Influence of Differential Stability of G Protein $\beta\gamma$ Dimers Containing the γ_{11} Subunit on Functional Activity at the M_1 Muscarinic Receptor, A_1 Adenosine Receptor, and Phospholipase C-β[†]

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Received March 11, 2006; Revised Manuscript Received July 21, 2006

ABSTRACT: $G\gamma_{11}$ is an unusual guanine nucleotide-binding regulatory protein (G protein) subunit. To study the effect of different Gβ-binding partners on γ_{11} function, four recombinant $\beta\gamma$ dimers, $\beta_1\gamma_2$, $\beta_4\gamma_2$, $\beta_1\gamma_{11}$, and $\beta_4\gamma_{11}$, were characterized in a receptor reconstitution assay with the G_q-linked M_1 muscarinic and the G_{i1}-linked A_1 adenosine receptors. The $\beta_4\gamma_{11}$ dimer was up to 30-fold less efficient than $\beta_4\gamma_2$ at promoting agonist-dependent binding of [^{35}S]GTPγS to either α_q or α_{i1}. Using a competition assay to measure relative affinities of purified $\beta\gamma$ dimers for α, the $\beta_4\gamma_{11}$ dimer had a 15-fold lower affinity for G_{i1} α than $\beta_4\gamma_2$. Chromatographic characterization of the $\beta_4\gamma_{11}$ dimer revealed that the $\beta\gamma$ is stable in a heterotrimeric complex with G_{i1} α; however, upon activation of α with $MgCl_2$ and GTPγS under nondenaturing conditions, the β_4 and γ_{11} subunits dissociate. Activation of purified G_{i1} α:$\beta_4\gamma_{11}$ with Mg^{+2}/GTPγS following reconstitution into lipid vesicles and incubation with phospholipase C (PLC)-β resulted in stimulation of PLC-β activity; however, when this activation preceded reconstitution into vesicles, PLC-β activity was markedly diminished. In a membrane coupling assay designed to measure the ability of G protein to promote a high-affinity agonist-binding conformation of the A_1 adenosine receptor, $\beta_4\gamma_{11}$ was as effective as $\beta_4\gamma_2$ when coexpressed with G_{i1} α and receptor. However, G_{i1} α:$\beta_4\gamma_{11}$-induced high-affinity binding was up to 20-fold more sensitive to GTPγS than G_{i1} α:$\beta_4\gamma_2$-induced high-affinity binding. These results suggest that the stability of the $\beta_4\gamma_{11}$ dimer can modulate G protein activity at the receptor and effector.

Guanine nucleotide-binding regulatory proteins (G proteins),[1] comprised of an α, β, and γ subunit, represent a principle mechanism utilized by cells to process extracellular stimuli via cell-surface receptors (*1*, *2*). Alternative splicing of 17 genes, followed by post-translational modification, results in at least 23 G protein α subunits, which have been shown to play key roles in determining specific signaling pathways from the receptor to effector (*2*). The 7 β and 12 γ isoforms (*3*) result in a formidable number of potential $\beta\gamma$ combinations (*4*, *5*). In addition to interactions with the receptor and effector, the functional effects of the $\beta\gamma$ dimer may be influenced by specific interactions between β and γ (*6*); for example, the combination of β_2 and γ_1 is not known to form a dimer (*7*). Thus, behavior of individual β and γ isoforms may be derived from their binding partner. Functionality of the γ isoforms is further distinguished by different isoprenoid lipid moieties at the C terminus; γ_1, γ_8, and γ_{11} are covalently modified with the 15 carbon farnesyl group (*8*, *9*), while γ_2, γ_3, γ_4, γ_5, γ_7, γ_9, γ_{10}, γ_{12}, and γ_{13} are modified with the 20 carbon geranylgeranyl group (*10*). The nature of this lipid modification has been shown to influence interactions with the lipid membrane (*11*, *12*) as well as activity at receptors (*13*−*15*) and effectors (*16*, *17*).

For example, in one study comparing coupling properties of $\beta\gamma$ dimers on four G_i-linked receptors, $\beta_1\gamma_{11}$ was more efficient than $\beta_1\gamma_2$ at stabilizing the high-affinity state of both the A_1 adenosine and 5-HT_{1A} receptors (*15*). This result suggests that the identity of the isoprenoid lipid at the C terminus of the γ subunit not only determines the hydrophobicity of the $\beta\gamma$ dimer but also influences the specificity of receptor:G protein interactions. However, experiments at effectors have concluded that $\beta_1\gamma_{11}$ was much less effective than $\beta_1\gamma_2$ at activating phospholipase C (PLC)-β, adenylyl cyclase II (*16*), or PtdIns 3-kinase p110γ (*18*). Furthermore, a study characterizing T-type calcium-channel regulation reported that the $\beta_2\gamma_2$, $\beta_2\gamma_{10}$, $\beta_2\gamma_{12}$, and $\beta_2\gamma_{13}$ dimers were able to inhibit calcium currents but the $\beta_2\gamma_{11}$ dimer was not (*19*).

[†] This work was supported by the National Institutes of Health Grant RO1-DK-19952.

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[1] Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; Sf9 cells, *Spodoptera frugiperda* cells; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; [^{3}H]-CPX, tritiated 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5′-*N*-ethylcarboximide-adenosine; [^{125}I]-ABA, N^6-(4-amino-3-^{125}iodo-benzyl)adenosine; AMP-PNP, 5′-adenylylimidodiphosphate; R-PIA, R-phenylisopropyladenosine; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethane-sulfonic acid; BSA, bovine serum albumin; Genapol C-100, polyoxyethylene (10) dodecyl ether; SDS, sodium dodecyl sulfate; RGS, regulators of G protein signaling.

10.1021/bi0604882 CCC: $33.50
Published on Web 08/29/2006

A study examining the role of β-subunit diversity in A_{2a} adenosine receptor coupling characterized each of the five β subunits purified with the γ_2 subunit. Strikingly, the $\beta_1\gamma_2$ dimer was much less efficacious than $\beta_2\gamma_2$, $\beta_3\gamma_2$, and $\beta_4\gamma_2$ at stimulating agonist-dependent GTPγS binding to α_s reconstituted with membranes expressing the A_{2a} adenosine receptor (*20*). This result was corroborated by the demonstration that $\beta_4\gamma_2$ was much more effective than $\beta_1\gamma_2$ at stabilizing the high-affinity state of the A_{2a} adenosine receptor in intact membranes (*21*). At the physiological level, the β_4 subunit may be involved in the A_{2a} adenosine receptor-mediated regulation of inflammation, because several cytokines have been shown to selectively upregulate both the A_{2a} adenosine receptor and the β_4 subunit in human dermal microvascular endothelial cells (*22*).

Both β_4 and γ_{11} subunits are widely distributed, and although expression of β_4 in the brain (*23*) is not matched by γ_{11}, these subunits are expressed together in the heart and kidney and are especially abundant in the lung (*15*, *24*, *25*). High receptor coupling efficiency of $\beta_1\gamma_{11}$ has been reported at two G_i-linked receptors (*15*), and we have observed similar high coupling efficiency with $\beta_1\gamma_{11}$ in a reconstitution assay with G_s α and the β-adrenergic receptor (data not shown). Further, our data characterizing the high coupling efficiency of $\beta_4\gamma_2$ (*20*) was mirrored by a study using the $G_{i/o}$-linked M_2 muscarinic receptor (*26*). An intriguing possibility was that dimers composed of both β_4 and γ_{11} would have synergistically high potency at coupling to the receptor.

Thus, the $\beta_4\gamma_{11}$ dimer was characterized both functionally and biochemically using the following techniques: (1) a panel of four purified $\beta\gamma$ dimers, $\beta_1\gamma_2$, $\beta_4\gamma_2$, $\beta_1\gamma_{11}$, and $\beta_4\gamma_{11}$, was evaluated for the ability to stimulate agonist-dependent GTPγS binding to G_q α or G_{i1} α following reconstitution with the M_1 muscarinic or A_1 adenosine receptors, respectively; (2) a competition assay was used to compare the affinities of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ for G_{i1} α; (3) the stability of the G_{i1} α:$\beta_4\gamma_{11}$ heterotrimer was compared to other heterotrimers under activating and nonactivating conditions using size-exclusion chromatography; (4) the ability of G_q α, $\beta_1\gamma_2$, $\beta_4\gamma_2$, $\beta_1\gamma_{11}$, and $\beta_4\gamma_{11}$ and heterotrimeric versions of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ to stimulate PLC-β was evaluated; (5) receptor:G protein interaction in an *Spodoptera frugiperda* (Sf9) cell membrane was measured by coexpressing the G_{i1} α:$\beta_4\gamma_2$ and G_{i1} α:$\beta_4\gamma_{11}$ heterotrimers with the A_1 adenosine receptor and comparing the ability of each heterotrimer to promote the high-affinity agonist-binding state of the A_1 adenosine receptor; and finally, (6) the capacity of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ to coprecipitate with G_{i1} α and tagged A_1 adenosine receptor from Sf9 cell digitonin extracts was examined.

Surprisingly, results presented here demonstrate that interactions between γ_{11} and different β isoforms can have profound effects on the efficiency of a $\beta\gamma$ dimer to couple to the receptor and effector. These differences appear to be mediated by the stability of the $\beta\gamma$ complex and, like the binding of $\beta\gamma$ to phosducin (*27*), may represent a mechanism for the attenuation of $\beta\gamma$ signaling.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses. Construction of baculoviruses encoding the β_1, β_4, γ_2, γ_{11}, G_q α, and G_{i1} α subunits and the $_{6HISFLAG}$-A_1 adenosine receptor has been described (*28*−*32*). Baculoviruses encoding the M_1 muscarinic receptor and the $_{\mathbf{6HIS}}$-G_{i1} α subunit were kind gifts from E. Ross (University of Texas, Southwestern Medical Center) and T. Kozasa (University of Illinois) via R. Neubig (University of Michigan), respectively.

Expression and Purification of Recombinant $\beta\gamma$ Dimers, Heterotrimeric G Proteins, and α Subunits in Sf9 Cells. To characterize their biochemical properties, specific $\beta\gamma$ combinations were purified both as dimers and as a heterotrimer with G_{i1} α. Recombinant baculoviruses encoding the desired combination of $_{\mathbf{6HIS}}$-G_{i1} α subunit and $\beta\gamma$ dimer were used to infect Sf9 cells at a multiplicity of infection (MOI) of 3, which were harvested 48−60 h after infection (*20*). The $\beta_1\gamma_2$, $\beta_4\gamma_2$, $\beta_1\gamma_{11}$, and $\beta_4\gamma_{11}$ dimers were purified using a modification of the G_{i1} α-affinity chromatography procedure described (*33*). Briefly, membranes were prepared from the Sf9 cell pellet, and heterotrimers containing the $_{\mathbf{6HIS}}$-G_{i1} α subunit were extracted with 0.1% Genapol. Extracts were then passed over a Ni^{2+} column and washed with Ni^{2+} base buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 5 and μM GDP) containing 17 μg/mL phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL pepstatin, leupeptin, and aprotinin, 5 mM imidazole, 0.1% Genapol, and 300 mM NaCl (total). A subsequent wash with Ni^{2+} base buffer containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was performed before elution of the protein with Ni^{2+} base buffer containing 50 mM $MgCl_2$, 10 mM NaF, 30 μM $AlCl_3$, and 1% cholate. The protein was concentrated using a Centricon 30 concentrator and exchanged twice to remove cholate, NaF, and $AlCl_3$ using Ni^{2+} base buffer containing 0.1% CHAPS, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). The activation-dependent purification of $\beta\gamma$ dimers is specific for properly folded, biologically viable G protein, resulting in a highly pure preparation of $\beta\gamma$ (*20*). The G_{i1} α:$\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ heterotrimer, for use in the α-affinity assay, was purified using a modification of this method. Instead of subunit elution by AlF_4^-, Ni^{2+} base buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 0.1% CHAPS, and 5 μM GDP) containing 200 mM imidazole was used to elute the heterotrimeric G protein. Imidazole was then reduced by repeated concentration and dilution with Ni^{2+} base buffer containing 1 mM EDTA and 1 mM DTT. The $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_1\gamma_2$, $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_4\gamma_2$, $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_1\gamma_{11}$, $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_4\gamma_{11}$, $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_2\gamma_{11}$, and $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_3\gamma_{11}$ heterotrimers, which were characterized by gel-filtration chromatography, were also purified using this method. All protein was aliquoted and stored at −80 °C; protein concentration was determined by densitometric analysis of silver-stained gels using ovalbumin as a standard curve. The $\beta\gamma$ dimer preparations were highly pure (see Figure 1A); heterotrimers were also highly pure (Figure 4A illustrates the α and β subunits of the representative $_{\mathbf{6HIS}}$-G_{i1} α:$\beta_1\gamma_2$ heterotrimer). Verification of proper post-translational processing of the γ subunit was accomplished by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (*8*). Mass spectra of the purified $\beta\gamma$ isoforms containing either γ_2 or γ_{11} demonstrated that the major mass in each spectrum was compatible with the predicted masses for the known γ sequences containing geranylgeranyl or farnesyl modifications, respectively (data not shown but see refs *8* and *20*). The methods for purification of G_q α and G_{i1} α have been described (*20*, *34*).

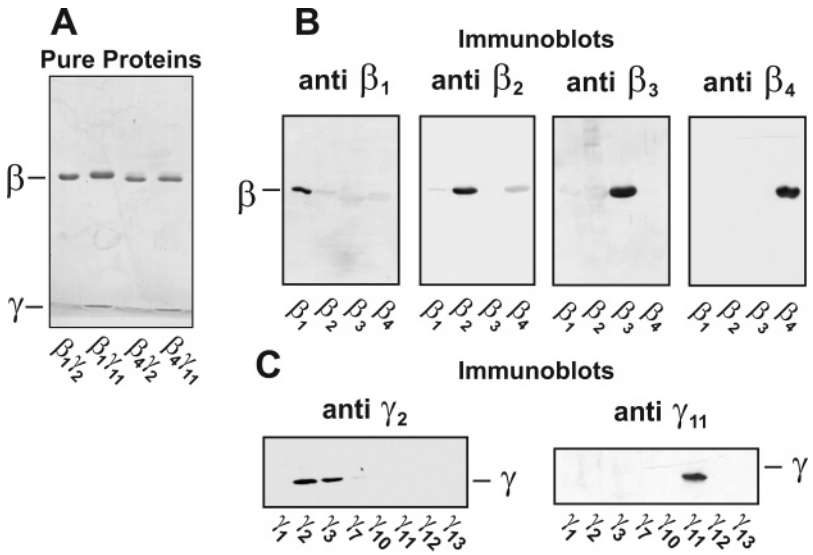

FIGURE 1: Purity of $\beta\gamma$ dimers and specificity of antibodies. (A) Polyacrylamide gel illustrating the purity of four $\beta\gamma$ dimer combinations. After coexpression with the $_{6HIS}$-G_{i1} α subunit in Sf9 cells, the heterotrimer was adsorbed to a Ni^{2+}−NTA column and washed and the $\beta\gamma$ dimers were selectively eluted with AlF_4^-. (B) Purified recombinant $\beta\gamma$ dimers (300 ng of $\beta_1\gamma_2$, $\beta_2\gamma_2$, and $\beta_4\gamma_2$; 900 ng of $\beta_3\gamma_2$) were separated on 12% polyacrylamide gels, transferred to nitrocellulose, and incubated with β-specific antibodies. At antibody dilutions of 1:1000, only minor cross-reactivity was observed with the β_2-specific antibody. (C) Purified recombinant $\beta_1\gamma_x$ dimers (100 ng) containing indicated γ isoforms were separated on 12% polyacrylamide gels and blotted with γ-specific antibodies; the γ_2 antibody cross-reacted only with γ_3, which has over 80% sequence similaritiy. The γ_{11} antibody did not react with any other isoform at the concentrations tested.

Preparation of Membranes Containing Recombinant M1 Muscarinic Receptors, A1 Adenosine Receptors, or A1 Adenosine Receptor:G Protein Combinations. Sf9 cell membranes expressing either the M_1 muscarinic receptor or the A_1 adenosine receptor from recombinant baculoviruses were stripped with urea as described (*34*, *35*), to remove or denature endogenous α and $\beta\gamma$ subunits (*36*). Membranes prepared from Sf9 cells expressing different combinations of G protein subunit isoforms and receptor were not stripped with urea, to preserve the receptor:G protein interactions. Harvested cells were resuspended in buffer [20 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethane-sulfonic acid (HEPES) at pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 μM GDP, 17 μg/mL PMSF, and 2 μg/mL leupeptin, pepstatin, and aprotinin] and lysed by nitrogen cavitation; the resulting lysate was centrifuged at 750*g* to pellet unbroken cells and nuclei. Membranes were then prepared from the supernatant by centrifugation at 28000*g* for 50 min at 4 °C. The membrane pellet was homogenized with buffer containing 20 mM HEPES at pH 8.0, 100 mM NaCl, 1% glycerol, 10 μM GDP, 17 μg/mL PMSF, and 2 μg/mL leupeptin, pepstatin, and aprotinin using a Potter−Elvehjem homogenizer, followed by several passages through a 28-guage needle. The volume was adjusted to give a final concentration of approximately 10 mg/mL, and aliquots were snap-frozen in liquid nitrogen and stored at −80 °C. The BCA assay using bovine serum albumin (BSA) as a standard was used to determine the total membrane protein concentration.

Preparation of Antibodies. The polyclonal antibody to the γ_{11} isoform (Sigma Genosys) was raised by immunization of rabbits with keyhole limpet hemocyanin (KLH) coupled to the γ_{11}-specific peptide PALHIEDLPEKEKC; specificity of this antibody is demonstrated in Figure 1C. The γ_2-specific antibody was purchased from CalBiochem, the β_1-, β_2-, β_3-, and β_4-specific antibodies were purchased from Santa Cruz. The M2 FLAG-specific antibody was purchased from Sigma. The $G_{i1/2}$ α antibody 0116 was a kind gift from Dr. D. Manning (*37*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. Proteins requiring concentration were precipitated with acetone (*38*). Subsequently, proteins were separated using either 12 or 15% polyacrylamide gels and visualized by staining with silver. The detection of receptors, γ subunits, or G proteins in membranes or precipitations utilized Western blotting. Briefly, proteins were transferred to nitrocellulose at 4 °C for 30 min at 20 V; γ blots were also subsequently baked at 70 °C for 1 h in a vacuum oven to retain γ protein on the blot (*39*). Antibody binding was detected by enhanced chemiluminescence.

Measurement of Agonist-Stimulated GTPγS Binding to G_q α or G_{i1} α after Reconstitution with βγ into Membranes Containing Either the M_1 Muscarinic Receptor or the A_1 Adenosine Receptor. Reconstitution experiments with membranes and G protein subunits were performed as described (*35*) with the following modifications. Aliquots of urea-treated Sf9 cell membranes containing the M_1 muscarinic receptor were pelleted by centrifugation and resuspended in 375 μL of GTPγS-binding buffer [25 mM HEPES at pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1% BSA, 50 nM GDP, and 1 μM 5′-adenylylimidodiphosphate (AMP-PNP)] with a 28-guage needle. The membrane suspension was reconstituted with 15 nM G_q α, and 30 μL aliquots were distributed into reaction tubes so that each tube contained 26 μg of total membrane protein from the M_1 muscarinic receptor preparation. The concentrations of $\beta\gamma$ were prepared by serial dilution with GTPγS-binding buffer containing 0.05% Genapol; a total of 2 μL of each $\beta\gamma$ concentration was added to the reaction tubes. After a 30 min incubation on ice and a 10 min preincubation at 25 °C, 8 μL of GTPγS-binding buffer containing [^{35}S]-GTPγS (approximately 1 000 000 dpm, 5 nM final) and carbachol (1 mM final) was added to each tube to start the 7 min

reaction. The reaction was terminated by vacuum filtration, and bound [^{35}S]-GTPγS was determined by scintillation counting; EC_{50} values that reflect the receptor coupling ability of each $\beta\gamma$ dimer were determined by plotting [^{35}S]-GTPγS binding as a function of $\beta\gamma$ and fitting the data to a sigmoidal binding isotherm generated by GraphPad Prism.

Considering differences in agonist and α-subunit specificity, a slight modification of this protocol was used to obtain dose−response experiments with $\beta\gamma$ and the A_1 adenosine receptor. Each reaction tube contained 42 μg of total membrane protein from the A_1 adenosine receptor preparation and approximately 10 nM purified G_{i1} α. In addition, to break down endogenous adenosine that is continuously generated in membrane preparations, adenosine deaminase was added to the membrane suspension before the 30 min incubation at a concentration of 14 units of activity/mL. Also, the GDP concentration in the GTPγS-binding buffer was raised to 500 nM to reduce nonspecific binding, and the A_1 adenosine receptor was activated with 100 nM R-phenylisopropyladenosine (R-PIA). Increases in $\beta\gamma$-dependent GTPγS binding were typically 2-fold for both receptor systems.

Measurement of Affinity of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ for G_{i1} α. An α:$\beta\gamma$-affinity assay was developed on the basis of the principle of competitive binding of $\beta\gamma$ for the α subunit. Our methodology is a variation of the flow cytometry study by Sarvazyan et al. (*40*); however, one major difference is that our protocol does not require labeling the α subunit, making the assay more amenable to use with other α subunits, such as G_s α and G_q α. In this assay, increasing concentrations of purified $\beta\gamma$ dimer are incubated with G_{i1} α:$\beta_1\gamma_{2\mathbf{FLAG6HIS}}$; affinity of the added $\beta\gamma$ for G_{i1} α is determined by how effectively it competes with $\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ for binding G_{i1} α. Approximately 800 ng of G_{i1} α:$\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ in a 26 μL volume was aliquoted into eppendorf tubes in Ni^{2+} competition buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 0.1% Genapol, and 10 μM GDP). Serial dilutions of purified γ dimers were made with this buffer, and a 4 μL volume of each $\beta_4\gamma_x$ concentration was added to the eppendorf tubes for a final volume of 30 μL. This reaction mixture was allowed to incubate at 30 °C for 1 h, during which time the added $\beta\gamma$ dimer could exchange with $\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ by binding to G_{i1} α. Concurrent with the incubation, magnetic Ni^{2+} beads (Qiagen) were prepared by aliquoting 30 μL of a bead slurry into 1.5 mL eppendorf tubes. Magnetic beads allowed almost complete immobilization of the pellet during aspiration of the supernatant, which permitted smaller reaction volumes and higher precision in the assay than nonmagnetic Ni^{2+} beads. Beads were washed once with 500 μL of Ni^{2+} competition buffer, and the supernatant was discarded after centrifugation; subsequently, 470 μL of Ni^{2+} competition buffer was used to resuspend the magnetic beads. After the 1 h incubation, the reaction mixture was added to the magnetic bead resuspension and gently mixed several times for 5 min at room temperature. After centrifugation, the magnetic beads were immobilized using a Qiagen magnet and the supernatant was removed. A total of 10 μL of SDS sample buffer was added to each of the pellets. Samples were boiled for 10 min, and proteins were separated using a 12% polyacrylamide−SDS gel. Silver-stained bands of the G_{i1} α subunit and the β subunit were quantified by scanning densitometry. In the experimental design, if the purified $\beta_4\gamma_x$ incubated with G_{i1} α:$\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ has a high affinity for α, more of the α subunit would form a heterotrimeric complex with the added $\beta_4\gamma_x$, which would reside in the supernatant after centrifugation of the reaction volume. Thus, the loss of an α subunit in the pellet as a function of the $\beta\gamma$ concentration in the assay provides a curve that defines an IC_{50} value that reflects α:$\beta\gamma$ affinity. Data are expressed as the densitometric ratio of α/β staining to control for differences in G_{i1} α:$\beta_1\gamma_{2\mathbf{FLAG6HIS}}$ binding to beads and protein losses during aspiration of the supernatant.

Characterization of G Protein Heterotrimers by Gel-Filtration Chromatography. Chromatography was performed on a Waters 650E Advanced Protein Purification System (Millipore) using a Superose 6 HR 10/30 column (Pharmacia) equilibrated with Superose 6 base buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% CHAPS, and 1 mM DTT) at a flow rate of 0.5 mL/min; all experiments were done at this flow rate and at 4 °C unless otherwise noted. Heterotrimeric G proteins were characterized under nonactivating conditions by comparing retention times of the subunits after injection onto the Superose 6 column; Superose 6 base buffer used for this chromatography was supplemented with 10 μM GDP. G proteins were activated by incubation in Superose 6 base buffer containing 5 mM $MgCl_2$ and 100 μM GTPγS for 45 min at 30 °C, followed by injection onto the Superose 6 column. Superose 6 base buffer containing 5 mM $MgCl_2$ was used to both equilibrate the column and resolve the G protein subunits from the activated G proteins. For both nonactivating and activating conditions, fractions were collected at 1 min intervals, analyzed by SDS−PAGE, and protein-visualized by staining with silver. Identity of the G protein γ subunit was verified by Western blotting with isoform-specific antibodies. Proteins of a known molecular weight, albumin (67 kDa), and ribonuclease A (13.7 kDa), were also characterized chromatographically and used as molecular-weight standards on the Superose 6 column.

Reconstitution of Purified G_q α, $\beta_1\gamma_2$, $\beta_1\gamma_{11}$, $\beta_4\gamma_2$, $\beta_4\gamma_{11}$, G_{i1}α $\beta_4\gamma_2$, and G_{i1}α $\beta_4\gamma_{11}$ with PLC-β. Recombinant turkey PLC-β was purified from baculovirus-infected Sf9 cells as described previously (*41*). Phospholipids at a molar ratio of 4:1 phosphatidylethanolamine (PE)/PIP_2 were used to create large multilamellar vesicles in a buffer containing 50 mM HEPES at pH 8.0, 80 mM KCl, 3 mM ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1 mM DTT as described (*18*). G protein $\beta\gamma$ dimers were reconstituted on ice for 30 min with [^{3}H]PIP_2-labeled phospholipid large unilamellar vesicles (LUVs) (final concentration of 100 μM PE/25 μM PIP_2 and 800 cpm/μL of [^{3}H]PIP_2) in a buffer consisting of 20 mM HEPES at pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl, and 0.04% CHAPS. A total of 5 ng of purified PLC-β in buffer containing 50 mM HEPES at pH 8.0, 67 mM KCl, 17 mM NaCl, 0.83 mM $MgCl_2$, 3 mM EGTA, 0.17 mM EDTA, 1 mM DTT, and 1 mg/mL BSA was then added, and the mixture was allowed to incubate for 10 min at 4 °C.

Two different protocols were used to activate G_q α or heterotrimers. In the *pre-reconstitution* protocol, G_q α, G_{i1}α $\beta_4\gamma_2$, and G_{i1}α $\beta_4\gamma_{11}$ were activated by incubation for 30 min at 30 °C in buffer consisting of 50 mM HEPES at pH 8.0, 1 mM EGTA, 3 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 1% Na-cholate, 1 mM DTT, and 1 mM GTPγS immediately prior to reconstitution with phospholipids

vesicles; in addition, 10 mM NaF and 20 μM $AlCl_3$ were included in the incubation with PLC-β. In the *post-reconstitution* protocol, $G_{i1}\alpha$ $\beta_4\gamma_2$ and $G_{i1}\alpha$ $\beta_4\gamma_{11}$ were reconstituted with phospholipid vesicles before activation using the same method for $\beta\gamma$ dimers; however, during the final 10 min of this incubation, 1 mM DTT and 1 mM GTPγS were included in the reaction mix. Similar to the *pre-reconstitution* protocol, the subsequent incubation with PLC-β also included 10 mM NaF and 20 μM $AlCl_3$. The PLC reaction was started by the addition of $CaCl_2$ (3 μM free Ca^{2+} final, 50 μL assay volume) and incubated for 15 min at 30 °C to release [^{3}H] IP_3. The reaction was stopped by the addition of 10% trichloroacetic acid (TCA), and the precipitated protein and intact [inositol-2-^{3}H]PIP_2 were removed by adding 10 mg/mL BSA to the samples and centrifugation at 7000 rpm for 5 min. The [^{3}H] IP_3 released into the aqueous solution was quantified by liquid scintillation counting.

Measurement of Antagonist and High-Affinity Agonist Binding to the A_1 Adenosine Receptor Coexpressed with G_{i1} α, β_4, and either γ_2 or γ_{11} in Sf9 Membranes. Membranes containing the A_1 adenosine receptor and specific G protein combinations were prepared by infection of Sf9 cells with baculoviruses for receptor, G_{i1} α, β_4, and either γ_2 or γ_{11}. The A_1 adenosine receptor agonist ABA was iodinated as described (*42*), and radioligand-binding experiments were performed as described (*21*). Briefly, the assays were performed in triplicate with 5 μg of membrane protein in a total volume of 0.1 mL of buffer (20 mM HEPES and 1 mM EDTA) with 1 unit/mL adenosine deaminase and 5 mM $MgCl_2$. Membranes were incubated with radioligands at room temperature for 3 h [for the agonist N^6-(4-amino-3-^{125}iodobenzyl)adenosine ([^{125}I]-ABA)] or 2 h [for the antagonist tritiated 8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-CPX)] in Millipore Multiscreen 96-well GF/C filter plates, and the assays were terminated by rapid filtration on a cell harvester (Brandel, Gaithersburg, MD) followed by 4 × 150 μL washes over 30 s with ice-cold 10 mM Tris-HCl at pH 7.4 and 10 mM $MgCl_2$. Nonspecific binding was measured in the presence of 50 μM 5′-*N*-ethylcarboximide-adenosine (NECA).

Precipitation of Receptor:G Protein Complexes from Digitonin Extracts of Sf9 Cell Membranes Containing the $_{6HISFLAG}$-A1 Adenosine Receptor, α_{i1}, and either $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$. The methodology for precipitation of receptor:G protein complexes is based on the study by Munshi et al. (*43*), which found that high concentrations of agonist in the absence of nucleotide resulted in a nucleotide free or "open" conformation of the G protein. This G protein conformation remains tightly bound to the high-affinity state of the A_1 adenosine receptor and allows for precipitation and characterization of receptors bound to their cognate G proteins. Briefly, Sf9 cells containing specific A_1 adenosine receptor:G protein combinations were lysed by nitrogen cavitation in the presence of receptor:G protein (RG) base buffer [25 mM HEPES at pH 7.2, 1 mM EGTA, 1 mM $MgCl_2$, 100 μM adenosine, 1% glycerol (w/v), 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, and 20 μg/mL benzamidine]. Membranes were prepared from the lysate as described above and resuspended with RG base buffer containing 1% digitonin (4:1 digitonin/membrane protein ratio) using a 21.5-guage needle, followed by stirring for 45 min at 4 °C. Extracts were prepared and immediately loaded onto Ni^{2+} columns equilibrated with RG base buffer and washed with three column volumes of RG base buffer containing 0.1% CHAPS. Protein was eluted with RG base buffer containing 0.1% CHAPS and 200 mM imidazole, concentrated, and characterized by SDS−PAGE and immunoblotting as described above.

Table 1: Comparison of the Ability of Different $\beta\gamma$ Isoforms To Couple G_q α to the M_1 Muscarinic Receptor and G_{i1} α to the A_1 Adenosine Receptor as Measured by the EC_{50} Values for Receptor:α:$\beta\gamma$ Coupling[a]

	M_1 muscarinic receptor	A_1 adenosine receptor
$\beta\gamma$ isoform	EC_{50} (nM) (range) (*n*)	EC_{50} (nM) (range) (*n*)
$\beta_1\gamma_2$	**0.8** (0.5 − 1.1) $n = 8$	**0.4** (0.3 − 0.6) $n = 2$
$\beta_1\gamma_{11}$	**0.2**[b] (0.01 − 0.2) $n = 7$	**1.1**[b] (0.9 − 1.4) $n = 2$
$\beta_4\gamma_2$	0.7 (0.4 − 1.1) $n = 3$	0.4 (0.3 − 0.6) $n = 3$
$\beta_4\gamma_{11}$	**12.0**[b] (9.4 − 15.3) $n = 3$	**11.8**[b] (9.2 − 15.2) $n = 3$
$\beta_2\gamma_2$	**0.9** (0.5 − 1.7) $n = 2$	nd
$\beta_3\gamma_2$	**1.1** (0.6 − 1.8) $n = 2$	nd
$\beta_1\gamma_3$	**0.8** (0.5 − 1.2) $n = 4$	nd
$\beta_1\gamma_{13}$	**0.7** (0.5 − 1.1) $n = 2$	nd
$\beta_1\gamma_{12}$	**0.7** (0.4 − 1.4) $n = 2$	nd

[a] EC_{50} values were determined by fitting the averaged data to single-site binding or competition curves as described in the Materials and Methods. Bold numbers indicate Ec_{50} values from the statistical fit, and numbers in parentheses represent the range of values within the 95% confidence interval. Statistical significance (indicated by the superscript) was determined using the F-statistic. nd = not determined. [b] Significantly different from EC_{50} values for other $\beta\gamma$ isoforms tested; $p < 0.001$.

Calculation and Expression of Results. In experiments using the M_1 muscarinic and A_1 adenosine receptors, data from all experiments were normalized as a percentage of maximal GTPγS binding as determined by the single-site binding curves generated by GraphPad Prism. Data were averaged for each $\beta\gamma$ isoform after normalization, and GraphPad Prism was used to obtain estimates of the EC_{50} values and statistical analysis of the binding curves, presented in Table 1. Statistical significance for differences among binding curves was determined using an *F* test; this technique is able to discern small but significant differences between two binding curves (*44*).

Competition binding experiments examining α:$\beta\gamma$ affinity used GraphPad Prism to generate inhibition curves for each of the experiments with the two $\beta\gamma$ dimers; the data were then normalized as percent inhibition of maximal α staining. Statistical significance for differences among binding curves was determined using the F-statistic (*44*).

For antagonist and agonist-binding isotherms, nonspecific binding and free radioligands were fit by least-squares regression to a straight line. The extrapolated fit value of nonspecific binding for each free concentration of radioligand was subtracted from the total binding to calculate specific binding. B_{max} and EC_{50} values were fit using nonlinear least-squares interpolation for single- or double-site binding

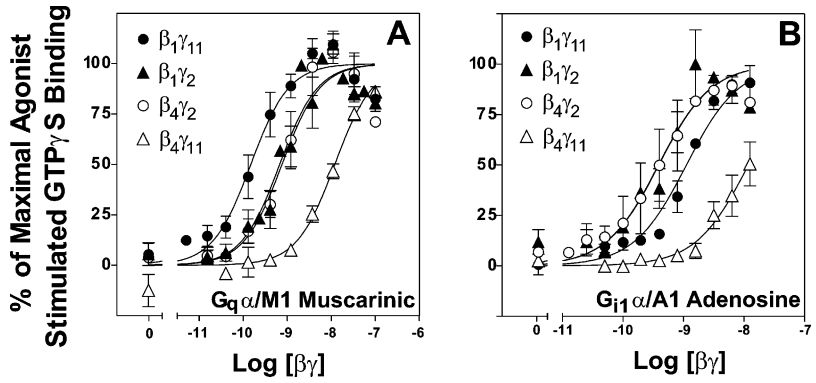

FIGURE 2: Comparison of the ability of different $\beta\gamma$ combinations to couple G_q α to the M_1 muscarinic receptor and G_{i1} α to the A_1 adenosine receptor. (A) Increasing concentrations of purified recombinant $\beta\gamma$ dimers were reconstituted with 15 nM G_q α into Sf9 cell membranes containing the M_1 muscarinic receptor, and then the ability of 1 mM carbachol to stimulate GTPγS binding to G_q α as a function of the $\beta\gamma$ concentration was measured as described in the Materials and Methods. The calculated EC_{50} value for the $\beta\gamma$ dimers shown as well as others can be found in Table 1. (B) Receptor-coupling experiments were performed as in A, except 10 nM G_{i1} α was used with the A_1 adenosine receptor. The ability of 100 nM R-PIA to stimulate GTPγS binding to G_{i1} α as a function of the $\beta\gamma$ concentration was measured as described in the Materials and Methods. EC_{50} values for these and other $\beta\gamma$ dimers are reported in Table 1.

models in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). The results of the two fits were compared with an *F* test. Data points in the Scatchard transformation were fit by linear regression to a straight line.

In experiments with PLC-β, data from all experiments were normalized as a percentage of maximal IP_3 production, as determined by the single-site binding curves generated by GraphPad Prism; EC_{50} values were then compared using the *F* test. V_{max} values were the mean level of IP_3 produced at maximal concentrations of α, $\beta\gamma$, or G protein isoform.

Materials. Reagents for Sf9 cell culture and purification of $\beta\gamma$ dimers have been described previously (*29*, *30*, *33*, *34*). GDP, imidazole, adenosine, HEPES, and anti-FLAG M2 agarose were from Sigma. FLAG peptide was synthesized at the University of Virginia Biomolecular Research Facility. Adenosine deaminase, CHAPS, and GTPγS were from Roche Molecular Biochemicals. A total of 10% polyoxyethylene (10) dodecyl ether (Genapol C-100) was from CalBiochem. Ni^{2+}−NTA Superflow resin and magnetic beads were from Qiagen. [^{35}S]-GTPγS was from New England Nuclear. Type HA 0.45 μm nitrocellulose filters and centricon 30 concentrators were from Millipore. Digitonin was from Wako. ABA was a gift from Susan Daluge of Glaxo-Wellcome (Research Triangle Park, NC). [$Na^{125}I$] was from Amersham Biosciences. NECA was from Research Biochemicals, Inc. (Natick, MA). [^{3}H]-CPX was from Perkin-Elmer (Boston, MA). [^{3}H]PIP_2 was from Perkin-Elmer Life Sciences. PE, phosphatidylinositol, PIP_2, and PIP_3 were purchased from Avanti Polar Lipids. The Superose 6 HR 10/30 column was from Pharmacia. All other materials were of the highest available purity.

RESULTS

Preparation of G Protein $\beta\gamma$ Subunits and Characterization of Antibodies. Purity of the $\beta\gamma$ dimers used in this study is demonstrated by the silver-stained 12% SDS−polyacrylamide gel in Figure 1A. These and other purified recombinant $\beta\gamma$ dimers were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with β- and γ-specific antibodies. Specificity of antibodies to β_{1-4} is shown in Figure 1B. All of the β antibodies were specific for their respective subunits; although the β_2 antibody showed slight cross-reactivity to the β_4 subunit, the β_1, β_3, and β_4 antibodies showed no cross-reactivity with other β isoforms at the protein levels tested (Figure 1B). Specificity of antibodies to γ_2 and γ_{11} is shown in Figure 1C. The γ_2 antibody displayed cross-reactivity only with γ_3, which is not unexpected, because γ_2 and γ_3 are members of the same γ subfamily, with over 80% similarity between sequences (*3*). The γ_{11} antibody did not cross-react with any other γ isoforms at the protein levels tested (Figure 1C).

Ability of Different $\beta\gamma$ Combinations To Support Coupling of G_q α to the M_1 Muscarinic Receptor. Sf9 membranes containing the M_1 muscarinic receptor were stripped with urea and reconstituted with purified G_q α and increasing concentrations of $\beta_1\gamma_2$, $\beta_4\gamma_2$, $\beta_1\gamma_{11}$, and $\beta_4\gamma_{11}$. These four $\beta\gamma$ dimers were tested for their ability to promote carbachol-dependent binding of GTPγS to G_q α. Figure 2A illustrates agonist-dependent nucleotide exchange as a function of the $\beta\gamma$ concentration; the most efficient $\beta\gamma$ dimer was $\beta_1\gamma_{11}$, with an EC_{50} of 0.2 nM (Table 1). Both $\beta_1\gamma_2$ and $\beta_4\gamma_2$ were similar in their ability to support receptor coupling, with EC_{50} values of 0.8 and 0.7 nM, respectively (Table 1). The weakest of the series was $\beta_4\gamma_{11}$, with an EC_{50} of 12 nM, over 10-fold lower than $\beta_1\gamma_2$ or $\beta_4\gamma_2$ and 24-fold lower than $\beta_1\gamma_{11}$ (Table 1). The activity of $\beta_4\gamma_{11}$ was markedly lower than five other $\beta\gamma$ combinations tested at this receptor, including $\beta_2\gamma_2$, $\beta_3\gamma_2$, $\beta_1\gamma_3$, $\beta_1\gamma_{12}$, and $\beta_1\gamma_{13}$, which all had EC_{50} values of around 1 nM (Table 1). This EC_{50} value for $\beta_4\gamma_{11}$ is also markedly lower than most EC_{50} values determined for purified $\beta\gamma$ dimers tested with the A_{2a} adenosine and β_1 adrenergic receptors (*20*).

Ability of Different $\beta\gamma$ Combinations To Support Coupling of G_{i1} α to the A_1 Adenosine Receptor. The same panel of $\beta\gamma$ dimers was characterized for the ability to promote agonist-dependent binding of GTPγS to G_{i1} α after reconstitution into Sf9 membranes containing the A_1 adenosine receptor using the specific agonist R-PIA. Effects of increasing concentrations of $\beta\gamma$ on agonist-dependent nucleotide binding to G_{i1} α are shown in Figure 2B. Both $\beta_1\gamma_2$ and $\beta_4\gamma_2$ displayed EC_{50} values of 0.4 nM; however, $\beta_1\gamma_{11}$ was slightly less efficient, with an EC_{50} value of 1.1 nM (Table 1). In a pattern reminiscent of the M_1 muscarinic receptor, the EC_{50} value of $\beta_4\gamma_{11}$ was also right-shifted at 11.8 nM

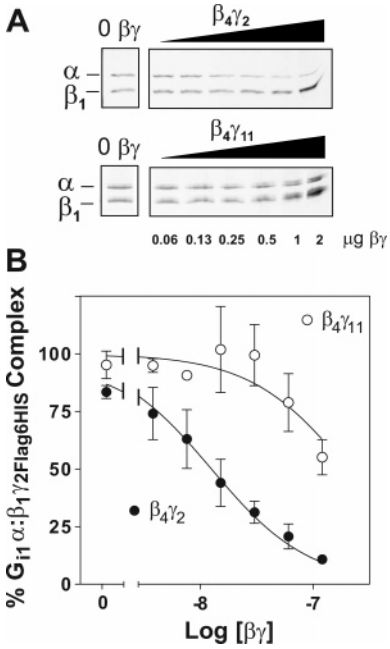

FIGURE 3: Relative affinities of the pure $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ dimers for G_{i1} α. (A) Silver-stained gels of α and β subunits after precipitation with magnetic Ni^{2+}−NTA beads. Increasing concentrations of the indicated $\beta\gamma$ were incubated with purified G_{i1} α:$\beta_1\gamma_{2\text{FLAG6HIS}}$, followed by sequestration of the tagged protein with magnetic Ni^{2+}−NTA beads, separation by SDS−PAGE, and staining with silver as described under the Materials and Methods. Note that increasing β staining with higher concentrations of $\beta_4\gamma_{11}$ is likely due to the dissociation of β_4 from γ_{11} and aggregation of the β subunit on the beads; this phenomenon was not observed to this extent with other $\beta\gamma$ dimers. (B) Apparent affinity as a measure of the ability of the added $\beta\gamma$ dimer to compete with $\beta_1\gamma_{2\text{FLAG6HIS}}$ for binding to the G_{i1} α subunit. Data are expressed as a ratio of the integrated density of the α and β_1 bands in A, to control for differences in pellet recovery. Calculated IC_{50} values are 13 nM for $\beta_4\gamma_2$ and 203 nM for $\beta_4\gamma_{11}$.

(Table 1), 30-fold lower in coupling efficiency than $\beta_1\gamma_2$ or $\beta_4\gamma_2$. Reconstitution experiments were also performed with $\beta_4\gamma_{11}$ and the A_{2a} adenosine receptor in an effort to characterize this $\beta\gamma$ combination at a G_s-linked receptor. In keeping with the data found with the M_1 muscarinic and A_1 adenosine receptors, the coupling efficiency of $\beta_4\gamma_{11}$ was shifted a log order to the right of $\beta_4\gamma_2$ (data not shown). Thus, similar results with $\beta_4\gamma_{11}$ in reconstitutions with a G_q-, G_i-, or G_s-linked receptor argue that it is not likely that the poor receptor coupling observed with $\beta_4\gamma_{11}$ is due to the receptor or α subunit but is a property of the $\beta_4\gamma_{11}$ dimer itself.

Comparison of the Affinity of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ for G_{i1} α. There are several explanations for the weak activity of $\beta_4\gamma_{11}$ at the two receptors tested (Figure 2), among them (1) low affinity of $\beta\gamma$ for the receptor, (2) low affinity of α:$\beta\gamma$ for the receptor, or (3) low affinity of $\beta\gamma$ for α. On a practical level, it is very difficult to distinguish between the first two explanations in a membrane; therefore, α:$\beta\gamma$ affinity was examined as a mechanism for decreased activity at the receptor. An affinity assay was developed to measure the competition of different $\beta\gamma$ dimers for binding to G_{i1} α:GDP in solution.

The results in Figure 3A illustrate silver-stained gels comparing pure G_{i1} α subunit affinity precipitated with the $\beta_1\gamma_{2\text{FLAG6HIS}}$ dimer after incubation with increasing concentrations of either $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ and magnetic Ni^{2+} beads. The top panel in Figure 3A demonstrates that, as increasing concentrations of $\beta_4\gamma_2$ are added to the incubation, less G_{i1} α precipitates with the $\beta_1\gamma_{2\text{FLAG6HIS}}$:$Ni^{2+}$ bead complex, presumably because it is forming a heterotrimer with the added $\beta_4\gamma_2$ dimer. In contrast, added $\beta_4\gamma_{11}$ dimer has little effect on the level of G_{i1} α precipitated with $\beta_1\gamma_{2\text{FLAG6HIS}}$ (bottom panel of Figure 3A). IC_{50} curves were derived from the data in Figure 3A for each $\beta\gamma$ tested; the $\beta_4\gamma_2$ dimer had an IC_{50} of 13 nM (Figure 3B), a value compatible with published measurements of affinity between G_{i1} α and $\beta_1\gamma_2$ (*40*). In contrast, the $\beta_4\gamma_{11}$ dimer had a 15-fold lower affinity for G_{i1} α than $\beta_4\gamma_2$, with an IC_{50} value of 203 nM (Figure 3B), mirroring the results of the receptor reconstitution experiments (parts A and B of Figure 2 and Table 1). Such a large difference in affinity is especially striking, considering that another study reported no differences in affinities of either $\beta_1\gamma_2$ or $\beta_1\gamma_{11}$ for G_{i1} α (*15*). These results suggest that the low coupling efficiency of purified $\beta_4\gamma_{11}$ at the A_1 adenosine receptor is a result of decreased affinity for G_{i1} α. Thus, it is likely that the lower affinity of $\beta_4\gamma_{11}$ for other α subunits, such as G_sα and G_qα, is responsible for the decreased ability to couple to the β-adrenergic and M_1 muscarinic receptors, respectively.

Stability of the G_{i1} α:$\beta_4\gamma_{11}$ Complex under Activating or Nonactivating Conditions. The results of the α:$\beta\gamma$-affinity experiments provide a mechanism for the differences observed between $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ in the receptor reconstitution experiments. However, purification of the $\beta_4\gamma_{11}$ dimer by coexpression of $_{6\text{HIS}}$-G_{i1} α in Sf9 cells requires a stable heterotrimer with low nanomolar α:$\beta\gamma$ affinity during the extraction and washing stages of purification. Elution of the $\beta_4\gamma_{11}$ dimer from the $_{6\text{HIS}}$-G_{i1} α was dependent upon G protein activation with aluminum, magnesium, and fluoride; this suggests that $_{6\text{HIS}}$-G_{i1} α:$\beta_4\gamma_{11}$ was a viable heterotrimer prior to activation. The loss of $\beta\gamma$ activity at this purification step suggests a requirement of the α subunit to stabilize the dimeric structure of $\beta_4\gamma_{11}$.

The premise that activation of $_{6\text{HIS}}$-G_{i1} α:$\beta_4\gamma_{11}$ destabilizes the $\beta_4\gamma_{11}$ dimer and leads to a loss of function was tested by comparing the elution profiles of purified $_{6\text{HIS}}$-G_{i1} α:$\beta\gamma$ heterotrimers by Superose 6 size-exclusion chromatography. This technique has previously been used to study G protein subunit association, because heterotrimeric G protein can be dissociated into its constituent subunits after activation with Mg^{+2} and AlF_4^- or GTPγS (*45*). While the α, β, and γ subunits can be purified as a heterotrimer under nonactivating conditions (*46*), coelution of the β and γ subunits as a dimer separately from α under activating conditions provided the cornerstone for the belief that the β and γ subunits exist as a functional dimer (*47*, *48*). In the present study, purified protein was incubated with GTPγS and Mg^{+2}, under activating conditions that favor maximal nucleotide exchange (*49*), and evaluated chromatographically. Figure 4 illustrates the experimental design with elution profiles of $_{6\text{HIS}}$-G_{i1} α:$\beta_1\gamma_2$ under two different conditions. In the presence of GDP and EDTA, the α, β, and γ subunits coelute as a heterotrimer at a position compatible with an 80 kDa protein, as judged by silver staining of the α and β subunits and Western blotting for γ (fractions 29−33 in Figure 4A). Purification of the heterotrimer with tagged α results in excess α subunit in

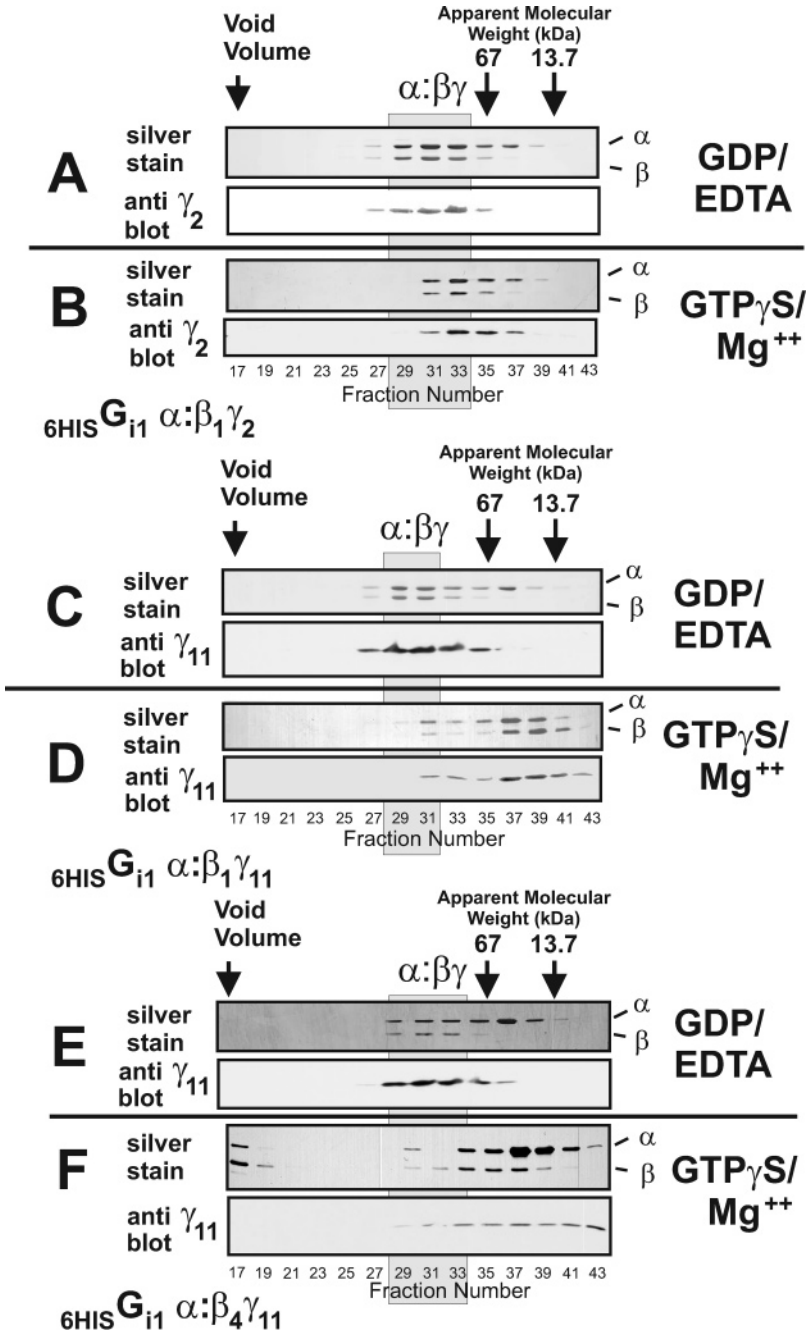

FIGURE 4: Size-exclusion chromatography of 6His-tagged heterotrimeric G proteins under nonactivating and activating conditions. (A) Purified recombinant $_{6HIS}$-G_{i1} α:$\beta_1\gamma_2$ was separated on a Superose 6 column calibrated with the molecular-weight standards albumin (67 kDa) and ribonuclease A (13.7 kDa) as described under the Materials and Methods. Elution fractions were separated on 12% polyacrylamide gels and stained with silver to identify retention times of α and β; identical fractions were also resolved on 12% polyacrylamide gels and blotted with a γ_2-specific antibody. Gray boxed area (fractions 29−33) indicates the predicted retention time for a heterotrimeric α:$\beta\gamma$ G protein. All subunits coeluted at an apparent molecular weight compatible with a heterotrimeric G protein. Because of the placement of the 6His tag, excess $_{6HIS}$-G_{i1} α was present in the G protein preparation and thus eluted as a monomer (fractions 35−37). (B) Chromatography and analysis was performed as in A, except that G protein was incubated with 100 μM GTPγS and 5 mM Mg^{+2} for 45 min at 30 °C; 5 mM $MgCl_2$ was also included in the chromatography buffer. All subunits coeluted; however, the retention time was shifted slightly to lower the apparent molecular weight. (C) Chromatography of $_{6HIS}$-G_{i1} α:$\beta_1\gamma_{11}$ as described in A, except the γ_{11} antibody was used for blotting fractions instead of the γ_2 antibody. Gray boxed area indicates the predicted retention time for a heterotrimeric α:$\beta\gamma$G protein (fractions 29−33). Under nonactivating conditions in C, all subunits coeluted at an apparent molecular weight compatible with a heterotrimeric G protein. Excess $_{6HIS}$-G_{i1} α present in the G protein preparation eluted as a monomer. (D) Chromatography of activated $_{6HIS}$-G_{i1} α:$\beta_1\gamma_{11}$ as described in B, except the γ_{11} antibody was used for blotting fractions instead of the γ_2 antibody. Both the α and $\beta\gamma$ eluted at a lower apparent molecular weight, suggesting subunit dissociation. A small fraction of $_{6HIS}$-G_{i1} α:$\beta_1\gamma_{11}$ coeluted with a retention time compatible with a heterotrimeric G protein, suggesting incomplete activation. (E) Chromatography of $_{6HIS}$-G_{i1} α:$\beta_4\gamma_{11}$ as described in C. Gray boxed area indicates the predicted retention time for a heterotrimeric G protein (fractions 29−33). Under nonactivating conditions, all subunits coeluted at an apparent molecular weight compatible with a heterotrimeric G protein. Excess $_{6HIS}$-G_{i1} α present in the G protein preparation eluted as a monomer. (F) Chromatography of activated $_{6HIS}$-G_{i1} α:$\beta_4\gamma_{11}$ as described in D. After activation, β_4 but not γ_{11} was observed in the void volume, suggesting a higher molecular-weight aggregate of β_4 alone. In contrast, γ_{11} but not β_4 was observed at a much lower apparent molecular weight, and most of G_{i1} α eluted at an apparent molecular weight compatible with a monomeric α subunit. These results demonstrate a physical separation between β_4 and γ_{11} that is directly related to the activation of the heterotrimer with GTPγS.

the preparation, which elutes as a monomer at fractions 35−37 (silver gel in Figure 4A). Activation of the $_{6HIS}$-G_{i1} α:$\beta_1\gamma_2$ protein with GTPγS/Mg^{+2} resulted in a shift of the G protein complex to a lower apparent molecular weight (Figure 4B). Similar results were observed in experiments with $_{6HIS}$-G_{i1} α:$\beta_4\gamma_2$ (data not shown). Although it appears that these chromatographic changes are the result of activation, it is difficult to discern the structural state of the G proteins by apparent size alone.

$_{6HIS}$-G_{i1} α:$\beta_1\gamma_{11}$ was also characterized chromatographically, to control for potentially different behavior derived from differential prenylation of the γ subunit. Under nonactivating conditions, results similar to $_{6HIS}$-G_{i1} α:$\beta_1\gamma_2$ were observed (Figure 4C). However, after activation, the elution peak of the β and γ subunits occurs in fraction 39 (Figure 4D), whereas the elution peak of the α subunit occurs in fraction 37. This result suggests that, in contrast to $_{6HIS}$-G_{i1} α:$\beta_1\gamma_2$, activation-dependent dissociation of $\beta_1\gamma_{11}$ from $_{6HIS}$-G_{i1} α occurs under the experimental conditions in Figure 4D.

Under nonactivating conditions, $_{6HIS}$-G_{i1} α:$\beta_4\gamma_{11}$ behaved similarly to $_{6HIS}$-G_{i1} α:$\beta_1\gamma_2$ and $_{6HIS}$-G_{i1} α:$\beta_1\gamma_{11}$ (Figure 4E), with both heterotrimeric protein and free α subunit observed as in the previous two figures. However, when activated, a significant fraction of the β_4 subunit eluted in the void volume in fraction 17, presumably as a high-molecular-weight aggregate (silver stain in Figure 4F). It is notable that there is no γ_{11} immunoreactivity in fraction 17 (immunoblot in Figure 4F). Equally remarkable was the presence of γ_{11} immunoreactivity in later fractions 41−43 (immunoblot in Figure 4F) with the absence of β_4 staining (silver stain in Figure 4F). These results indicate that β_4 dissociates from γ_{11} upon activation of heterotrimeric G protein.

Efforts to understand the relationship between γ_{11} and other β isoforms were made using purified heterotrimeric complexes of $_{6HIS}$-G_{i1} α:$\beta_2\gamma_{11}$ and $_{6HIS}$-G_{i1} α:$\beta_3\gamma_{11}$. In experiments addressing the chromatographic behavior of G_{i1} α:$\beta_2\gamma_{11}$ and G_{i1} α:$\beta_3\gamma_{11}$ under nonactivating and activating conditions, both heterotrimers displayed similar chromatographic behavior as G_{i1} α:$\beta_1\gamma_{11}$ (data not shown). Thus, it appears that $\beta_2\gamma_{11}$ and $\beta_3\gamma_{11}$ can form viable heterotrimers with G_{i1} α, with intermediate stabilities between G_{i1} α:$\beta_1\gamma_{11}$ and G_{i1} α:$\beta_4\gamma_{11}$.

Effect of Different Activating Conditions on the Ability of Purified G_q α, $\beta_1\gamma_2$, $\beta_1\gamma_{11}$, $\beta_4\gamma_2$, $\beta_4\gamma_{11}$, $G_{i1}\alpha$:$\beta_4\gamma_2$, and $G_{i1}\alpha$:$\beta_4\gamma_{11}$ To Stimulate PLC-β. Activation of PLC-β was measured to evaluate the downstream signaling properties of the G protein isoforms characterized at receptors (Figure 2). These experiments also examined the functional impact of the activation-dependent instability of G_{i1} α:$\beta_4\gamma_{11}$ observed in Figure 4. A comparison of the pure $\beta_1\gamma_2$, $\beta_4\gamma_2$, and G_q α (activated pre-reconstitution) demonstrated similar activity at PLC, with EC_{50} values in the low nanomolar range (Figure 5A and Table 3). Because farnesylated $\beta\gamma$ dimers have been shown to have lower activity at PLC-β (*16*), the effects of pure $\beta_1\gamma_{11}$ and $\beta_4\gamma_{11}$ were compared with the results shown in Figure 5B. Although EC_{50} values were similar (Table 3), V_{max} values for $\beta_1\gamma_{11}$ and $\beta_4\gamma_{11}$ were reduced approximately 40 and 70%, respectively, from $\beta_1\gamma_2$ (compare parts A and B of Figure 5 and Table 3). Thus, the $\beta_4\gamma_{11}$ dimer appears to have an activity typical of a farnesylated $\beta\gamma$ dimer at PLC-β.

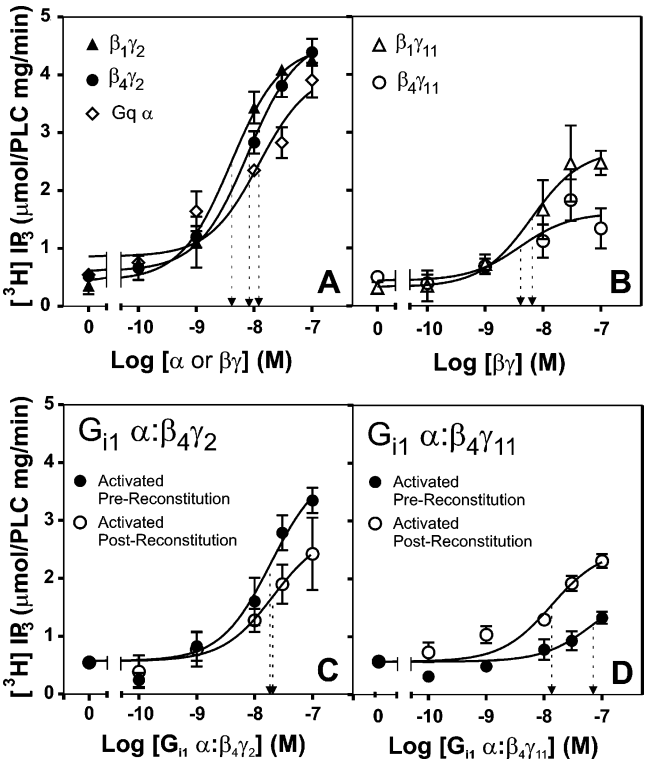

FIGURE 5: Effect of different activating conditions on the ability of purified G_q α, $\beta_1\gamma_2$, $\beta_1\gamma_{11}$, $\beta_4\gamma_2$, $\beta_4\gamma_{11}$, $G_{i1}\alpha$:$\beta_4\gamma_2$, and $G_{i1}\alpha$:$\beta_4\gamma_{11}$ to stimulate PLC-β. (A) Purified G_q α, $\beta_1\gamma_2$, and $\beta_4\gamma_2$ were reconstituted with PLC-β into phospholipid vesicles, and IP_3 production was measured as described in the Materials and Methods; G_q α was activated as described in the Results. (B) Purified $\beta_1\gamma_{11}$ and $\beta_4\gamma_{11}$ were reconstituted with PLC-β into phospholipid vesicles as in A. (C) Purified G_{i1} α:$\beta_4\gamma_2$ was activated either pre-reconstitution or post-reconstitution as described in the Materials and Methods and reconstituted with PLC-βas in A. (D) Purified G_{i1} α:$\beta_4\gamma_{11}$ was activated either pre-reconstitution or post-reconstitution as described in the Materials and Methods and reconstituted with PLC-βas in A. EC_{50} and V_{max} values can be found in Table 3. Arrows in each panel indicate the concentration of the protein that resulted in half-maximal activity.

The ability of $\beta_4\gamma_{11}$ to reconstitute receptor:α:$\beta\gamma$ interactions was markedly diminished when purified from a $G_{i1}\alpha$:$\beta_4\gamma_{11}$ heterotrimer (parts A and B of Figure 2). To determine the influence of the physical state of $\beta_4\gamma_{11}$ on its activity at the effector PLC-β, purified $G_{i1}\alpha$ $\beta_4\gamma_{11}$ heterotrimer was reconstituted into phospholipid vesicles with PLC-β either before or after heterotrimer activation and compared with the activity of proteins known to stimulate PLC-β (Figure 5A) and $\beta_4\gamma_{11}$ in Figure 5B. Activation of $G_{i1}\alpha$:$\beta_4\gamma_{11}$ before reconstitution with PLC-β into vesicles (preactivation) was used to approximate the conditions for activation-dependent purification of $\beta_4\gamma_{11}$, however, without chromatographic separation of $G_{i1}\alpha$ from $\beta_4\gamma_{11}$. In an effort to more closely resemble G protein activation *in vivo*, the $G_{i1}\alpha$:$\beta_4\gamma_{11}$ heterotrimer was activated after reconstitution of PLC-β into vesicles (postactivation). These conditions could potentially result in higher activity, because of the stabilization of the heterotrimer from the presence of PLC-β and phospholipid vesicles. This notion was supported by the results presented in parts C and D of Figure 5. The two different activation conditions had no effect on the activity of $G_{i1}\alpha$:$\beta_4\gamma_2$ on PLC-β; both EC_{50} and V_{max} values were the same (Figure 5C and Table 3). In contrast, the activation of $G_{i1}\alpha$:$\beta_4\gamma_{11}$ pre-reconstitution resulted in approximately a 5-fold decrease

Table 2: Characterization of the Affinity and Number of Binding Sites for the A1 and A_{2a} Adenosine Receptor Expressed with G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ in Sf9 Cells[a]

	A_1 adenosine receptor: α_{i1}:$\beta_4\gamma_2$		A_1 adenosine receptor: α_{i1}:$\beta_4\gamma_{11}$	
conditions	EC_{50} (nM)	B_{max} (fmol/mg of protein)	EC_{50} (nM)	B_{max} (fmol/mg of protein)
antagonist [^{3}H]-CPX	nd	**2578** $n = 3$	nd	**2234** $n = 3$
high-affinity agonist [^{125}I]-ABA	**0.08** (0.07−0.09)	**2094** $n = 5$	**0.06** (0.06−0.07)	**2104** $n = 5$

[a] Ec_{50} values were determined by fitting the averaged data to single-site binding or competition curves as described in the Materials and Methods. Bold numbers indicate Ec_{50} values from the statistical fit, and numbers in parentheses represent the range of values within the 95% confidence interval. nd = not determined.

Table 3: Comparison of the Ability of Different α, $\beta\gamma$, and G Protein Isoforms To Stimulate Turkey PLC-β Activity in a Reconstitution Assay[a]

	activation of avian PLC-β	
G protein isoform	EC_{50} (nM) (range) (n)	V_{max} [^{3}H] IP_3 [μmol (mg of PLC)$^{-1}$ min^{-1}] (range)
$\beta_1\gamma_2$	**4.0**; (2.8 − 5.7) $n = 2$	**4.3**[b]; (4.1 − 4.5)
$\beta_1\gamma_{11}$	**6.3**; (2.5 − 16.0) $n = 3$	**2.5**; (1.8 − 3.2)
$\beta_4\gamma_2$	**7.8**; (5.9 − 10.3) $n = 7$	**4.4**[c]; (3.2 − 5.6)
$\beta_4\gamma_{11}$	**4.0**; (0.9 − 17.8) $n = 5$	**1.3**; (−0.2 − 2.9)
G_q α	**12.1**; (7.4 − 20.0) $n = 3$	**3.9**; (2.9 − 5.0)
G_{i1} α:$\beta_4\gamma_2$ activated pre-reconstitution	**19.1**; (12.6 − 28.8) $n = 3$	**3.3**; (2.6 − 4.1)
G_{i1} α:$\beta_4\gamma_2$ activated post-reconstitution	**19.1**; (13.5 − 27.1) $n = 3$	**2.4**; (0.3 − 4.6)
G_{i1} α:$\beta_4\gamma_{11}$ activated pre-reconstitution	**71.7**[d]; (41.0 − 125.5) $n = 4$	**1.3**[e]; (0.9 − 1.7)
G_{i1} α:$\beta_4\gamma_{11}$ activated post-reconstitution	**13.4**; (8.9 − 20.2) $n = 4$	**2.3**; (1.8 − 2.8)

[a] EC_{50} values were determined by fitting the averaged data to a single-site binding as described in the Materials and Methods. Bold numbers indicate EC_{50} values from the statistical fit, and numbers in parentheses represent the range of values within the 95% confidence interval. Statistical significance (indicated by the superscript) for EC_{50} values was determined using the F-statistic. V_{max} values represent the level of [^{3}H] IP_3 produced at the maximal concentration (100 nM) of protein used in the reconstitutions. Basal activity in the PLC-β assay was approximately 0.4 μmol of [^{3}H] IP_3 (mg of PLC)$^{-1}$ min^{-1}. $\beta\gamma$ isoforms and G_q α were analyzed as one data set, and G_{i1} α:$\beta_4\gamma_2$ and G_{i1} α:$\beta_4\gamma_{11}$ were analyzed individually, comparing alternative activation conditions. [b] Significantly higher from the V_{max} value for $\beta_1\gamma_{11}$ and $\beta_4\gamma_{11}$; $p < 0.05$. [c] Significantly different from the V_{max} value for $\beta_4\gamma_{11}$; $p < 0.05$. [d] Significantly different from the EC_{50} value for G_{i1} α:$\beta_4\gamma_{11}$ activated post-reconstitution; $p < 0.0001$. [e] Significantly different from the V_{max} value for G_{i1} α:$\beta_4\gamma_{11}$ activated post-reconstitution; $p < 0.05$.

in potency of the activation of PLC-β (13 nM postactivation and 72 nM preactivation) and approximately a 44% decrease in the V_{max} (Figure 5D and Table 3). These data suggest at least two explanations for why the activity of $G_{i1}\alpha$:$\beta_4\gamma_{11}$ at PLC-β was lower when activated prior to reconstitution into vesicles. One explanation is incomplete activation of the heterotrimer under these conditions; this seems unlikely, because $\beta_4\gamma_{11}$ can be purified in comparable quantities as $\beta_4\gamma_2$ under similar activating conditions and $G_{i1}\alpha$:$\beta_4\gamma_{11}$ appears to be especially sensitive to activating conditions (Figure 5C). The second explanation is that the effect of activation of $G_{i1}\alpha$:$\beta_4\gamma_{11}$ before reconstitution on PLC-β activity [V_{max}, 1.3 μmol of [^{3}H] IP_3 (mg of PLC)$^{-1}$ min^{-1}, Table 3] was due to the dissociation of $\beta_4\gamma_{11}$ from G_{i1} α and subsequent separation of β_4 from γ_{11}; if this were the case, these conditions would approximate the conditions of the reconstitution of purified $\beta_4\gamma_{11}$ with PLC-β [V_{max}, 1.3 μmol of [^{3}H] IP_3 (mg of PLC)$^{-1}$ min^{-1}, Table 3]. Regardless, these data demonstrate that the $\beta_4\gamma_{11}$ dimer is a viable signaling molecule at both the receptor and effector, and the physical state of the $\beta_4\gamma_{11}$ dimer with respect to its cognate Gα subunit appears to have a large influence on interactions with these signaling molecules.

Comparison of the Ability of G_i Proteins Containing $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ To Stabilize the High-Affinity State of the A_1 Adenosine Receptor. To further evaluate the weak activity of $\beta_4\gamma_{11}$ at receptor coupling, an intact membrane assay system was used to study receptor:G protein interactions. In this assay, G protein coupling promotes the high-affinity agonist-binding conformation of the receptor, providing a useful indicator of the functional receptor:G protein interaction. The A_1 adenosine receptor is especially well-suited to this assay, because there is about 100-fold difference between the high- and low-affinity agonist-binding states of this receptor (*50*). Thus, whereas binding of the antagonist provides a measure of the total number of receptors, independent of G protein coupling, high-affinity agonist-binding sites indicate the number of receptors coupled to G proteins. The total number of receptors in membranes prepared from Sf9 cells expressing the A_1 adenosine receptor, G_{i1} α, and either $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ was characterized using the specific antagonist [^{3}H]-CPX; similar receptor densities were observed in both combinations of G proteins (Table 2). In addition, immunoblotting with specific antibodies revealed similar amounts of β_4 subunit in each membrane preparation as well as specific expression of both γ_2 and γ_{11} isoforms (Figure 6A). Similar agonist-dependent GTPγS binding in both membrane preparations also suggests similar levels of G_{i1} α (data not shown). Thus, these two membrane preparations represent two unique A_1 adenosine receptor:G protein combinations in a native membrane environment; the lack of receptor G protein interaction would be clearly indicated by the absence of high-affinity agonist-binding sites, illustrated clearly in an earlier study from this laboratory (*51*).

The formation of the high-affinity binding state of the A_1 receptor was examined using the specific agonist [^{125}I]-ABA; this binding state is promoted by the formation of a ternary complex with heterotrimeric G protein; thus, the binding assay is a measure of the ability of different $\beta\gamma$ dimers to bind both G_{i1} α and the A_1 receptor (*50*, *52*). Figure 6B shows that ABA binding is saturable, with similar B_{max} values (2000 fmol/mg) for membranes containing A_1 adenosine receptor and either G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ (Table 2). These B_{max} values, when compared to the B_{max} values for antagonist binding (Table 2), demonstrate that most of the receptors expressed with either G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ are coupled. Scatchard analysis of the binding data in Figure 6C and Table

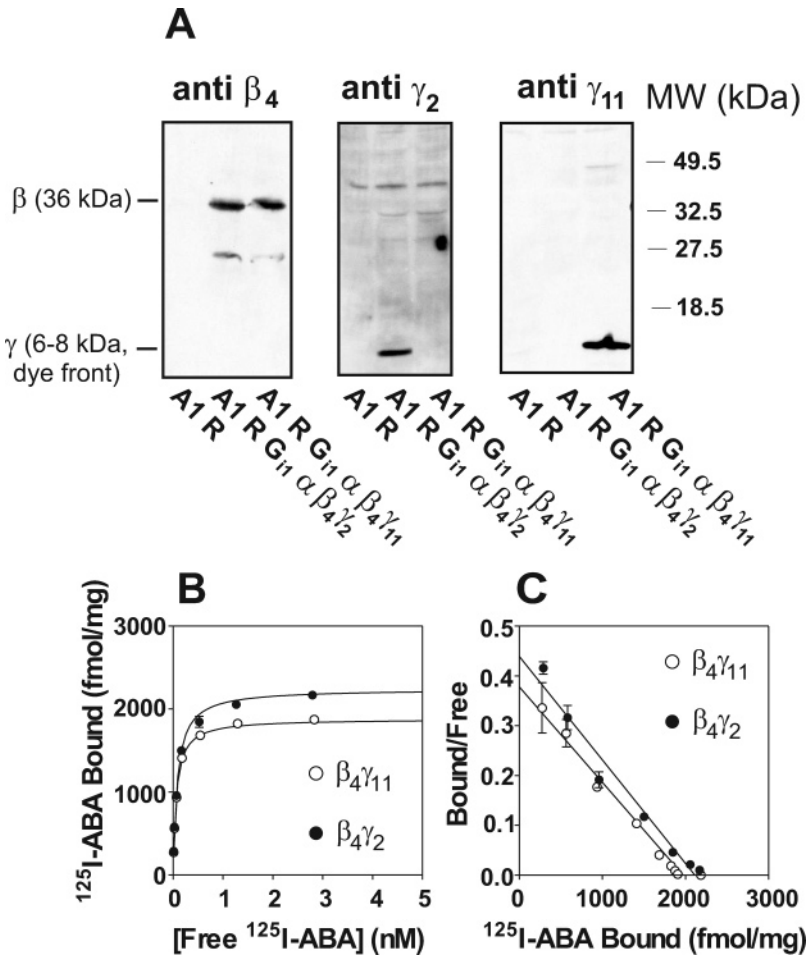

FIGURE 6: Agonist-binding properties of the A_1 adenosine receptor after expression with G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ in Sf9 cells. (A) Sf9 cell membranes (20 μg) expressing the A_1 adenosine receptor alone or with G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ were separated on 12% polyacrylamide gels and blotted with antibodies specific for β_4, γ_2, and γ_{11}. None of the β or γ subunits shown in Figure 4A was detected in control cells (A_1 receptor alone). (B) Binding of the specific agonist [^{125}I]-ABA in Sf9 membranes containing A_1 adenosine receptor and either G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ with results expressed as a hyperbolic binding isotherm; B_{max} and IC_{50} values are reported in Table 2. (C) Scatchard plot of the [^{125}I]-ABA binding to the A_1 adenosine receptor shown in B; note the presence of only one binding site in either membrane preparation.

2 demonstrates that the agonist ABA has a single binding site and EC_{50} values (0.08 nM, G_{i1} α:$\beta_4\gamma_2$ co-infection; 0.06 nM, G_{i1} α:$\beta_4\gamma_{11}$ co-infection) consistent with published values (*42*). These data suggest that the $\beta_4\gamma_{11}$ dimer, when expressed with α subunit and receptor, is fully capable of contributing to the formation of a functional receptor:α:$\beta\gamma$ complex.

Ability of $\beta_4\gamma_2$ and $\beta_4\gamma_{11}$ To Form Ternary Complexes with α_{i1} and the A_1 Adenosine Receptor. A critical test of the functional $\beta\gamma$ dimer coupling to the receptor is biochemical interaction, as measured by the detergent extraction and precipitation of a receptor:G protein complex. To this end, membranes of Sf9 cells infected with the $_{6HisFLAG}$-A_1 adenosine receptor, α_{i1}, and either $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ were extracted with digitonin and receptor:G protein complexes were purified by Ni−NTA chromatography and concentrated. Equal volumes of the purified receptor precipitations containing either G_{i1} α:$\beta_4\gamma_2$ and G_{i1} α:$\beta_4\gamma_{11}$ were separated by SDS−PAGE, transferred to nitrocellulose as described in the Materials and Methods, and blotted with antibodies specific for the receptor, α, β, and γ isoforms. The FLAG-tagged A_1 adenosine receptor, G_{i1} α, and β_4 (Figure 7A) were observed in each receptor precipitation. Immunoblots with the γ_2 and γ_{11} antibodies demonstrate that these subunit isoforms were specifically expressed and suggest that the γ isoforms are indeed capable of contributing to the formation of a receptor:G protein complex (Figure 7A). A densitometric comparison of the FLAG and β_4 immunoreactivities in the two precipitations (Figure 7B) suggests similar receptor:γ stoichiometries. These data, along with data from Figure 6, strongly support the conclusion that $\beta_4\gamma_{11}$, like $\beta_4\gamma_2$, is able to form a $\beta\gamma$ dimer that interacts normally and with high affinity to G_{i1} α and the A_1 adenosine receptor in a native membrane environment (Figure 6) and binds with high enough affinity to G_{i1} α and the A_1 adenosine receptor to be successfully purified (Figure 7).

Although the β_4 and γ_{11} combination was demonstrated to form a ternary complex with G_{i1} α and the A_1 adenosine receptor in Sf9 cells, direct evidence of $\beta_4\gamma_{11}$ dimer formation in mammalian cells was lacking. To address this issue, FLAG-tagged β_4 and HA-tagged γ_{11} constructs were expressed in HEK-293T cells. Membranes from these cells were extracted with Genapol, and $_{FLAG}\beta_4\gamma_{11HA}$ dimers precipitated with FLAG antibody. These experiments provided results similar to those in Figure 7A, demonstrating $\beta_4\gamma_{11}$ dimer formation in a mammalian cell, although the

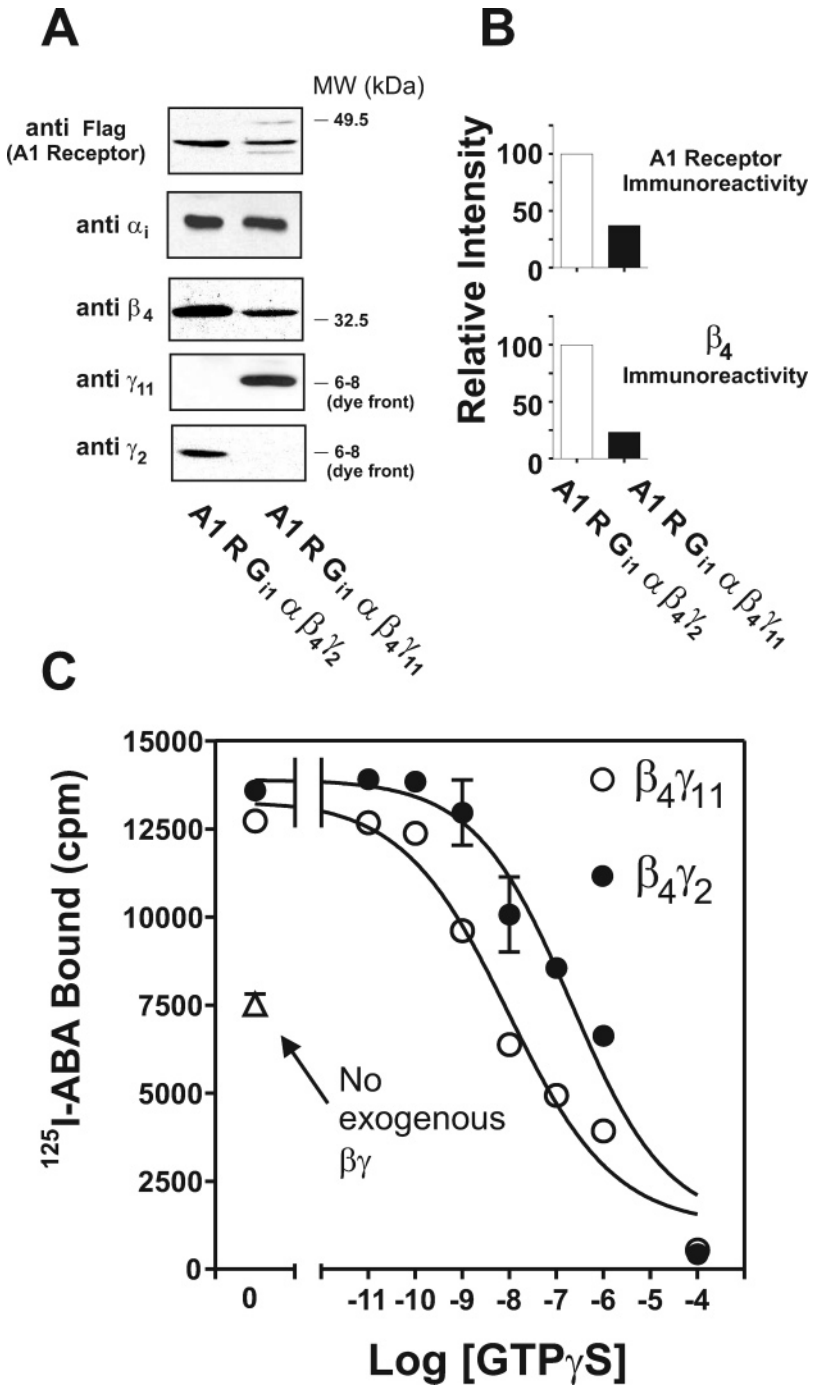

FIGURE 7: Precipitation of receptor:G protein complexes and GTPγS sensitivity of their high-affinity binding sites. (A) Complexes containing the $_{6HIS}$-A_1 adenosine receptor, α_{i1}, and either $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ were precipitated from digitonin extracts of Sf9 cell membranes as described in the Materials and Methods. Equal volumes of purified receptor:G_{i1} α:$\beta_4\gamma_2$ and receptor:G_{i1} α:$\beta_4\gamma_{11}$ were separated using 12% SDS−polyacrylamide gels, transferred to nitrocellulose, and blotted with antibodies specific for the $_{6HISFLAG}$-A_1 adenosine receptor (M2 FLAG), $G_{i1/2}$ α, β_4, γ_{11}, and γ_2. (B) Immunoreactive bands for the A_1 adenosine receptor and β_4 in A were quantified by scanning densitometry; levels of these proteins in the A_1 adenosine receptor G_{i1} α:$\beta_4\gamma_{11}$ precipitation were expressed as a percentage of the receptor and β_4 levels in the A_1 adenosine receptor G_{i1} α:$\beta_4\gamma_2$ precipitation. This quantitation shows that, for the A_1 adenosine receptor G_{i1} α:$\beta_4\gamma_{11}$ precipitation, densities of the A_1 receptor (FLAG) and β_4 immunoreactive bands are approximately 25−35% of the analogous bands in the A_1 adenosine receptor G_{i1} α:$\beta_4\gamma_2$ precipitation, suggesting similar G protein:receptor stoichiometries between the two precipitations in A. (C) High-affinity binding of the A_1-adenosine-specific agonist ABA was characterized in Sf9 membranes containing the A_1 adenosine receptor and G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ in the presence of increasing concentrations of GTPγS; note the same high level of ABA binding in the absence of GTPγS, consistent with parts B and C of Figure 6. Membranes containing the A_1 adenosine receptor and G_{i1} α:$\beta_4\gamma_{11}$ were over 20-fold more sensitive to GTPγS, with an IC_{50} value of 9.5 nM (95% confidence interval, 4.7−18.9 nM), than membranes containing the receptor and G_{i1} α:$\beta_4\gamma_2$, with an IC_{50} value of 208 nM (95% confidence interval, 89.9−481.3 nM). Also note that decreases in high-affinity binding sites drop below levels seen without exogenous $\beta\gamma$, indicating some receptor coupling in the absence of virally expressed $\beta\gamma$. The experiments were performed in triplicate. Data from separate experiments produced similar results.

stoichiometry of β_4 to γ_{11} was less than that of the $\beta_4\gamma_2$ control, likely because of the absence of a stabilizing α subunit (data not shown).

Sensitivity of the A_1 Adenosine Receptor:G Protein Ternary Complex Containing $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ to GTPγS. Having established that A_1 receptor:G protein complexes containing

$\beta_4\gamma_{11}$ can form using two experimental systems, we wanted to further characterize differences between the A_1 receptor:G_{i1} α:$\beta_4\gamma_{11}$ and A_1 receptor:G_{i1} α:$\beta_4\gamma_2$ complexes. Activation of G protein with GTPγS leads to dissociation of the G protein from the receptor, resulting in a lower affinity binding state of the A_1 adenosine receptor. Successful formation of A_1 receptor:G protein ternary complexes was demonstrated by the increase in the number of high-affinity A_1 receptor-binding sites in membranes after coexpression of either the $\beta_4\gamma_2$ or $\beta_4\gamma_{11}$ dimer with the A_1 receptor and G_{i1} α in Sf9 cells (Figure 7C). This result is consistent with a previous study from this laboratory, which characterized the influence of G proteins on the high-affinity binding state of the A_1 adenosine receptor (*51*). The stability of ternary complexes containing different G proteins as measured by the loss of high-affinity agonist binding can be quantified using increasing concentrations of GTPγS. Thus, the sensitivity of A_1 receptor ternary complexes containing either G_{i1} α:$\beta_4\gamma_2$ or G_{i1} α:$\beta_4\gamma_{11}$ to increasing concentrations of GTPγS was compared. Ternary complexes containing the $\beta_4\gamma_{11}$ dimer were up to 20-fold more sensitive to GTPγS (IC_{50} = 9.5 nM) than those containing $\beta_4\gamma_2$ (IC_{50} = 208 nM, Figure 7C). This result suggests that the stability of an A_1 adenosine receptor:G protein complex containing $\beta_4\gamma_{11}$ is much more sensitive to activating conditions than the analogous receptor:G protein complex containing $\beta_4\gamma_2$. Thus, activation of a $G_{i1}$$\alpha$:$\beta_4\gamma_{11}$ heterotrimer may facilitate the release of α from the receptor more readily than activation of a $G_{i1}$$\alpha$:$\beta_4\gamma_2$ heterotrimer.

DISCUSSION

The biological impact of β- and γ-subunit heterogeneity on G protein signaling is just beginning to be appreciated. After a role for the β subunit in receptor coupling was first proposed (*53*), subsequent studies have confirmed the original finding and have suggested that the isoform identity of the β subunit is critical to receptor:G protein signaling. For example, we have recently demonstrated that the A_{2a} adenosine receptor is able to discriminate among different $\beta\gamma$ isoforms in signaling via G_s α, with $\beta_4\gamma_2$ increasing the coupling efficiency 12-fold over $\beta_1\gamma_2$ (*20*). A parallel study also used high-affinity agonist binding of the A_{2a} adenosine receptor as a measure of receptor:G protein ternary complex formation, concluding that $\beta_4\gamma_2$ had a 5-fold higher affinity for the G_s α:A_{2a} adenosine receptor complex than $\beta_1\gamma_2$ (*21*). Both of these papers argue that the β_4 subunit is much more efficient than the β_1 subunit at coupling G_s α to the A_{2a} adenosine receptor. Evidence that these results may not be limited to the G_s-linked A_{2a} adenosine receptor was provided by a study that demonstrated that $\beta_4\gamma_2$ was more efficient than $\beta_1\gamma_2$ at promoting agonist-dependent nucleotide exchange at the M_2 muscarinic receptor (*26*). Approaches similar to the original antisense techniques of Kleuss et al. (*54*) are also demonstrating the importance of β-isoform specificity in receptor signaling. Specific gene silencing by siRNA via lentiviral vectors has shown that ablation of the β_2 subunit abolishes the C5a-mediated chemotactic response in macrophages (*55*); ablation of the β_1 subunit had no effect on this signaling system, suggesting that G protein heterotrimers coupling to the C5a receptor contain β_2 but not β_1.

Progress has also been made with respect to the influence of the G protein γ isoform toward receptor signaling. Early work suggested that the nature of the prenyl group at the C terminus of the γ subunit influenced receptor coupling, with more efficient coupling at the A_1 adenosine receptor associated with the geranylgeranyl lipid. Substitution of this lipid on the γ_2 subunit with the farnesyl moiety significantly reduced receptor coupling, and replacing the farnesyl group on the γ_1 subunit with geranylgeranyl increased receptor coupling (*14*). Conversely, the farnesyl lipid was found to be more effective than the geranylgeranyl lipid at coupling the $\beta_1\gamma_1$ dimer to rhodopsin (*13*). Later studies implicated the primary sequence of the γ subunit as a determinant of the receptor:G protein interaction. For example, in a panel of geranylgeranylated $\beta_1\gamma$ dimers containing various γ isoforms, the $\beta_1\gamma_5$ and $\beta_1\gamma_{10}$ dimers were notably weaker in their ability to stimulate the high-affinity state of the α_{2A}-adrenergic receptor (*56*). Moreover, in a comparison of G proteins containing the γ_5 or γ_7 subunits; G_o α:$\beta_1\gamma_7$ displayed a 2-fold increase over G_o α:$\beta_1\gamma_5$ in its ability to undergo M_2 muscarinic receptor-dependent hydrolysis of GTP (*57*). Intriguingly, the γ_{11} subunit, which is widely expressed and modified by the farnesyl lipid (*58*, *59*), has been shown to be more efficient than other geranylgeranylated $\beta\gamma$ dimers at promoting the high-affinity agonist-binding state of the A_1 adenosine and 5-HT_{1A} receptors (*15*).

These reports of differential activity of β and γ isoforms at the receptor suggest the importance of G protein subunit heterogeneity; however, the biological activity of a G protein β or γ subunit must be evaluated in the context of the functional dimer. For example, a study comparing the ability of purified β dimers to support high-affinity agonist binding to the α_{2A}-adrenergic receptor found that $\beta_1\gamma_5$ was significantly less effective than $\beta_1\gamma_{11}$ (*56*). One conclusion that could be drawn from this result is that the γ_5 subunit does not couple well to the α_{2A}-adrenergic receptor. However, the study also found that the $\beta_3\gamma_5$ dimer was just as effective as $\beta_1\gamma_{11}$ in supporting the high-affinity state of the receptor (*56*), providing an excellent example of how the combination of β and γ ultimately dictates interactions with receptors.

Because the combination of the β_4 and γ_{11} subunits proved to have the lowest efficiency at promoting nucleotide exchange in reconstitution experiments with the M_1 muscarinic and A_1 adenosine receptors, the possibility that β_4 and γ_{11} may represent an incompatible dimer combination, similar to $\beta_2\gamma_1$ (*7*), was explored by transfection of HEK-293T cells with tagged β_4 and γ_{11}. However, both the $\beta_4\gamma_{11}$ dimer and the $\beta_4\gamma_2$ dimer were able to be purified from Genapol extracts of membrane preparations from these cells after transfection (see the Results), suggesting that $\beta_4\gamma_{11}$ is a viable signaling molecule in a mammalian cell.

To identify the molecular reasons for low activity of purified $\beta_4\gamma_{11}$ at the receptor, the affinity between α and $\beta\gamma$, a prerequisite for the G protein:receptor interaction, was examined. In comparison to the $\beta_4\gamma_2$ dimer, $\beta_4\gamma_{11}$ had a much lower affinity for G_{i1} α. This low affinity was not easily reconciled with the purification protocol, which includes the extraction of protein from membranes with 0.1% Genapol and column washes with buffer containing 0.1% CHAPS. All of these steps suggest a $_{6HIS}$-G_{i1} α:$\beta_4\gamma_{11}$ heterotrimer that remains tightly bound until activation-dependent elution of the dimer from the immobilized $_{6HIS}$-G_{i1} α. One possible explanation was that a conformational change occurred in

the $\beta_4\gamma_{11}$ dimer after activation and dissociation from $_{6HIS}$-G_{i1} α.

To probe any conformational changes occurring in $\beta_4\gamma_{11}$, gel-filtration chromatography was used to analyze the physical properties of the $\beta_4\gamma_{11}$ dimer in the form of a heterotrimeric G protein before and after activation. Initial clues that the $\beta_4\gamma_{11}$ dimer was structurally divergent came from chromatographic separations of purified $\beta_4\gamma_{11}$, which indicated that the β_4 subunit had aggregated into a higher molecular-weight structure (data not shown). The assumption was made that, because the $_{6HIS}$-G_{i1} α:$\beta_4\gamma_{11}$ heterotrimer was able to undergo conformational change and release $\beta_4\gamma_{11}$ during purification, it was a viable protein. Indeed, the hydrodynamic properties of this heterotrimer under nonactivating conditions were similar to the well-characterized G_{i1} α:$\beta_1\gamma_2$ and G_{i1} α:$\beta_1\gamma_{11}$ (parts A and C of Figure 4). However, incubation of the protein with Mg^{+2} and GTPγS resulted in an elution profile that clearly shows the dissociation of β_4 from γ_{11}. Dissociation of all three G protein subunits would lead to signaling via activated GTP-bound α but no effective $\beta\gamma$ signal. In addition, the loss of $\beta\gamma$ structural integrity would preclude further receptor coupling in a mechanism with functional parallels to the absorption of the $\beta\gamma$ dimer by phosducin (*27*) and thus terminate the G protein activation/deactivation cycle for such heterotrimers containing β_4 and γ_{11}.

There are various examples in the literature of weak affinity between β and γ subunits. The β_5 subunit has been reported to weakly associate with γ_2, a condition that is exacerbated by the presence of certain detergents (*60*, *61*). Trypsin protection studies have also suggested that the β_3 isoform is weaker than the β_1 or β_2 isoforms in its interaction with γ subunits (*62*). Weak affinity of the γ_{11} subunit for β has been implied experimentally, because purification or immunoprecipitation of γ_{11} containing dimers has proven difficult (*59*, *63*). In addition, *in vitro* translation experiments revealed that the closely related γ_1 isoform was limited in its ability to dimerize with different β isoforms (*6*, *7*).

The physical separation of β_4 and γ_{11} observed chromatographically explains the loss of functional activity in several of the assays used to characterize $\beta_4\gamma_{11}$. For example, dissociation of β_4 from γ_{11} subsequent to activation-dependent elution from a G_{i1} α column accounts for the low affinity of $\beta_4\gamma_{11}$ for α_{i1} observed in the competition assay (Figure 3). Further, this suggests that dissociation of β_4 from γ_{11} is not easily reversed under the experimental conditions used. This mechanism is supported by data in Figure 2, in which purified $\beta_4\gamma_{11}$ has a low activity in receptor reconstitution experiments. Perhaps the most direct examination of the functional consequences of dissociation of β_4 from γ_{11} is in the reconstitution experiments with PLC-β and the G_{i1} α:$\beta_4\gamma_{11}$ heterotrimer (parts C and D of Figure 5). Activity of G_{i1} α:$\beta_4\gamma_{11}$ on PLC-β decreased when G protein activation preceded reconstition into vesicles, suggesting a result similar to that of the chromatography experiment with activated G_{i1} α:$\beta_4\gamma_{11}$: dissociaton of β_4 from γ_{11} in solution. The higher activity of G_{i1} α:$\beta_4\gamma_{11}$ under conditions when it was allowed to reconstitute with phosholipid vesicles and PLC-β prior to activation may reflect a more stable environment for $\beta_4\gamma_{11}$.

The stability of the G_{i1} α:$\beta_4\gamma_{11}$ heterotrimer under nonactivating conditions was mirrored functionally by the observation that, when this G protein is assembled in an Sf9 cell, it is able to promote the high-affinity state of the A_1 adenosine receptor as well as the G_{i1} α:$\beta_4\gamma_2$ heterotrimer. Dissociation of β_4 from γ_{11} also influences the interpretation of differences in GTPγS sensitivity of A_1 receptor:G_{i1} α:$\beta_4\gamma_2$ and A_1 receptor:G_{i1} α:$\beta_4\gamma_{11}$ complexes in Figure 7C. High-affinity agonist binding is a sensitive indicator of the formation of the receptor:G protein complex, and GTPγS will destabilize the complex by changing the conformation of the G protein by binding the α subunit and potentially inducing subunit dissociation (*50*). However, dissociation of β_4 from γ_{11} as a consequence of GTPγS binding could also destabilize the receptor:G protein complex and provide a mechanism for the differences in GTPγS sensitivity. Because a $\beta_4\gamma_{11}$ dimer could be purified from mammalian HEK-293T cells, the fact that the A_1 receptor:G_{i1} α:$\beta_4\gamma_{11}$ complex was found to be much less stable than A_1 receptor:G_{i1} α:$\beta_4\gamma_2$ in a membrane environment provides evidence that the mechanism of activation-dependent dissociation of $\beta\gamma$ dimers containing γ_{11} could affect signaling *in vivo*. For example, G proteins containing β_4 and γ_{11} may be more sensitive than other G proteins and activate with lower levels of stimulus at the receptor. In addition, dissociation of β_4 and γ_{11} could result in a null $\beta\gamma$ signal, channeling signaling information through the α subunit. Thus, a heterotrimer containing β_4 and γ_{11} could productively interact with an activated receptor, and the duration of α signaling would depend upon other factors, such as the presence of regulators of G protein signaling (RGS) proteins or nearby free $\beta\gamma$ dimers of higher stability to recycle with the receptor. These results also raise the question of the fate of monomeric β_4 and γ_{11} in a cell and whether these subunits reassociate with each other or neighboring $\beta\gamma$ subunits. Future studies will be needed to address these issues.

These results for the first time attribute the reduced affinity of a $\beta\gamma$ dimer containing γ_{11} to critical steps in the G protein activation cycle: nucleotide exchange or subunit dissociation caused by nucleotide exchange. The significance of this finding is bolstered by the conclusion of a recent study that tracked the movement of fluorescently tagged α, β, and γ subunits in cells after receptor activation (*64*). A major finding of the study was that $\beta_1\gamma_{11}$ physically separated from α and translocated from the plasma membrane to the Golgi as a result of α-subunit activation (*64*); while this does not directly address the issue of $\beta_4\gamma_{11}$ dissociation *in vivo*, it is an important concept that receptor activation of G proteins containing the $\beta_1\gamma_{11}$ dimer can result in the spatial segregation of α and $\beta\gamma$ signaling. The conclusions from our study build upon these novel findings, in that in addition to spatial segregation of G protein signaling, dissociation of particular $\beta\gamma$ dimers, such as $\beta_4\gamma_{11}$, upon G protein activation could result in temporal regulation of G protein signaling. This broad range of functionalities of the γ_{11} subunit, exquisitely dependent upon its β-subunit partner, suggests a prominent role for γ_{11} in G protein signaling.

ACKNOWLEDGMENT

We thank Dr. E. Ross and Dr. T. Kozasa for the baculoviruses encoding the M_1 muscarinic receptor and the $_{6HIS}$-G_{i1} α subunit, respectively; purified PLC-β was a kind gift from Dr. K. Harden. We thank Dr. D. Manning for the $G_{i1/2}$ α antibody. We thank Dr. Margaret Lindorfer for the generous donation of the Superose 6 column used in this

study. We thank Dr. Miller Jones for the γ_{11} antibody, and Drs. Fraydoon Rastinejad, Wladek Minor, and Shuren Wang for helpful discussions on modeling the $\beta_4\gamma_{11}$ interactions. We also thank Melinda Ring for expert technical assistance, and Dr. Nicholas Sherman of the W. M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia, for mass spectrometric analysis. We acknowledge the University of Virginia Pratt Committee for its generous support of the Biomedical Research Facility.

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BI0604882