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Regions in the G Protein γ Subunit Important for Interaction with Receptors and Effectors

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ABSTRACT

G$\beta\gamma$ dimers containing the γ_{11} or γ_1 subunits are often less potent and effective in their ability to regulate effectors compared with dimers containing the γ_2 subunit. To explore the regions of the γ subunit that affect the activity of the $\beta\gamma$ dimer, we constructed eight chimeric γ subunits from the γ_1 and γ_2 subunits. Two chimeras were made in which the N-terminal regions of γ_1 and γ_2 were exchanged and two in which the C-terminal regions were transposed. Another set of chimeras was made in which the *CAAX* motifs of the chimeras were altered to direct modification with different prenyl groups. All eight γ chimeras were expressed in Sf9 cells with the β_1 subunit, G$\beta\gamma$ dimers were purified, and then they were assayed in vitro for their ability to bind to the Gα_{i1} subunit, to couple Gα_{i1} to the A1 adenosine receptor, to stimulate phospholipase C-β, and to regulate type I or type II adenyl cyclases. Dimers containing the C-terminal sequence of the γ_2 subunit modified with the geranylgeranyl lipid had the highest affinity for $G_{i1}\alpha$ (range, 0.5–1.2 nM) and were most effective at coupling the $G_{i1}\alpha$ subunit to receptor. These dimers were most effective at stimulating the phosphatidylinositol-specific phospholipase C-β isoform and inhibiting type I adenyl cyclase. In contrast, $\beta\gamma$ dimers containing the N-terminal sequence of the γ_2 subunit and a geranylgeranyl group are most effective at activating type II adenyl cyclase. The results indicate that both the N- and C-terminal regions of the γ subunit impart specificity to receptor and effector interactions.

Among the best characterized signal transduction systems are the pathways used by receptors coupled to heterotrimeric G proteins. The G protein $\beta\gamma$ subunit plays an important role in these pathways. It is required for the interaction between the receptor and the α subunit to initiate GDP/GTP exchange and also stabilizes the basal state of the receptor-α-GDP-$\beta\gamma$ complex, which forms the high-affinity ligand binding conformation of receptors (Cabrera-Vera et al., 2003). Once released from the activated α subunit, the $\beta\gamma$ subunit can regulate more than 20 effectors, including PLC-β, adenylyl cyclase, ion channels (Hildebrandt, 1997; Cabrera-Vera et al., 2003), PtdIns 3-kinase (Kerchner et al., 2004), and guanine nucleotide exchangers for small GTP binding proteins (Welch et al., 2002). The free $\beta\gamma$ dimer can also participate in regulatory events by binding to cytoplasmic proteins such as the β-adrenergic receptor kinase or phosducin (Cabrera-Vera et al., 2003). It is noteworthy that the multiple isoforms of the $\beta\gamma$ dimer can differentially regulate effectors (Hildebrandt, 1997; Cabrera-Vera et al., 2003), making it necessary to understand the regions of these subunits that interact with their targets.

To date, seven β and 12 γ subunits have been identified in mammalian systems (Downes and Gautam, 1999); thus, the $\beta\gamma$ dimers, which can be assembled from the known subunits, yield a large number of potentially unique complexes that differ in their interactions with receptors and effectors (Hildebrandt, 1997; Cabrera-Vera et al., 2003). Whereas the β_1–β_4 subunits are more than 85% identical in amino acid sequence, the sequence identity of the γ subunits ranges from 10 to 70% (Downes and Gautam, 1999). The diversity of either the β or γ subunit can impart specificity to the interactions of the dimer with receptors (Richardson and Ro-

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ABBREVIATIONS: PLC-β, phosphatidylinositol-specific phospholipase C-β isoform; PtdIns, phosphatidylinositol 4,5-bisphosphate; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Genapol C-100, polyoxyethylene (10) dodecyl ether.

bishaw, 1999; Hou et al., 2000; McIntire et al., 2001) or effectors (Cabrera-Vera et al., 2003), and the type of prenyl group on the γ subunit is clearly important in the interactions with both receptors (Kisselev et al., 1995; Yasuda et al., 1996) and effectors (Myung et al., 1999). Multiple experiments suggest that the γ subunit is divided into three functionally different regions. The central region of all γ subunits is believed to be involved in the interactions between the β and γ subunits. For example, mutagenesis data indicate that a 14 amino acid sequence (amino acids 36–49 on γ_1 or 33–46 on γ_2) is critical for the interaction with the β_1 subunit (Lee et al., 1995), and the crystal structure of the $\beta\gamma$ dimer confirms that 17 of the 27 residues in the γ subunit that interact with the β subunit are centrally located (Sondek et al., 1996). The N- and C-terminal regions of the γ subunits are the least conserved (Sondek et al., 1996; Downes and Gautam, 1999), and several lines of evidence suggest that these regions are important for the interaction with receptors (Yasuda et al., 1996; Hou et al., 2000) and effectors (Yasuda et al., 1996, 1998; Hildebrandt, 1997; Akgoz et al., 2002; Cabrera-Vera et al., 2003).

The γ subunits segregate into five groups: γ_1, γ_{11}, and γ_8 comprise subfamily I; γ_2, γ_3, γ_4, and γ_9, subfamily II; γ_7 and γ_{12}, subfamily III; γ_5 and γ_{10}, subfamily IV; and γ_{13} forms its own family (Downes and Gautam, 1999). Experiments show that $\beta\gamma$ dimers containing the γ_1 or the γ_{11} subunit are less potent in activating PLC-β and type II adenyl cyclase than dimers containing the γ_2 subunit (Myung et al., 1999). Whereas the expression of γ_1 is restricted to the visual system, γ_{11} and γ_8 are widely expressed (Morishita et al., 1998; Downes and Gautam, 1999). These three γ subunits of subfamily I are modified with the farnesyl lipid (Downes and Gautam, 1999), and the distribution of charged amino acids in the N and C termini is quite different from that in the other nine γ subunits.

To examine how the N- and C-terminal domains of the γ subunit and its prenyl group affect the interaction with α subunits, receptors, and effectors, we constructed eight chimeric γ subunits from the γ_1 and the γ_2 subunits. These two γ subunits were used for the chimeras because the N and C termini of these proteins are representative of the major differences between the γ subunits in subfamily I and the other nine γ subunits. Two chimeras were made in which the N-terminal amino acids of γ_1 and γ_2 were exchanged. Two other chimeras were made in which the C-terminal 23 amino acids of γ_1 and γ_2 were exchanged. Finally, another set of chimeras was made in which the *CAAX* motifs in the four chimeras were altered to direct modification with different prenyl groups. All eight γ chimeras were expressed in Sf9 cells with the β_1 subunit and purified. Each dimer was tested using five in vitro assays chosen to probe major facets of $\beta\gamma$ activity: the ability to bind to the $G\alpha_{i1}$ subunit, to couple $G\alpha_{i1}$ to the A1 adenosine receptor, to stimulate PLC-β, or to regulate type I and II adenylyl cyclases. The in vitro results indicate that the γ subunit's C terminus and its prenyl group are important for coupling α subunits to the A1 adenosine receptor, for the activation of PLC-β, and for the inhibition of type I adenylyl cyclase. In contrast, the γ subunit's N terminus is very important for the activation of type II adenylyl cyclase.

Materials and Methods

Strategy for Construction of the Chimeric γ Subunits. The γ_1 and γ_2 subunits have a conserved set of three residues approximately 20 amino acids from the N terminus (QLK) and the C terminus (DPL). The strategy for making the chimeras was based on creating new cDNAs for the γ_1 and γ_2 subunits, which had restriction sites engineered into the QLK and DPL sequences and are similar to those published previously (Jian et al., 2001). These sites are highlighted in Fig. 1A. In addition, note the similarities in the N- and C-terminal regions of the γ_1 and γ_{11} subunits. Thus, whereas the chimeras were made with regions from γ_1 because the cDNA for γ_1 contained a convenient restriction site in the DPL sequence, the regions were selected to be representative of γ_{11} (Fig. 1). An AflII site was used to create the QLK sequence and a BamHI site to code for the DPL sequence in each molecule. The nomenclature used to describe these chimeric γ subunits is shown in Fig. 1B. The protein was divided into three regions by the QLK and DPL sites, designated as AAA for γ_1 and BBB for γ_2. If the C-terminal prenyl group was switched from the native modification, the amino acids in the mutant *CAAX* motif are indicated (Yasuda et al., 1996). For example, $\gamma_{2\text{-L71S}}$ indicates the native γ_2 subunit modified to change only the normal C-20 geranylgeranyl to the C-15 farnesyl. A designation of γ_{BBA} indicates a chimeric γ subunit in which the C terminus of the γ_1 subunit (molecule A) is added to the N-terminal and middle regions of the γ_2 subunit (molecule B) at the conserved sequence. The prenyl group of this chimera would be farnesyl. A designation of $\gamma_{BBA\text{-S74L}}$ would indicate the same chimeric γ subunit mutated to code for the addition of geranylgeranyl at the C terminus.

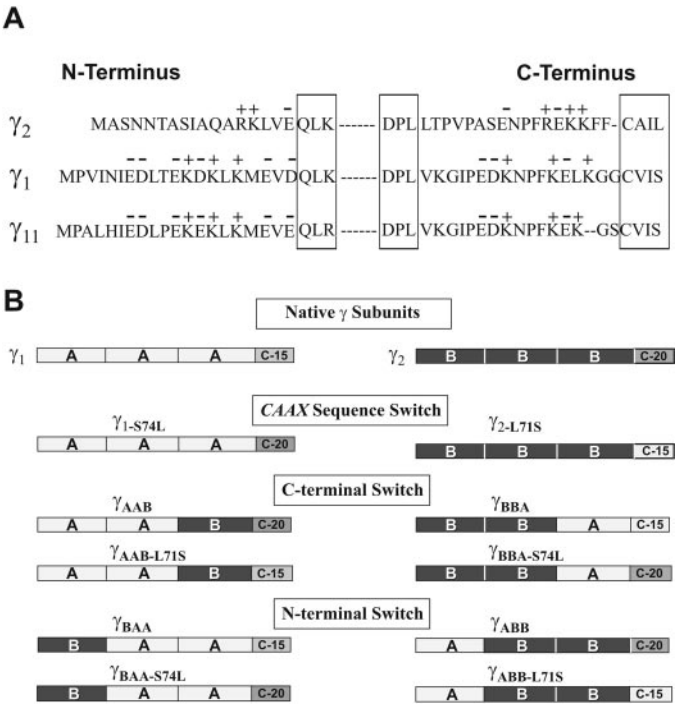

Fig. 1. Strategy for constructing the chimeric γ subunits. A, comparison of the primary sequences and charged amino acids in the N- and C-terminal regions of the γ_1 and γ_{11} subunits with those of γ_2. The QLK and DPL sites and the C-terminal *CAAX* motif are highlighted. The center section of the molecules is omitted. B, schematic view of the chimeric γ subunits. The sequence of the γ_1 subunit is indicated in white and the γ_2 subunit in gray. Both of the γ_1 and γ_2 subunits are divided into three regions at the QLK and DPL sites. The molecules were designated as AAA for the γ_1 subunit and BBB for the γ_2 subunit. The farnesyl group (C-15) on the γ_1 subunit is indicated in light gray, and the geranylgeranyl group (C-20) on the γ_2 subunit is indicated in dark gray. The nomenclature used in the text to identify each chimera is indicated above the schematic representation.

The γ_1 cDNA was excised from a pEV plasmid (HindIII/BamHI) and subcloned into the pGem7zF+ plasmid using the same restriction sites; it was then excised with HindIII/Asp700 for subcloning into the pAlter-1 mutagenesis plasmid at the HindIII and SmaI sites. The γ_2 cDNA in pGem4z was excised by digestion with XbaI and SmaI and was subcloned into pAlter-1 using the same restriction sites. To construct the chimeras, identical restriction sites were created in the QLK and DPL sequences in both the γ_1 and γ_2 cDNAs; an AflII site was engineered to code for the QLK sequence, and a BamHI site was engineered to code for the DPL sequence. Because the BamHI restriction site already existed in the γ_1 cDNA in the site corresponding to the DPL residues, a BamHI site was added to the γ_2 cDNA through site-directed mutagenesis by altering one base in the γ_2 sequence (GAA to GAG, encoding glutamic acid) using the Morph Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime → 3 Prime, Boulder, CO), taking care to conserve the protein sequence. The AflII restriction site for the QLK sequence was engineered into both the γ_1 and γ_2 subunits using the same mutagenesis kit. The resulting γ_1 and γ_2 cDNAs in the pAlter-1 plasmid containing the new AflII and BamHI sites were used to make the C- or N-terminal chimeric γ subunits.

Construction of N- and C-Terminal γ Chimeras and Production of Recombinant Baculoviruses. The γ_1 and γ_2 C-terminal chimeras (γ_{AAB} and γ_{BBA}) were constructed by digesting the γ_1 and γ_2 cDNAs in the pAlter-1 plasmid with BamHI and NheI. The four resulting DNA fragments contain the C termini of γ_1 and γ_2 and cDNAs encoding for the intact N terminus and middle regions of the γ_1 and γ_2 proteins. The C-terminal cDNA fragments were gel-purified and ligated back into either the cDNA encoding the N terminus and middle regions of the γ_1 or γ_2 as desired. This protocol generated the constructs containing the γ_{AAB} and γ_{BBA} chimeras. To exchange the N termini of the γ_1 and γ_2 molecules, the respective cDNAs in the pAlter-1 vector were digested with AflII and NheI, and the N-terminal cDNA fragments were gel-purified and ligated back into the cDNA encoding the middle regions and C terminus of either γ_1 or γ_2 as desired. This protocol generated the constructs containing γ_{BAA} and γ_{ABB} chimeras. It was desirable to subclone the γ cDNAs into the XbaI site in the baculovirus transfer vector pVL1393. Thus, the four γ chimeras in the pAlter-1 plasmid were subcloned into the linear pCNTR plasmid, which added the XbaI restriction sites to the end of each chimera. Each of the four chimeras was subcloned into the pVL1393 baculovirus transfer vector by excising them from pCNTR with XbaI and ligating the fragments into pVL1393 at its XbaI sites. The four γ chimeras containing altered *CAAX* sequences—$\gamma_{AAB\text{-}L71S}$ (farnesyl), $\gamma_{BBA\text{-}S74L}$ (geranylgeranyl), $\gamma_{BAA\text{-}S74L}$ (geranylgeranyl), and $\gamma_{ABB\text{-}L71S}$ (farnesyl)—were constructed using polymerase chain reaction on the cDNAs in pVL1393 as described previously (Lindorfer et al., 1996). Each of the eight pVL1393 transfer vectors containing the cDNA for a chimeric γ subunit was sequenced to ensure fidelity. Recombinant baculoviruses encoding for the eight γ chimeras were produced by cotransfecting each recombinant plasmid DNA with linear wild-type BaculoGold viral DNA into Sf9 cells and purified by one round of plaque purification (Graber et al., 1994). The recombinant baculoviruses encoding the α_s, α_{i1}, β_1, γ_1, $\gamma_{1\text{-}S74L}$, γ_2, and $\gamma_{2\text{-}L71S}$ subunits have been described previously (Graber et al., 1994; Lindorfer et al., 1996).

Expression and Purification of G Protein α and βγ Subunits. G protein α subunits were overexpressed in bacteria- (Sarvazyan et al., 1998) and baculovirus-infected Sf9 insect cells (Graber et al., 1994). Bacterially expressed, myristoylated α_{i1} was purified from *Escherichia coli* (BL21/DE3) by following the method of Mumby and Linder (1994). The amount of α_{i1} protein was determined using the Bradford assay. The $G_{i1}\alpha$ subunits expressed in Sf9 insect cells were purified to homogeneity as described previously (Graber et al., 1994), and the $G_s\alpha$ subunit used in the adenylyl cyclase assays was prepared from a 0.1% (w/v) CHAPS extract of crude cell lysates (McIntire et al., 2001). The specific activity of GTP binding of these preparations was ~11 to 15 nmol/mg of α subunit, as measured by guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding (Graber et al., 1994; Sarvazyan et al., 1998). Bovine brain βγ dimer was isolated from brain cortex synaptosomal membranes as described previously (Sarvazyan et al., 1998). The membranes were a gift from Dr. T. Ueda (University of Michigan, Ann Arbor, MI). Recombinant βγ subunits were prepared from baculovirus-infected Sf9 insect cells. Sf9 cells were coinfected with the appropriate recombinant baculoviruses encoding the β_1 subunit and native or chimeric γ subunits in Sf9 cells at a multiplicity of infection of 3 and harvested 48 h after infection. All βγ subunits were extracted from frozen cell pellets with 0.1% Genapol C-100, and the detergent-extracted βγ dimers were purified on a DEAE column followed by affinity chromatography on a $G_{i1}\alpha$-agarose column (Graber et al., 1996). The βγ combinations used in this study are properly folded because they were purified on α subunit affinity column, have a high affinity for the α subunit (see Fig. 2), and were released after activation with AlF_4^-. The purified βγ dimers were resolved on 12% polyacrylamide gels and stained with silver, and the concentration of the purified βγ dimers estimated using ovalbumin standards. All preparations of the dimers were highly pure, and examples of their purity have been published previously (McIntire et al., 2001). The experiments shown under *Results* were performed using at least two different preparations of each βγ dimer with consistent results.

Analysis of the Post-Translational Processing of the γ Subunit by Mass Spectrometry. To confirm that the proper chimeric γ subunits were being expressed and that their C termini were properly modified, the molecular masses of the γ subunits in the 12 βγ dimers used in this study were determined using matrix-assisted laser desorption ionization mass spectrometry as described previously (Lindorfer et al., 1996). The molecular mass of each chimeric γ subunit was estimated by the University of Wisconsin GCG program. The results summarized in Table 1 indicate that each chimeric γ subunit was faithfully expressed and had a fully processed C terminus. For example, the γ_{BAA} protein in the sample was composed of one species with a molecular mass of 7818 Da. This result is consistent with the predicted amino acid sequence of the chimera and processing of the molecule by removal of the three C-terminal amino acids (−VIS), the addition of a farnesyl lipid to the C-terminal cysteine, the addition of a carboxylmethyl group to the C terminus, the removal of the N-terminal methionine, and acetylation of the resulting N-terminal alanine. The observed prenyl modification of the γ subunits in this study is consistent with the electrospray mass spectrometric analysis of previous samples, which show that more than 90% of the purified protein is properly modified (Lindorfer et al., 1996). Finally, the data in Table 1, combined with that in Figs. 2 to 5, show that the use of the conserved QLK and DPL sites, which are located in the two helical regions of the γ subunit (Sondek et al., 1996), to construct the chimeras seems to generate fully functional molecules. These chimeras combine with β subunits, purify efficiently over an α subunit affinity column, and interact well with receptors and effectors (Figs. 2–5).

Expression of A1 Adenosine Receptors, Phospholipase C-β, and Type I and II Adenylyl Cyclase. A recombinant baculovirus encoding the A1 adenosine receptor was used to overexpress the receptor in Sf9 insect cells, and membranes were purified as described previously (Yasuda et al., 1996). Sf9 insect cell membranes overexpressing recombinant type I or II adenylyl cyclase were prepared as described previously (McIntire et al., 2001). Recombinant turkey PLC-β was overexpressed in Sf9 cells and purified as described previously (Myung et al., 1999).

Assay of the Activity of βγ Dimers. Different facets of the activity of each βγ dimer were measured in a set of in vitro assays chosen to probe the known protein-protein interaction domains of the dimer. Each of these assays was selected for its ability to highlight a known function of the βγ dimer and because the outcome was directly dependent on βγ activity. The affinity of the βγ dimer for the α subunit reflects interactions of the top and sides of the β subunit with the switch II and C-terminal regions of the α subunit (Cabrera-

Vera et al., 2003). The prenyl group on the C-terminal region of the γ subunit is also important for binding the α subunit (Iniguez-Lluhi et al., 1992). The affinity of the $\beta\gamma$ dimers for $G\alpha_{i1}$ was measured in solution with pure proteins by flow cytometry using fluorescein-labeled bacterially expressed myristoylated α_{i1} and biotinylated bovine brain $\beta\gamma$ as described previously (Sarvazyan et al., 1998). In brief, 1 nM biotinylated brain $\beta\gamma$ was prebound to streptavidin-coated polystyrene beads (SVP-60-5; Spherotech Inc., Libertyville, IL) and incubated with 1 nM fluorescein-labeled, myristoylated α_{i1} together with varying amounts of recombinant $\beta\gamma$ in 20 mM HEPES, pH 8, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1.2 mM $MgCl_2$, 0.1% Lubrol, and 10 μM GDP. After a 30-min incubation at room temperature, samples were analyzed with a Becton Dickinson FACScan (BD Biosciences, San Jose, CA) capturing events on the forward scatter, side scatter, and fluorescein channels. Histograms of fluorescein fluorescence from singlet bead populations were obtained, and mean channel numbers were calculated using LYSIS II software (BD Biosciences).

The ability of each particular $\beta_1\gamma_x$ dimer to support coupling of the $G_{i1}\alpha$ subunit to the A1 adenosine receptor was used to monitor the interactions of the N-terminal, central, and C-terminal regions of the 10 γ subunit chimeras with the receptor. In this assay, the receptor is expressed in an Sf9 cell membrane in the absence of G proteins, causing it to be in its low-affinity agonist binding conformation (Yasuda et al., 1996). Reconstitution of the G protein heterotrimer into the membrane re-establishes the high-affinity agonist binding conformation of the receptor as measured by a radioligand binding assay using an agonist ligand (Fig. 2A) (McIntire et al., 2002). On the other hand, if an excess of α subunit and graded amounts of a $\beta\gamma$ dimer are reconstituted into the membrane, the amount of the receptor returned to the high-affinity agonist binding state as measured using a low concentration of the agonist ligand reflects the affinity of the receptor-α subunit complex for the $\beta\gamma$ dimer (McIntire et al., 2002). Because only the γ subunit was different in the experiments shown in Fig. 2B, the assay provides a measure of the ability of the dimers containing the different chimeric γ subunits to interact with the receptor and α subunit. This value is expressed as the EC_{50} value of receptor coupling shown in Fig. 2B. The ability of the three regions of the γ subunit to interact with PLC-β was measured by reconstituting dimers containing the chimeric γ subunits and pure PLC-β into synthetic lipid vesicles (Myung et al., 1999). Sf9 cells infected with recombinant baculoviruses for adenyl cyclases can be used to monitor the direct interaction of the $\beta\gamma$ dimer with type I and type II adenyl cyclase (Taussig et al., 1994; McIntire et al., 2001).

To measure type I or II adenylyl cyclase activity, Sf9 membranes overexpressing the desired enzyme (10 μg of membrane protein/assay tube) were reconstituted with the purified guanosine 5′-3-*O*-(thio)triphosphate-activated $G_s\alpha$ subunit and varying concentrations of pure $\beta\gamma$ dimers containing either a native or chimeric γ subunit and held on ice for 30 min (McIntire et al., 2001). Each assay was incubated at 30°C for 7 min, and the reaction was terminated with 1 ml of 0.11 N HCl. The samples were prepared for the assay of cAMP and the assay performed as described previously (McIntire et al., 2001). The activated $G_s\alpha$ subunit was used as the coactivator of type I and type II cyclase in these experiments because the $\beta\gamma$ dimer does not increase the activity of type II cyclase in the absence of $G_s\alpha$ or in the presence of forskolin (Taussig et al., 1994), and the $\beta\gamma$ dimer only modestly inhibits type I cyclase if it is preactivated with forskolin or Ca^{2+}/calmodulin (Taussig et al., 1994). Pilot experiments with our Sf9 membranes confirmed both of these findings (data not shown).

Calculation and Expression of Results. Experiments presented under *Results* are the average of three or more similar experiments. Data expressed as concentration-response curves were fit to sigmoid curves using the fitting routines in the Prism software (GraphPad Software Inc., San Diego, CA). The EC_{50} and V_{max} values shown in Tables 2 and 3 were taken from these fits. Statistical differences between the fitted curves were determined using all of the individual data points from multiple experiments to calculate the *F* statistic (Motulsky and Ransnas, 1987).

Materials. All reagents used in the culture of Sf9 cells and for the expression and purification of G protein $\beta\gamma$ subunits have been described previously in detail (Graber et al., 1996). The baculovirus transfer vector pVL1393 was purchased from Invitrogen (Carlsbad, CA); BaculoGold viral DNA was from PharMingen (San Diego, CA); 10% Genapol C-100 and phosphatidylinositol 4,5-bisphosphate were from Calbiochem (San Diego, CA); phosphatidylethanolamine (bovine heart) was from Avanti Polar Lipids (Alabaster, AL); inositol-2-[^{3}H]phosphatidylinositol 4,5-bisphosphate was from NEN Life Science Products (Boston, MA); CHAPS was from Roche Molecular Biochemicals (Indianapolis, IN); bovine serum albumin (fatty acid-free) was from Sigma (St. Louis, MO); and the pCNTR shuttle vector and the Morph Site-Specific Plasmid DNA Mutagenesis Kit were from 5 Prime → 3 Prime, Inc. All other reagents were of the highest purity available.

TABLE 1

Molecular mass of recombinant, chimeric γ subunits

The molecular weights of the γ subunits in the dimers used in this study were measured by mass spectrometry as described under *Materials and Methods*. Molecular masses were determined on two independent preparations of each $\beta\gamma$ dimer and were in agreement. The data presented are from one determination.

γ Subunits	Calculated Mass	Observed Mass
		Da
Native		
γ_1	8332	8330
γ_2	7751	7751
Exchanged prenyl group		
$\gamma_{1\text{-S74L}}$	8400	8400
$\gamma_{2\text{-L71S}}$	7683	7681
C-terminal chimeras		
γ_{AAB}	8551	8552
$\gamma_{AAB\text{-L71S}}$	8483	8482
γ_{BBA}	7532	7531
$\gamma_{BBA\text{-S74L}}$	7600	7602
N-terminal chimeras		
γ_{BAA}	7818	7818
$\gamma_{BAA\text{-S74L}}$	7886	7888
γ_{ABB}	8265	8265
$\gamma_{ABB\text{-L71S}}$	8197	8,199

Results

The $\beta_1\gamma_1$ and $\beta_1\gamma_{11}$ dimers are measurably less active than the $\beta_1\gamma_2$ dimer in its ability to activate K^+ channels (Cabrera-Vera et al., 2003), PLC-β (Myung et al., 1999), type II adenylyl cyclase (Myung et al., 1999), and PtdIns 3-kinase (Kerchner et al., 2004), or to inhibit type I adenylyl cyclase (McIntire et al., 2001). Because the dimers tested in these experiments all contained the β_1 subunit, differences in the primary amino acid sequence of the γ subunits, in their prenyl modification or both must explain the differences in activity. Whereas it was initially attractive to ascribe the unique properties of the γ_1 subunit to its specialized role in visual transduction (Cabrera-Vera et al., 2003), the fact that γ_{11} and γ_8 have similar amino acid sequences and prenyl modifications (Fig. 1A) and are widely expressed (Downes and Gautam, 1999) makes it important to understand the reasons for the differential activity of this subfamily of γ subunits. Figure 1A presents the differences in the N and C termini of the γ_1, γ_{11}, and γ_2 subunits. Note that the N-terminal 20 amino acids of the very similar γ_1 and γ_{11} subunits have six negatively charged amino acids, whereas this region of the γ_2 subunit has only one negative charge, and that the C-terminal amino acids of the γ subunits also show marked differences in charge distribution. Thus, differences

in the amino acid sequences and charges in the N- and C-terminal regions of these proteins may be important for the signaling specificity of the βγ dimer. To address this issue, we constructed a set of four chimeric γ subunits in which the N- and C-terminal amino acids of γ_1 and γ_2 were exchanged. To complete the set, an additional four mutants with altered prenyl modifications was prepared. Figure 1B presents the eight chimeras that were made and the nomenclature used to describe them. The 12 γ subunits shown in Fig. 1B were expressed with β_1 subunit, and the dimers were purified from baculovirus-infected Sf9 insect cells by α_{i1}-agarose affinity chromatography. The activity of the complete set of βγ dimers was examined in a panel of in vitro assays measuring the affinity of the dimer for the $G_{i1}\alpha$ subunit, the ability to support coupling of the α subunit to the bovine A1 adenosine receptor, and the ability to activate PLC-β and regulate type I or type II adenylyl cyclases.

Receptor Coupling. The C-terminal domain of the mammalian γ subunit and its prenyl group are known to be important for the interaction of the βγ dimer with α subunits and receptors (Yasuda et al., 1996; Azpiazu et al., 1999). The affinity of the α subunit for the βγ dimer is an important component of the interaction of the heterotrimer with the receptor (Sarvazyan et al., 1998), but this parameter is not commonly measured. Moreover, the differences noted with the different forms of the γ subunit can be minimized when high concentrations of the α and βγ subunits are reconstituted into vesicles or membranes (Fig. 2A). For these reasons, we performed experiments to compare the affinity of dimers containing the chimeric γ subunits for the $G_{i1}\alpha$ subunit with the EC_{50} values determined for the ability of the dimer to support the high-affinity agonist binding state of the A1 adenosine receptor. Figure 2A shows the ability of various concentrations of the $G\alpha_{i1}$:$\beta_1\gamma_2$ heterotrimer to reconstitute the high-affinity agonist binding conformation of the adenosine A1 receptor overexpressed in Sf9 cell membranes (Yasuda et al., 1996). Four sets of membranes were reconstituted with increasing amounts of heterotrimer, and the amount of total agonist ligand bound was plotted as a function of ligand concentration. Note that concentrations of heterotrimer in the 40 nM range shift the affinity of the receptor for the agonist (amino-benzyladenosine) from approximately 30 (○, no G protein) to 0.3 nM (▼, 40 nM $G\alpha_{i1}$:$\beta_1\gamma_2$). An intermediate shift in the affinity of the receptor is caused by 2 to 6 nM heterotrimer. When the concentration of the heterotrimer is in the 2 to 6 nM range, it is possible to measure differences in the ability of various βγ combinations to interact with the receptor-α subunit complex and support high-affinity binding (McIntire et al., 2002). Therefore, we measured the ability of the six C-terminal chimeras to support the establishment of the high-affinity agonist binding conformation of bovine A1 adenosine receptors at the 6 nM heterotrimer. The results were expressed as the EC_{50} value of the interaction and compared with the results with the affinity of the βγ dimers for the $G_{i1}\alpha$ subunit measured in solution. The data in Fig. 2B show that the K_d (*x*-axis) of the βγ dimer for the $G_i\alpha$ subunit varies approximately 20-fold from 0.4 nM ($\beta_1\gamma_2$) to 7.7 nM ($\beta_1\gamma_{BAA}$). Note that the EC_{50} value for supporting coupling of the $G_{i1}\alpha$ subunit to the receptor (*y*-axis) varies 5- to 6-fold from 0.4 nM ($\beta_1\gamma_2$) to approximately 2.2 nM ($\beta_1\gamma_{BAA}$). All βγ dimers containing γ subunits modified with the farnesyl group (□) had lower affinities for the $G_{i1}\alpha$ subunit and lower EC_{50} values for supporting receptor coupling than did those containing the geranylgeranyl moiety. These results confirm and extend the concept that the C-terminal region of the γ subunit and its prenyl group are very important for the interaction of the dimer with α subunits and receptors in this assay. Moreover, the data show that there is a rough correlation between the EC_{50} value for receptor coupling and the affinity of the α and βγ subunits themselves.

Phospholipase C-β. Our previous experiments show that the activity of the $\beta_1\gamma_1$ and $\beta_1\gamma_{11}$ dimers on PLC-β is significantly less than that of the $\beta_1\gamma_2$ dimer, and the activity is affected by the type of prenyl group on the γ subunit (Myung et al., 1999). To determine how the N- and C-terminal regions of the γ subunit affected the interaction of the βγ dimer with PLC-β, dimers containing the 12 γ subunits shown in Fig. 1B were tested for their ability to stimulate avian PLC-β in vitro. The data in Fig. 3A show that $\beta_1\gamma_2$ markedly activates PLC-β with an EC_{50} value of approximately 5 nM (■) and that $\beta_1\gamma_1$ (○) is approximately 5-fold less potent (EC_{50} value of 25 nM; Table 2). The activity of the $\beta_1\gamma_1$ dimer can be made equal to the $\beta_1\gamma_2$ dimer by replacing the central and C-terminal amino acids of the γ_1 subunit with those from the γ_2 subunit (△, $\beta_1\gamma_{ABB}$). A chimeric γ subunit containing the first 50 amino acids of the γ_1 subunit and the C terminus of the γ_2 subunit ($\beta_1\gamma_{AAB}$) is also as equally effective as $\beta_1\gamma_{ABB}$ (Table 2). These results suggest that the C-terminal 23 amino acids of the γ_2 subunit are important for potent activation PLC-β in vitro, a result confirmed by the data in Fig. 3C, which shows that replacing the last 23 amino acids and the geranylgeranyl group of γ_2 with the amino acids and farnesyl group of γ_1 ($\beta_1\gamma_{BBA}$) yields a dimer with activity very similar to that of $\beta_1\gamma_1$. The amino acids in the central region do not seem to affect the interaction with PLC-β, because $\beta_1\gamma_{BAA}$ has an activity equal to that of $\beta_1\gamma_{BBA}$ and $\beta_1\gamma_1$ (Table 2).

Chimeric γ subunits with switched prenyl groups indicate that the differences in the activity of the dimers shown in Fig. 3, A and C, are due to both the differences in the C-terminal 23 residues and the composition of the prenyl group. Note that switching the prenyl group on the $\beta_1\gamma_2$ dimer from farnesyl to geranylgeranyl ($\beta_1\gamma_{BAA\text{-}S74L}$) provides a dimer with intermediate activity on PLC-β (◆, Fig. 3B). Exchanging the prenyl group on $\beta_1\gamma_1$ ($\beta_1\gamma_{1\text{-}S74L}$) produces a similar result (●). In fact, all dimers containing γ subunits with exchanged prenyl groups were intermediate in either their EC_{50} value or their maximal activity on PLC-β (Table 2). These results indicate that the βγ dimers containing the C-terminal sequence of the γ_2 subunit modified with a geranylgeranyl group are more potent and effective in activating PLC-β in vitro than those with the C-terminal region of the γ_1 subunit and/or modified with farnesyl group.

Type I Adenylyl Cyclase. The data in Fig. 4 present the ability of five representative βγ dimers to inhibit type I adenylyl cyclase. Figure 4A shows that the $\beta_1\gamma_2$ dimer inhibited type I adenylyl cyclase with an IC_{50} value of 15 nM (■) and was approximately 2-fold more potent and effective than the $\beta_1\gamma_1$ dimer (IC_{50} value of 36 nM; Table 2). However, a chimeric γ subunit with the C terminal 23 amino acids of the γ_1 subunit and its prenyl group exchanged with those of the γ_2 subunit (γ_{AAB}) produces a γ subunit with activity equal to that of the γ_2 subunit (▲, Fig. 4A). In contrast, replacing the C terminus of γ_2 and the geranylgeranyl group of γ_2 with the

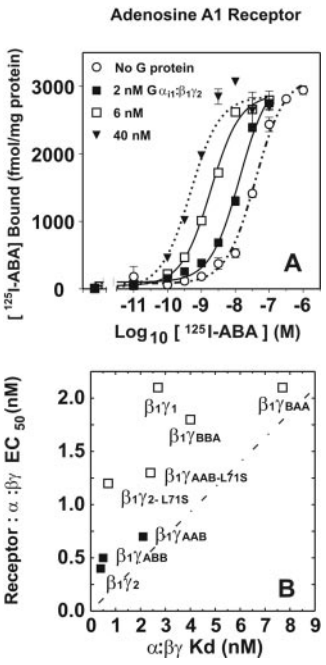

Fig. 2. Comparison of the ability of native $\beta\gamma$ dimers and those containing chimeric γ subunits to support the high-affinity agonist binding state of the A1 adenosine receptor. A, Sf9 cell membranes expressing recombinant, bovine A1 adenosine receptors were reconstituted with the $G_{i_1}\alpha$ subunit and the $\beta\gamma$ dimer at concentrations of 0, 2, 6, or 40 nM. The ratio of receptor $\alpha/\beta\gamma$ was approximately 1:25. The amount of agonist binding to the receptor was measured with graded concentrations of ^{125}I-amino-benzyladenosine as described under *Materials and Methods*. The figure indicates the recovery of high-affinity agonist binding conformation of the receptor as a function of the total amount of G protein heterotrimer reconstituted into the membrane. Each data point is an average of three similar experiments performed in triplicate. B, a plot of the affinity of the $G_{i_1}\alpha$ subunit for eight different $\beta\gamma$ dimers measured in the flow cytometer against the EC_{50} values for the formation of the high-affinity agonist binding state of the receptor measured at 6 nM $G_{i_1}\alpha$ and concentrations of $\beta\gamma$ ranging from 0 to 100 nM. Each data point is an average of three similar experiments performed in triplicate. Details of both assays are found under *Materials and Methods*. □, dimers with farnesyl moieties; ■, dimers with geranylgeranyl moieties.

C terminus of γ_1 and the farnesyl group (γ_{BBA}) produces a dimer with activity similar to that of $\beta_1\gamma_1$ (△, Fig. 4C). The data in Table 2 indicate that other dimers in which the C terminus of the γ_1 subunit is intact and modified with farnesyl were roughly equal to the native $\beta_1\gamma_1$ dimer in their ability to inhibit type I adenylyl cyclase (e.g., $\beta_1\gamma_{BAA}$ or $\beta_1\gamma_{BBA}$). These observations indicate that the C-terminal region of γ_2 is most critical for the inhibition of type I adenylyl cyclase in vitro. As was the case with PLC-β, both the C terminal amino acids and the prenyl group seem to be important for the interaction of the $\beta\gamma$ dimer with type I cyclase. Note that if the prenyl group on the $\beta_1\gamma_{AAB}$ dimer is changed to farnesyl ($\beta_1\gamma_{AAB\text{-}L71S}$), the activity was intermediate between that of β_1g_2 and $\beta_1\gamma_1$ (▲, Fig. 4B). Similar data were obtained with the six other dimers containing γ subunits with modified prenyl groups (Table 2). Overall, the data in Figs. 3 and 4 demonstrate that the C-terminal amino acid sequence of the γ_2 subunit and its native geranylgeranyl group are important determinants for the interaction of the $\beta\gamma$ subunit with effectors such as PLC-β and type I adenylyl cyclase.

Type II Adenylyl Cyclase. The $\beta_1\gamma_2$ subunit activates type II adenylyl cyclase more than 10-fold with a nanomolar EC_{50} value, and surprisingly, neither the $\beta_1\gamma_1$ nor the $\beta_1\gamma_{11}$ dimer activates type II adenylyl cyclase well (Myung et al., 1999). In addition, phosphorylation of the γ_{12} subunit in the $\beta_1\gamma_{12}$ dimer significantly inhibits its ability to stimulate type II adenylyl cyclase (Yasuda et al., 1998). The phosphorylation site has been determined to be at Ser[1] in the N terminus of the molecule (Asano et al., 1998). There are more negative charges in the N-terminal region of γ_1 or γ_{11} compared with the γ_2 subunit (Fig. 1A), potentially explaining the inability of dimers containing the γ_1 or γ_{11} subunits to activate type II adenylyl cyclase. Taken together, these results suggest that the introduction of negative charges in the N-terminal regions of the γ subunit inhibit the interaction of the dimer

TABLE 2

Comparison of the ability of $\beta\gamma$ dimers containing native or chimeric γ subunits to activate PLC-β or to inhibit type I adenylyl cyclase.

The EC_{50}, IC_{50}, or V_{max} values for PLC obtained with the indicated $\beta\gamma$ dimers were determined by fitting each data set to sigmoid curves as described under *Materials and Methods*. V_{inhib} is the level of cAMP production observed with 10^{-7} M of the indicated $\beta\gamma$ dimer. Control levels of ACI activity were 0.9 nmol cAMP/mg protein/min. Values are the average of three similar experiments expressed as means ± S.E.M.

Type of Chimeras and $\beta\gamma$ Dimers	Phospholipase C-β		Type I Adenylyl Cyclase	
	EC_{50}	V_{max}	IC_{50}	V_{inhib}
	nM	*μmol/mg PLC/min*	*nM*	*nmol/mg protein/min*
Chimeras with activity similar to $\beta_1\gamma_2$				
$\beta_1\gamma_2$	5.2 ± 1.1	3.40 ± 0.07	15.4 ± 1.4	0.26 ± 0.06
$\beta_1\gamma_{ABB}$	6.2 ± 1.2†	3.24 ± 0.13†	18.1 ± 2.2†	0.34 ± 0.24†
$\beta_1\gamma_{AAB}$	7.8 ± 1.1†	3.16 ± 0.09†	14.2 ± 1.3†	0.39 ± 0.03†
Chimeras with activity similar to $\beta_1\gamma_1$				
$\beta_1\gamma_1$	24.6 ± 1.2	2.14 ± 0.16	36.4 ± 2.6	0.58 ± 0.09
$\beta_1\gamma_{BBA}$	51.2 ± 1.3*	2.57 ± 0.29*	96.0 ± 15.1*	0.48 ± 0.28*
$\beta_1\gamma_{BAA}$	25.8 ± 1.3*	1.95 ± 0.15*	60.7 ± 6.6*	0.49 ± 0.25*
$\beta_1\gamma_{AAB\text{-}L71S}$	19.1 ± 1.3§	2.32 ± 0.13§	24.6 ± 1.5§	0.54 ± 0.03§
Chimeras with intermediate activity				
$\beta_1\gamma_{1\text{-}S74L}$	15.9 ± 1.2§	3.21 ± 0.20§	18.5 ± 1.7§	0.53 ± 0.04§
$\beta_1\gamma_{2\text{-}L71S}$	15.0 ± 1.2§	2.83 ± 0.14§	26.2 ± 2.6§	0.43 ± 0.15§
$\beta_1\gamma_{BBA\text{-}S74L}$	12.2 ± 1.5§	2.94 ± 0.22§§	16.2 ± 2.2§	0.51 ± 0.08
$\beta_1\gamma_{BAA\text{-}S74L}$	12.2 ± 1.2§	2.34 ± 0.22§§	22.9 ± 1.9§	0.37 ± 0.14
$\beta_1\gamma_{ABB\text{-}L71S}$	15.6 ± 1.3§	2.66 ± 0.17§	26.1 ± 2.2§	0.62 ± 0.11§

† Significant differences in responses to $\beta\gamma$ dimers in comparison with native $\beta_1\gamma_1$, $p < 0.0001$.
* Significant differences in responses to $\beta\gamma$ dimers in comparison with native $\beta_1\gamma_2$, $p < 0.0001$.
§ Significant differences in responses to $\beta\gamma$ dimers containing γ subunits with altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups, $p < 0.0001$.
§§ Significant differences in responses to $\beta\gamma$ dimers containing γ subunits with altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups, $p < 0.001$.

with the type II adenylyl cyclase. Thus, we examined the ability of βγ dimers containing N-terminal chimeric γ subunits to activate type II adenylyl cyclase.

The data in Fig. 5 present the ability of six representative βγ dimers to activate type II adenylyl cyclase performed using concentrations of dimers ranging from 0.1 to 100 nM. As expected (Yasuda et al., 1998), the $\beta_1\gamma_2$ dimer (■) activated type II adenylyl cyclase with an estimated EC_{50} value of 13 nM and was far more potent and effective in activating this effector enzyme than the $\beta_1\gamma_1$ dimer (Fig. 5A). Adding the N-terminal region of the γ_2 subunit to the γ_1 subunit and changing its prenyl group to geranylgeranyl ($\beta_1\gamma_{BAA\text{-}S74L}$) generated a molecule with activity equal to that of $\beta_1\gamma_2$ (◆, Fig. 5A). A similar result was observed with $\beta_1\gamma_{BBA\text{-}S74L}$ (Table 3). In contrast, a dimer with the poor activity of $\beta_1\gamma_1$ could be created using a chimeric γ subunit containing the

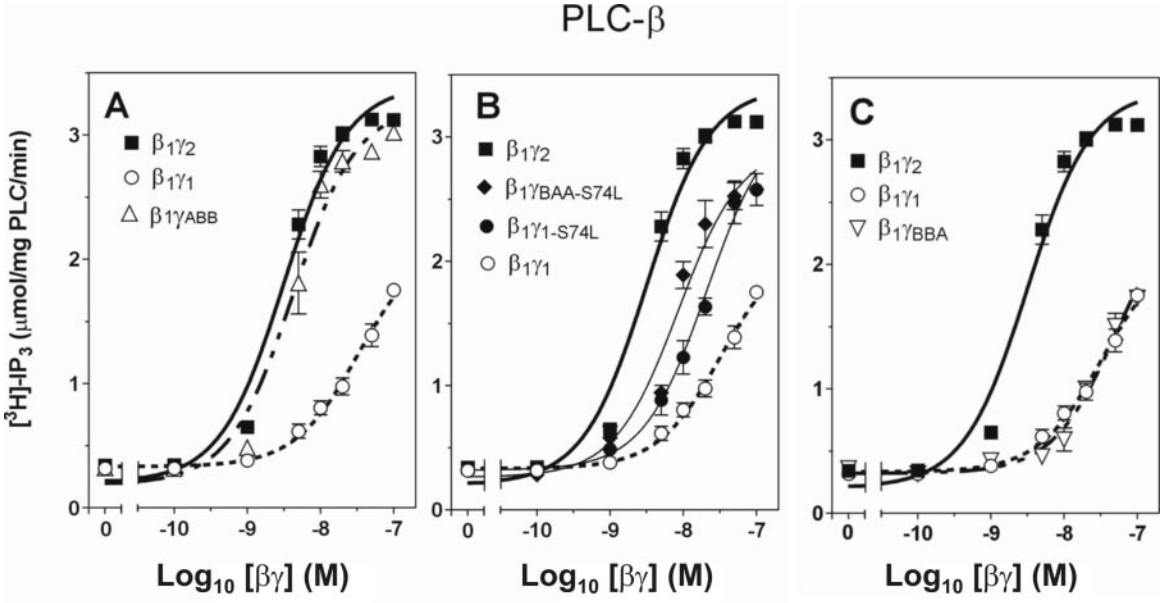

Fig. 3. Comparison of the ability of native βγ dimers and those containing chimeric γ subunits to stimulate PLC-β. A, the indicated concentrations of βγ dimers were reconstituted with recombinant turkey PLC-β in phospholipid vesicles containing [^{3}H]phosphatidylinositol bisphosphate, and PLC-β activity was measured as described under *Materials and Methods*. The activity of $\beta_1\gamma_{ABB}$ (△) was compared with $\beta_1\gamma_2$ (■, thick solid line) and $\beta_1\gamma_1$ (○, thick broken line). The effect of the $\beta_1\gamma_{ABB}$ dimer was statistically different from $\beta_1\gamma_1$ ($p < 0.0001$; Table 2) but not from $\beta_1\gamma_2$. B, an analogous experiment performed with $\beta_1\gamma_{1\text{-}S74L}$ and $\beta_1\gamma_{BAA\text{-}S74L}$ and compared with the effects of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. See Table 2 for the activity of dimers containing other chimeric γ subunits. C, an analogous experiment performed with $\beta_1\gamma_{BBA}$ and compared with the effect of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. The effect of the $\beta_1\gamma_{BAA}$ dimer was statistically different from $\beta_1\gamma_2$ but not from $\beta_1\gamma_1$ ($p < 0.0001$; Table 2). Each data point is an average of three independent experiments, each performed in duplicate.

TABLE 3

Comparison of the ability of βγ dimers containing native or chimeric γ subunits to activate type II adenylyl cyclase

The EC_{50} and V_{max} values obtained with each βγ dimer were determined by fitting each data set to sigmoid curves as described under *Materials and Methods*. Values are the average of three similar experiments expressed as means ± S.E.M.

Type of Chimeras and βγ Dimers	Type II Adenylyl Cyclase	
	EC_{50}	V_{max}
	nM	*μmol/mg protein/min*
Chimeras with activity similar to $\beta_1\gamma_2$		
$\beta_1\gamma_2$	13.3 ± 1.2	29.05 ± 1.72
$\beta_1\gamma_{BAA\text{-}S74L}$	15.6 ± 1.2§	27.8 ± 1.74§
$\beta_1\gamma_{BBA\text{-}S74L}$	18.2 ± 1.4§	24.07 ± 1.85§
Chimeras with activity similar to $\beta_1\gamma_1$		
$\beta_1\gamma_1$	46.7 ± 1.9	6.31 ± 0.65
$\beta_1\gamma_{ABB\text{-}L71S}$	48.7 ± 2.1§	6.89 ± 1.09§
$\beta_1\gamma_{AAB}$	61.8 ± 2.0*	9.13 ± 2.31*
$\beta_1\gamma_{AAB\text{-}L71S}$	93.3 ± 11.9§	25.91 ± 9.45§
$\beta_1\gamma_{1\text{-}S74L}$	33.1 ± 1.6§§	8.39 ± 1.68
Chimeras with intermediate activity		
$\beta_1\gamma_{BAA}$	24.6 ± 1.3†	13.52 ± 1.11†
$\beta_1\gamma_{BBA}$	35.7 ± 1.2††	14.05 ± 1.07†
$\beta_1\gamma_{2\text{-}L71S}$	41.7 ± 1.3§	16.19 ± 1.3§
$\beta_1\gamma_{ABB}$	25.2 ± 1.3*	23.17 ± 1.66††

§ Significant differences in responses to βγ dimers containing γ subunits with altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups, $p < 0.0001$.
* Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_2$, $p < 0.0001$.
§§ Significant differences in responses to βγ dimers containing γ subunits with altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups, $p < 0.001$.
† Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_1$, $p < 0.0001$.
†† Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_1$, $p < 0.001$.

N-terminal 23 amino acids of γ_1 and a farnesyl group ($\beta_1\gamma_{AAB\text{-}L71S}$; ●, Fig. 5C). These results suggest that the N-terminal region and the geranylgeranyl group are very important for the activation of type II adenylyl cyclase in vitro. Indeed, Fig. 5B shows that the $\beta_1\gamma_{BAA}$ dimer, which contained the N-terminal amino acids of γ_2 and the farnesyl modification, was intermediate in the activation of type II adenylyl cyclase. Indeed, the data in Table 3 show that every chimeric γ subunit, which is intermediate in activity on type II cyclase, is missing either the N-terminal amino acids of γ_2 and/or the geranylgeranyl group. These results indicate that the $\beta\gamma$ dimers containing the N-terminal sequence of the γ_2 subunit, which has one negatively charged amino acid, are more potent and effective in activating type II adenylyl cyclase than those with the N-terminal region of the γ_1 subunit, which has six negatively charged amino acids. A C terminus modified with the geranylgeranyl group is also important. Similar results are observed with the effector PtdIns 3-kinase in that dimers containing γ_1 or γ_{11} weakly activate the enzyme (Kerchner et al., 2004).

Discussion

There are three major findings of this in vitro study. First, $\beta\gamma$ dimers with a γ subunit containing the C-terminal 23 amino acid sequence of the γ_2 subunit and modified with a geranylgeranyl group have the highest affinity for the $G_{i1}\alpha$ subunit and are the most effective in coupling of the $G_{i1}\alpha$ subunit to bovine A1 adenosine receptors (Fig. 2). These data provide an important demonstration of the correlation between the affinity of the α subunit for the $\beta\gamma$ dimer and the ability of the complex to support high-affinity receptor-G protein coupling. Second, dimers with these attributes are most effective at activating PLC-β and inhibiting type I adenylyl cyclase in vitro (Figs. 3 and 4). Finally, $\beta\gamma$ dimers containing the N-terminal 19 residues of the γ_2 subunit are far more effective at activating type II adenylyl cyclase than are those containing the N-terminal 22 residues of the γ_1 subunit. The full activity on type II adenylyl cyclase also required modification of the γ subunit with a geranylgeranyl group, but experiments with dimers such as $\beta_1\gamma_{1\text{-}S74L}$ and $\beta_1\gamma_{11\text{-}S73L}$ (Table 2), which are modified with the geranylgeranyl lipid, indicate that the C-terminal prenyl group is of secondary importance to the N-terminal amino acid residues in the activation of this effector (Myung et al., 1999).

The finding that the C-terminal region of the γ subunit and its prenyl group is much more important than the N terminus for the interaction with α subunits and receptors confirms and extends a large amount of data obtained with rhodopsin in the visual system (Jian et al., 2001), the A1 adenosine receptor (Yasuda et al., 1996), the 5-hydroxytryptamine-1A receptor (Butkerait et al., 1995), the α_{2A} receptor (Richardson and Robishaw, 1999; Lim et al., 2001), and bombesin receptors (Jian et al., 1999). Experiments performed with a similar set of chimeric γ subunits made between γ_1 and γ_2 were tested for their ability to support coupling between rhodopsin and the $G_t\alpha$ subunit (Jian et al., 2001). These experiments also indicated that the C terminus of the γ_2 subunit and its geranylgeranyl group contribute to better coupling between receptor and the heterotrimer (Jian et al., 2001). Moreover, experiments using synthetic peptides to probe the rhodopsin:γ subunit interaction found that the C-terminal 12 amino acids of the γ subunit (DKNPFKELKGGC in γ_1 or SENPFREKKFFC in γ_2) are clearly involved in interacting with α subunits and the receptor. When the γ_1 peptide was altered to change Phe^{64} to threonine and Leu^{67} to serine, it markedly decreased the interac-

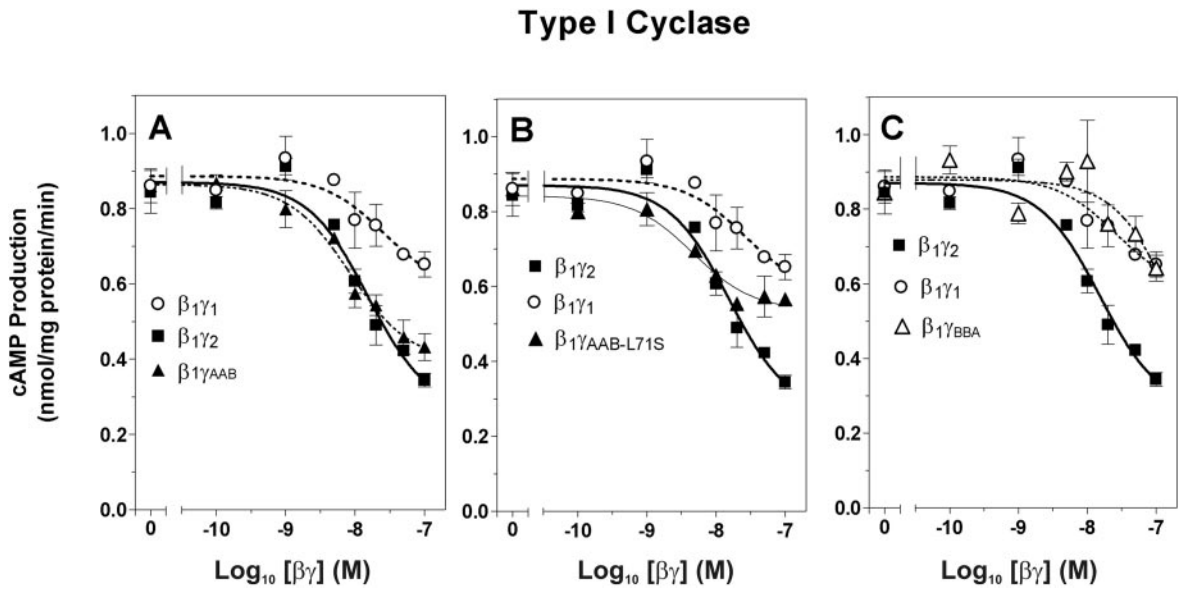

Fig. 4. Comparison of the ability of native $\beta\gamma$ dimers and those containing chimeric γ subunits to inhibit type I adenylyl cyclase. A, Sf9 cells were infected with a recombinant baculovirus encoding the type I adenylyl cyclase, membranes were prepared, and the cyclase reaction was performed with the indicated concentrations of $\beta\gamma$ dimers. cAMP was measured as described under *Materials and Methods*. The ability of $\beta_1\gamma_{AAB}$ (▲) to inhibit type I cyclase was compared with $\beta_1\gamma_2$ (■, thick solid line) and $\beta_1\gamma_1$ (○, thick broken line). The effect of $\beta_1\gamma_{AAB}$ was statistically significant different from $\beta_1\gamma_1$ but not from $\beta_1\gamma_2$ (Table 2). Each data point is an average of three independent experiments, each performed in duplicate. B, an analogous experiment performed with $\beta_1\gamma_{AAB\text{-}L71S}$ (▲) and compared with the effect of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. Each data point is an average of three independent experiments, each performed in duplicate. C, an analogous experiment performed with $\beta_1\gamma_{BBA}$ (open triangles) and compared with the effect of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. The effect of the $\beta_1\gamma_{BBA}$ dimer was statistically different from $\beta_1\gamma_2$ but not from $\beta_1\gamma_1$ ($p < 0.0001$; Table 2). Each data point is an average of three independent experiments, each performed in duplicate.

tion with rhodopsin (Azpiazu et al., 1999). Taken together, the results indicate that the C terminus of the mammalian γ subunit is very important for the interaction between G protein-coupled receptors and the heterotrimer. The receptor also seems to contact multiple domains in the α subunit and a region near the C terminus of the β subunit (Cabrera-Vera et al., 2003). Thus, each subunit in the heterotrimer is used for efficient activation of the α subunit.

Whereas data obtained with mammalian cells point to a role for the C terminus of the γ subunit in coupling βγ dimers to receptors, studies with the *Saccharomyces cerevisiae* pheromone mating pathway provide evidence that the C-terminal region of the yeast γ subunit is not important in receptor coupling. Using yeast strains in which the Ste18 locus (the only yeast γ subunit) was disrupted and pheromone signaling was inhibited, a variety of mutated γ subunits was tested for their ability to rescue signaling. Yeast γ subunits with a variety of C-terminal sequences, mutated by alanine scanning or by deletion of up to eight amino acids immediately preceding the CAAX box, were all able to rescue pheromone signaling (Chinault and Blumer, 2003). These data suggest that this region of the yeast molecule was not important for signaling through yeast G protein coupled receptors in vivo (Chinault and Blumer, 2003). More studies with mammalian cells are needed to reconcile these differences, although the diversity of the Gβ and Gγ subunits expressed in the mammalian genome will complicate these studies.

Whereas the C termini of γ subunits contain sequences capable of interacting with multiple receptors, subtle differences in the residues and charges must be important in determining specificity. For example, the $\beta_1\gamma_1$ dimer couples the G_{i1} α subunit to the α_2-adrenergic receptor poorly (Richardson and Robishaw, 1999), whereas the $\beta_1\gamma_{11}$ dimer couples well (Lim et al., 2001), yet there are only two differences in the C-terminal 12 amino acids of γ_1 and γ_{11} (Fig. 1A). Experiments performed with receptors reconstituted with G protein heterotrimers using various protocols show relatively modest differences in the interaction of the various γ subunits with receptors (Yasuda et al., 1996; Hou et al., 2001; Lim et al., 2001; Akgoz et al., 2002). However, antisense experiments performed with intact cells suggest an exquisite selectivity between receptors, α subunits, and certain βγ combinations (Kleuss et al., 1993). Thus, despite considerable effort, the factors dictating the specificity that must exist in intact cells remain elusive. These data, combined with the studies performed in yeast, suggest that additional work with mammalian cells is needed to reconcile these differences. The diversity of the Gβ and Gγ subunits in the mammalian genome will make these studies complex, but the observation that mice with the γ_7 subunit knocked out have an altered phenotype (Schwindinger et al., 2003) offers hope that these studies will provide interesting results.

The data in Fig. 3 indicate that, as with receptors, both the prenyl group and the C-terminal amino acids of the γ subunit contribute significantly to the interaction of the dimer with PLC-β in vesicles. This finding is consistent with experiments showing that native dimers containing the γ_1 or γ_{11} subunits have reduced activity on this effector (Myung et al., 1999). In addition, experiments performed with dimers containing the γ_5 subunit modified to contain a shortened or extended C terminus are less effective at activating PLC-β (Akgoz et al., 2002). A possible explanation for the role of the prenyl group in the activation of effectors may be provided by the hypothesis that the prenyl group on the γ subunit interacts with the C-terminal region of the β subunit and participates in a conformational change that, in part, determines

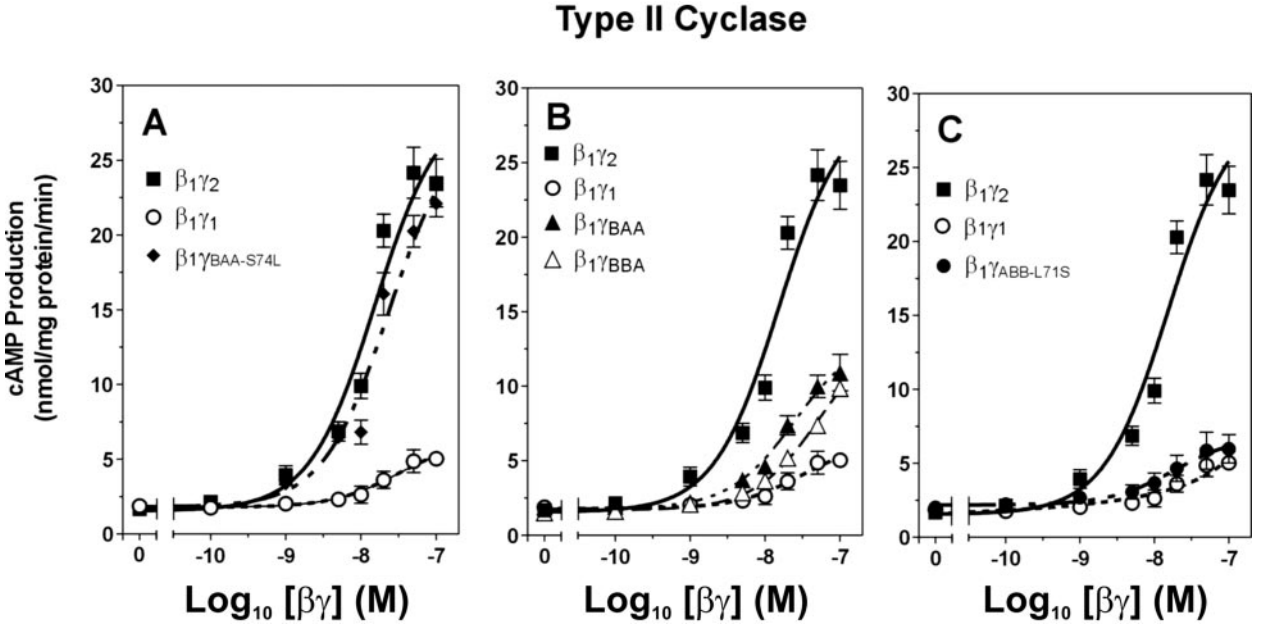

Fig. 5. Comparison of the ability of native βγ dimers and those containing chimeric γ subunits to stimulate type II adenylyl cyclase. A, Sf9 cells were infected with a recombinant baculovirus encoding the type II adenylyl cyclase, membranes were prepared, and the cyclase reaction was performed with the indicated concentrations of βγ dimers as described under *Materials and Methods*. The ability of $\beta_1\gamma_{BAA\text{-}S74L}$ (♦) to activate type II adenylyl cyclase was compared with $\beta_1\gamma_2$ (■, thick solid line) and $\beta_1\gamma_1$ (○, thick dotted line). The effect of $\beta_1\gamma_{BAA\text{-}S74L}$ was statistically significantly different from $\beta_1\gamma_1$ (Table 3). B, an analogous experiment performed with $\beta_1\gamma_{BAA}$ and $\beta_1\gamma_{BBA}$ and compared with the effects of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. Each data point is an average of three independent experiments, each performed in duplicate. C, an analogous experiment performed with $\beta_1\gamma_{ABB\text{-}L71S}$ (●) and compared with the effects of $\beta_1\gamma_1$ and $\beta_1\gamma_2$. The difference between the effect of $\beta_1\gamma_{ABB\text{-}L71S}$ and $\beta_1\gamma_2$ was statistically significant (Table 3). Each data point is an average of three independent experiments, each performed in duplicate.

the activity of the dimer at effectors (Myung and Garrison, 2000). On the other hand, the prenyl group may interact directly with PLC-β, as suggested by experiments with prenylated peptides (Akgoz et al., 2002). It is also possible that both mechanisms come into play. A more complete understanding of the role of this region of the β and γ subunits in these interactions awaits structural information or biophysical experiments determining the exact sites of contact between these regions of the dimer and receptors or effectors.

A surprising finding is that the C-terminal region of the γ subunit seems to predominate in the inhibition of type I adenylyl cyclase, whereas the N-terminal region of the molecule is the most important for the activation of type II adenylyl cyclase. However, the regulation of adenyl cyclase is complex, with multiple binding sites identified for the known regulatory molecules (Taussig et al., 1994). The differences identified for regulation by the γ subunit in the dimer are in keeping with these findings. The observations that the N-terminal domain of γ is important for the activation of type II cyclase are consistent with earlier experiments showing that phosphorylation of the γ_{12} subunit in the $\beta_1\gamma_{12}$ dimer with protein kinase C decreases its ability to stimulate type II adenylyl cyclase (Yasuda et al., 1998). The phosphorylation site in γ_{12} is Ser^1 at the N terminus (Asano et al., 1998), suggesting that negative charges in this region decrease the interaction of the dimer with type II cyclase. The γ_1, γ_8, and γ_{11} subunits contain six negatively charged amino acids in their N terminus (Fig. 1A); thus, dimers containing these γ subunits might be predicted to interact poorly with type II cyclase. Indeed, dimers containing γ_1, γ_{11}, or chimeras such as $\gamma_{ABB\text{-}L71S}$ or γ_{ABB} are not able to activate the enzyme well (Table 3) (Myung et al., 1999). Together, these results strongly suggest that negatively charged amino acids in the N terminus of the γ subunit lead to an inability to activate type II adenylyl cyclase in vitro. The observation that inhibition of the type I isoform of adenyl cyclase is quite dependent on the sequences in the opposite end of the γ subunit indicates that the different isoforms of adenyl cyclase must bind to different regions of the β and γ subunits in the $\beta\gamma$ dimer. In keeping with this finding, mutations on the top surface of the β subunit that dramatically inhibit the activation of type II cyclase do not alter the inhibition of type I cyclase (Li et al., 1998). Thus, the precise determinants of the interaction of the type I and type II cyclases with $\beta\gamma$ dimers seem to be different.

The isoform of the β subunit is also important in the interaction of $\beta\gamma$ dimers with effectors, and certain regions on the top surface of the β subunit have been identified as critical for interaction of the $\beta\gamma$ dimer with effectors (Ford et al., 1998). A major concept arising from these studies is that whereas different effectors may interact with distinct or overlapping regions of the β subunit, certain regions in the β subunit interacting with effectors are in the domain covered by the α subunit in the heterotrimer (Ford et al., 1998). Thus, formation of the basal state of the α:$\beta\gamma$-receptor complex is an efficient mechanism to terminate $\beta\gamma$ signaling. The data in Fig. 2B show that the C terminus of the γ subunit and its prenyl group are important determinants of the affinity of the α subunit for the $\beta\gamma$ dimer. Thus, this domain of the γ subunit may be important for the rapid formation of a high-affinity α:$\beta\gamma$ complex, which speeds reversal of the effects of the $\beta\gamma$ dimer on targets such as PLC-β or adenylyl cyclase.

The present study also demonstrates that the nature of the N and C terminal regions of the γ subunit imparts significant specificity to $\beta\gamma$ signaling. Thus, $\beta\gamma$ dimers containing γ subunits from subfamily I such as γ_1 or γ_{11} (and probably γ_8, although it has not been studied extensively) seem to have unique signaling properties in that they are unable to activate type II adenylyl cyclase or other important effectors, such as phosphatidylinositol-(4,5)-3-kinase (Kerchner et al., 2004). The distribution of γ_1 is restricted to retinal rods (Fung, 1983), and the original report of γ_8 (termed γ_c) suggested that it was restricted to cone cells (Ong et al., 1995). However, the γ_{11} subunit is widely expressed (Morishita et al., 1998), and it now seems that γ_8 is also widely expressed (Downes and Gautam, 1999). Thus, it is important to define the signaling properties of dimers containing these farnesylated γ subunits in multiple cellular systems. The appealing concept that certain $\beta\gamma$ dimers may associate selectively with distinct α subunits and/or receptors has been used to account for the diversity of these subunits in the G protein family (Hildebrandt, 1997; Richardson and Robishaw, 1999; McIntire et al., 2001; Cabrera-Vera et al., 2003). This hypothesis also predicts that unique $\beta\gamma$ dimers will be released upon receptor activation in intact cells, allowing a given receptor to release a $\beta\gamma$ dimer with selective signaling properties. If the $\beta\gamma$ dimers released were from subfamily I, they might have very selective signaling properties at effectors. The data in this report support this idea; however, future experiments will need to concentrate on the functions of this diverse family of proteins in intact cellular systems.

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