ1γ2-mediated conditional stimulation of these enzymes.

Next, we compared the ability of Gβγ to conditionally stimulate the human, rat, and canine isoforms of ACV and ACVI. The human, rat, and canine isoforms of ACV are similar in their N-terminal regions. Although the human and rat ACV isoforms have similar N-terminal regions, the N-terminal domain of the canine ACV is very dissimilar. As shown in Fig. 3A, Gβγ conditionally stimulated hACV and rat ACV, but not...
Regulation of ACV and ACVI by Gβγ Subunits

FIGURE 2. Gβγ enhances the activity of Gαs- and forskolin-stimulated hACV and hACVI. Panel A, concentration-dependent activation of hACV and hACVI by Gβγ in the presence of Gαs. Membranes (6 μg) from Sf9 cells expressing hACV or hACVI were preincubated with Gβγ, at the indicated concentrations and then stimulated with 40 nM Gαs, for 15 min at room temperature. Panels B and C, membranes (6 μg) from Sf9 cells expressing hACVI were preincubated with 100 nM Gβγ, or buffer for 2 min and then stimulated with different concentrations of Gαs (panel B) or forskolin (Fsk, panel C) for 15 min at room temperature. AC activities were monitored as described under “Materials and Methods.” Data presented are the mean ± S.E. of triplicate determinations and are representative of three similar experiments.

FIGURE 3. Gβγ enhances the activity of Gαs-stimulated human, rat, and canine ACVI isoforms and human and rat, but not canine, ACV. Panel A, membranes (10 μg of protein) from Sf9 cells expressing hACV, rat ACV (rACV), or canine ACV (cACV) were assayed for AC activities in the presence of 40 nM Gαs, with or without the addition of Gβγ (100 nM) as described in Fig. 1. Panel B, COS-7 cell were transfected with either empty vector, pcDNA3-hACV, or pcDNA1-rACV. Canine ACV was expressed in Sf9 cells. Membranes from Sf9 cells or COS-7 cells (10 μg protein) were analyzed for AC activity as described in Fig. 1. The mean ± S.E. of triplicate determinations are shown.

canine ACV, that was expressed in Sf9 cells. Moreover, Gβγ conditionally stimulated human, rat, and canine isoforms of ACVI (Fig. 3B). The data in Fig. 3A show the specificity of the Gβγ effects among the ACV isoforms and are consistent with the role of the N-terminal segments of human and rat ACV in the conditional stimulation by Gβγ subunits.

Because the two-hybrid assay showed that the N termini of hACV and hACVI interact with Gβγ subunit, we examined the role of the N-terminal segment of hACV in mediating the regulation by Gβγ. For this purpose, initially the entire N-terminal region (aa 1–151) of hACV was deleted, and the wild type and mutant (hACVIΔN) enzymes were expressed in Sf9 cells. Although hACVIΔN showed a much lower activity as compared with full-length hACVI (Fig. 4A), its stimulated activity was about 4-fold greater than the corresponding control activity in membranes from mock-infected cells. Nonetheless, because the expression of hACVIΔN in Sf9 cells was greater than full-length hACVI, it would appear that the N terminus of hACVI is necessary for the proper folding or insertion of the enzyme in the membrane. Notwithstanding the low activity of hACVIΔN, whereas both forskolin and Gαs stimulated hACVIΔN, Gβγ did not further enhance its activity (Fig. 4A); in parallel experiments, the activity of full-length hACVI in the presence of Gαs was enhanced by Gβγ (Fig. 4A). Because of the low activity of hACVI in which the entire N terminus was deleted, we tested two other deletion mutants of hACVI in which either the first 76 residues (hACVIΔ77) or amino acids 77–151 (hACVIΔ77–151) of the N terminus were deleted. The forskolin- or Gαs-stimulated activities of both these forms of hACVI were similar to the full-length enzyme (Fig. 4B). However, only the full-length and hACVIΔ77–151, forms of the enzyme were conditionally stimulated by Gβγ (Fig. 4B). These data show that residues 77–151 in the N terminus of hACVI are necessary for stimulation of enzyme activity by Gβγ.

Next we investigated the role of endogenous Gβγ subunits in receptor-mediated activation of hACV in intact cells. In these experiments, the C terminus of G protein-coupled receptor kinase 2 (GRK2-CT) that contains the Gβγ binding domain was overexpressed in COS-7 cells to sequester the Gβγ subunits that are released upon activation of β-adrenergic receptors by isoproterenol. This approach has previously been used to examine the role of Gβγ subunits in regulation of effectors such as phospholipase C-β, ACII, ACVIII, and activation of the mitogen-activated protein kinase cascade (32, 43, 44). As shown in Fig. 5A, in COS-7 cells, isoproterenol increased cAMP accumulation in a concentration-dependent manner by ~3-fold over basal values. The overexpression of hACV in these cells increased isoproterenol-stimulated cAMP formation. Importantly, the expression of GRK2-CT markedly decreased the ability of isoproterenol to stimulate cAMP accumulation in COS-7 cells transfected with hACVI but not in empty vector transfected cells (Fig. 5A). The inability of GRK2-CT to alter isoproterenol-stimulated activity in empty vector-transfected cells suggests that the endogenous forms of ACs in COS-7 cells are not regulated by Gβγ subunits. Using reverse transcription-PCR, one of the endogenous AC isoforms...
Regulation of ACV and ACVI by \( \beta \gamma \) Subunits

In COS-7 cells has been reported to be \( \beta \gamma \)-stimulated ACVII (originally mislabeled as ACXI) (45). However, the inability of GRK2-CT to alter the endogenous AC activity (basal or isoproterenol-stimulated) in COS-7 cells suggests that ACVII does not contribute the majority of AC activity in these cells. Moreover, GRK2-CT did not alter the ability of forskolin to increase cAMP accumulation in both control and hACVI overexpressing cells (Fig. 5B), demonstrating that the GRK2-CT does not alter AC activity in a nonspecific manner. Please note that although \( \beta \gamma \) subunits stimulate forskolin-activated hACVI activity in vitro (Fig. 1), in the absence of an agonist that would activate G proteins, in intact cells the forskolin-stimulated activity is independent of \( \beta \gamma \), hence the lack of an effect of GRK2-CT. The inability of GRK2-CT to inhibit forskolin-stimulated activity in intact cells also demonstrates that the endogenous activation of G proteins, and therefore, free \( \beta \gamma \) subunits is low. This notion is further validated by the experiment shown in Fig. 5C. When COS-7 cells were co-transfected with hACVI and constitutively active (Q213L) mutant of \( \alpha \) subunits, GRK2-CT did not inhibit cAMP formation (Fig. 5C). These data also show that the GRK2-CT does not inhibit hACVI activity in a nonspecific manner.

The studies in Fig. 5A show that \( \beta \gamma \) subunits contribute toward isoproterenol-stimulated hACVI activity in intact cells. Because \( \beta \)-adrenergic receptors have also been shown to couple to \( G_1 \) (46), we next examined if the \( \beta \gamma \) could be derived from activation of \( G_1 \). For this purpose, COS-7 cells expressing either hACVI alone or in combination with GRK2-CT were treated with pertussis toxin before stimulation with isoproterenol. To ensure that \( G_1 \) was indeed inactivated in these cells, a parallel set of experiments were performed where cells transfected with hACVI and M2 muscarinic receptor were treated with or without pertussis toxin. As shown in Fig. 5D, pertussis toxin treatment eliminated the ability of the M2 muscarinic receptor agonist carbacol to inhibit hACVI activity in intact cells, demonstrating complete inhibition of \( G_1 \) activity. However, pertussis toxin treatment did not alter the ability of GRK2-CT to attenuate isoproterenol-stimulated hACVI activity (Fig. 5E), indicating that the \( \beta \gamma \) subunits derived from a source(s) other than \( G_1 \) contribute to the \( \beta \)-adrenergic receptor agonist-mediated stimulation of hACVI activity.

**DISCUSSION**

It is now well established that the cytosolic, C1 and C2 domains of the various mammalian, membrane-bound, adenylyl cyclase isoforms are sufficient to reconstitute enzyme activity that can be regulated by a number of modulators including \( \alpha \) and \( G_0 \) (5, 9). However, it is becoming apparent that other regions of the AC molecule also participate in the regulation of its enzyme activity. For example, the N-terminal residues of ACVIII has been shown to play a major role in the regulation of its activity by protein kinase C and \( G_0 \) (22, 23) and is also necessary for its interactions with Snapin (24), although the role of this latter interaction remains unknown. Similarly, the N terminus of ACVIII is necessary for modulation of its activity by capacitative \( Ca^{2+} \) entry in intact cells (25). In this report we have identified the G protein \( \beta_1 \) and \( \beta_2 \) subunits as binding partners for the N-terminal regions of hACV and its closely related isoform, hACVI. For hACV, the G protein \( \beta \) subunits are identified as the first binding partners that interact with the N-terminal region of the enzyme.

In cells, \( \beta \) and \( G_\gamma \) subunits form an obligate heterodimer. Upon receptor-mediated activation and dissociation of heterotrimeric G protein components, the \( \beta \gamma \) subunits have been shown to regulate the activities of several effectors including phospholipase C-\( \beta \), GRK2, and certain isoforms of adenylyl cyclases such as ACI, ACII, ACIII, ACIV, ACVII, and ACVIII (13, 32). Among the AC isoforms, \( \beta \gamma \) stimulates the activity of ACII, ACIV, and ACVII, provided some active \( G_\alpha \) is present (13). This conditional (i.e. in presence of \( G_\alpha \)) stimulation of ACII, ACIV, and ACVII by \( \beta \gamma \) subunits is similar to our findings that \( \beta \gamma \) subunits stimulate the activities of hACV and hACVI in the presence of \( G_\alpha \). The difference between our findings and those with ACII is that forskolin-stimulated activities of hACV and hACVI can be further enhanced by \( \beta \gamma \) subunits (Figs. 1 and 2), whereas in the case of ACII, forskolin-stimulated activity is only weakly enhanced by \( \beta \gamma \) (40). Thus, even the conditional stimulation of the different isoforms by \( \beta \gamma \) subunits varies among the different AC isoforms. The \( \beta \gamma \) interaction sites on ACII reside on the C1b and C2 regions (18, 19, 42), and the sequence (PFAHL) on the C1b region that binds \( \beta \gamma \) is conserved in ACIV and ACVII (19). Interestingly, we have previously shown that \( \beta \gamma \) interactions with ACI also...
Regulation of ACV and ACVI by Gβγ Subunits

![Image](https://example.com/figure5.pdf)

**FIGURE 5.** Gβγ participate in stimulation of hACVI activity by isoproterenol. Panels A and B, COS-7 cells were transfected to express hACVI or GRK2-CT either separately or together and then stimulated with isoproterenol at the indicated concentrations (panel A) or 10 μM forskolin (Fsk) (panel B). Panel C, cells were transfected to express hACVI, Gs, Q213L, or GRK2-CT either separately or together. The expression of GRK2-CT and hACVI was monitored by Western blotting using anti-GRK2 antibody or anti-ACVI antibody, respectively. Panel D, cells were transfected to coexpress hACVI and M2 muscarinic receptor and treated with or without pertussis toxin (PTX, 25 ng/ml) for 6 h before being stimulated with forskolin (Fsk, 10 μM) and/or carbachol (Cch, 100 μM). Where present, carbachol was added immediately before the addition of forskolin. Panel E, cells were transfected with hACVI alone or together with GRK2-CT and treated with or without pertussis toxin as described in panel D before being exposed to 3 μM isoproterenol. Data presented are the mean ± S.E. of triplicates. A representative of three experiments is shown. n.s., not significantly different. *, p < 0.05.

The Gβγ subunits include the C1b and C2 regions of this enzyme (9), although ACI is inhibited by Gβγ subunits, whereas ACII is stimulated by Gβγ. The N-terminal segments of hACV and hACVI do not contain the consensus Gβγ binding sequence (PFALH) found on ACII, ACIV, and ACVII.

Besides of its importance in Gβγ-mediated activation of hACV and hACVI, deletion of the entire N terminus of hACVI markedly diminishes its activity, as previously shown for ACI (11). This region is likely important in proper folding and/or insertion of the enzyme in the membrane. However, smaller deletions in the N-terminal region do not alter hACVI activity (Fig. 4), identifying the region encompassed by residues 77–151 as necessary for Gβγ-mediated stimulation of activity.

Using overexpression of ACV, ACVI, and Gβγ subunits, Bayewitch et al. (47) reported that Gβγ subunits inhibit ACV and ACVI in COS-7 cells. This is opposite to our in vitro findings using ACV and ACVI from different species and purified Gβγ subunits. Because the rabbit isoforms of ACV and ACVI used by Bayewitch et al. (47) have significant similarity in their N termini with the human and rat isoforms, the opposite findings cannot be explained by the species of origin. More likely, the effects that Bayewitch et al. (47) observed by overexpression of Gβγ subunits in intact cells may be due to some indirect effect. As mentioned above, Gβγ modulates many other effectors such as phospholipase Cβ, phosphatidylinositol 3-kinase, ion channels, etc., which in turn increases intracellular Ca^{2+} concentration, and thereby inhibit the Ca^{2+}-sensitive ACV or ACVI. Although as shown here the Gβγ subunits would also be expected to stimulate ACV and ACVI, the net effect on cAMP accumulation would be dependent upon their positive and negative effects and, it would appear that in the experiments of Bayewitch et al. (47) the inhibitory effects (nonspecific) of overexpressed Gβγ overcame their stimulatory actions. Likewise, unlike our findings (Fig. 3B), Premont et al. (48) showed that Gβγ subunits do not alter the activity of rat ACVI. However, it should be noted that in their experiments, membranes of HEK293 cells expressing rat ACVI were stimulated by GTPγS (Fig. 2B in Ref. 48). Because GTPγS activates a number of G proteins besides Gs, it is possible that the Gβγ released from these other G proteins had already stimulated ACVI activity, and no further effects of exogenously added Gβγ could be observed. This may also explain why Premont et al. (48) observed a 3-fold increase in the activity of ACII by exogenously added Gβγ, whereas we (not shown) and others (19) have observed a 9-fold increase in ACII activity by Gβγ. Thus, the seemingly contradictory findings of Premont et al. (48) and
those reported here may be due to the different experimental approaches used.

Gβγ subunits also regulate hACVI activity in intact cells. Thus, the sequestration of endogenous Gβγ by GRK2-CT decreases the efficacy of the β-adrenergic receptor agonist, isoproterenol, to stimulate cAMP accumulation in hACVI overexpressing cells but not in control cells (Fig. 5). The lack of an effect of GRK2-CT on isoproterenol-stimulated activity of endogenous ACs in COS-7 cells suggests that the expression of Gβγ-stimulated ACVII that has been detected in these cells by reverse transcription-PCR (45) is low. Moreover, the lack of an effect of GRK2-CT on forskolin-stimulated or constitutively active Gα subunits of heterotrimeric G proteins interact with the N termini of hACV and ACVI by Gβγ subunits, provided that some Gα subunits or forskolin is present. This regulation is similar to the conditional stimulation of ACII, ACIV, and ACVII by Gβγ. We have also shown that residues 77–151 in the N terminus of ACV are important for Gβγ-mediated conditional stimulation of this enzyme. Moreover, we show that endogenous Gβγ is most likely derived from Gα, and not Gγ, play an important role in the stimulation of hACV by agonists that activate Gα and that Gα, by itself is not sufficient for the full activation of hACVI in intact cells.

Acknowledgments—We thank Dr. Heidi Hamm, Vanderbilt University, for the generous gift of purified GST-phosducin. We also thank Dr. Adriano Marchese, Loyola University Chicago, for the anti-GRK2 antibody 3A10 and M2-muscarinic receptor and GRK2-CT cDNAs. Dr. Adriano Marchese, Loyola University Chicago, for the anti-GRK2 antibody 3A10 and M2-muscarinic receptor and GRK2-CT cDNAs. We also thank Dr. Heidi Hamm, Vanderbilt University, for the generous gift of purified GST-phosducin. We also thank Dr. Adriano Marchese, Loyola University Chicago, for the anti-GRK2 antibody 3A10 and M2-muscarinic receptor and GRK2-CT cDNAs.

REFERENCES
1. Smit, M. I., and Iyengar, R. (1998) Adv. Second Messenger Phosphoprotein Res. 32, 1–21
2. Defer, N., Best-Belpomme, M., and Hanoune, J. (2000) Am. J. Physiol. Renal Physiol. 279, 400–416
3. Patel, T. B., Du, Z., Pierre, S., Cartin, L., and Scholich, K. (2001) Gene (Amst.) 269, 13–25
4. Tang, W. J., and Gilman, A. G. (1992) Cell 70, 869–872
5. Tang, W. J., and Gilman, A. G. (1995) Science 268, 1769–1772
6. Yan, S. Z., Hahn, D., Huang, Z. H., and Tang, W. J. (1996) J. Biol. Chem. 271, 10941–10945
7. Scholich, K., Barbier, A. J., Mullenix, J. B., and Patel, T. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2915–2920
8. Scholich, K., Wittkopp, C., Barbier, A. J., Mullenix, J. B., and Patel, T. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9602–9607
9. Wittkopp, C., Scholich, K., Yigzaw, Y., Stringfellow, T. M., and Patel, T. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9551–9556
10. Cali, J. J., Parekh, R. S., and Krupinski, J. (1996) J. Biol. Chem. 271, 1089–1095
11. Tang, W. J., Krupinski, J., and Gilman, A. G. (1991) J. Biol. Chem. 266, 8595–8603
12. Iyengar, R. (1993) FASEB J. 7, 768–775
13. Sunahara, R. K., Dessauver, C. W., and Gilman, A. G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 461–480
14. Iwami, G., Kawabe, T., Ebina, T., Cannon, P. J., Homcy, C. J., and Ishikawa, Y. (1995) J. Biol. Chem. 270, 12481–12484
15. Chen, Y., Harry, A. L., Li, S., Smith, M. I., Bai, X., Magnusson, R., Pieron, J. P., Weng, G., and Iyengar, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14100–14104
16. Jacobowitz, O., and Iyengar, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10630–10634
17. Watson, P. A., Krupinski, J., Kempsinski, A. M., and Frankenfeld, C. D. (1994) J. Biol. Chem. 269, 28893–28898
18. Weitmann, S., Schultz, G., and Kleuss, C. (2001) Biochemistry 40, 10853–10858
19. Diel, S., Klass, K., Wittig, B., and Kleuss, C. (2006) J. Biol. Chem. 281, 288–294
20. Wu, Z., Wong, S. T., and Storms, D. R. (1993) J. Biol. Chem. 268, 23766–23768
21. Levin, L. R., and Reed, R. R. (1995) J. Biol. Chem. 270, 7573–7579
22. Lai, H. L., Lin, T. H., Kao, Y. Y., Lin, W. J., Hwang, M. J., and Chern, Y. (1999) Mol. Pharmacol. 56, 644–650
23. Kao, Y. Y., Lai, H. L., Hwang, M. J., and Chern, Y. (2004) J. Biol. Chem. 279, 34440–34448
24. Chou, J. L., Huang, C. L., Lai, H. L., Hung, A. C., Chien, C. L., Kao, Y. Y., and Chern, Y. (2004) J. Biol. Chem. 279, 46271–46279
25. Gu, C., and Cooper, D. M. (1999) J. Biol. Chem. 274, 8012–8021
26. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
27. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Science 252, 802–808
28. Rhees, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
29. Logothetis, D. E., Kurachi, Y., Galper, I., Neer, E. J., and Clapham, D. E. (1987) Nature 325, 321–326
30. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriaza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
31. Vanhaesebroeck, B., Leveers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends Biochem. Sci. 22, 267–272
32. Steiner, D., Saya, D., Schallmich, E., Simonds, W. F., and Vogel, Z. (2006) Cell. Signal. 18, 62–68
33. Salim, S., Sinnarajah, S., Kehrl, H. J., and Dessauver, C. W. (2003) J. Biol. Chem. 278, 15842–15849
34. Chen-Goodspeed, M., Lukan, A. N., and Dessauver, C. W. (2005) J. Biol. Chem. 280, 1808–1815
35. Kozasa, T., and Gilman, A. G. (1995) J. Biol. Chem. 270, 1734–1741
36. Gao, X., and Patel, T. B. (2005) Mol. Pharmacol. 67, 42–49
37. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
38. Iniguez-Lluhi, J. A., Simon, M. I., Robishaw, J. D., and Gilman, A. G. (1992) J. Biol. Chem. 267, 23409–23417
39. Muller, S., Straub, A., Schroder, S., Bauer, P. H., and Lohse, M. J. (1996) J. Biol. Chem. 271, 11781–11786
40. Tang, W. J., and Gilman, A. G. (1991) Science 254, 1500–1503
41. Gao, B. N., and Gilman, A. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10178–10182
42. Chen, J., De Vivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J.,
Regulation of ACV and ACVI by Gβγ Subunits

Logothetis, D. E., Hildebrandt, J. D., and Iyengar, R. (1995) Science 268, 1166–1169
43. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
44. Crespo, P., Cachero, T. G., Xu, N., and Gutkind, J. S. (1995) J. Biol. Chem. 270, 25259–25265
45. Premont, R. T. (1994) Methods Enzymol. 238, 116–127
46. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Nature 390, 88–91
47. Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F., and Vogel, Z. (1998) FASEB J. 12, 1019–1025
48. Premont, R. T., Chen, J., Ma, H. W., Ponnapalli, M., and Iyengar, R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9809–9813
49. Zhong, J., Hume, J. R., and Keef, K. D. (2001) J. Physiol. (Lond.) 531, 105–115