The G Protein β Subunit Is a Determinant in the Coupling of Gs to the β1-Adrenergic and A2a Adenosine Receptors*

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The signaling specificity of five purified G protein βγ dimers, β1γ2, β2γ2, β3γ2, β4γ2, and β5γ2, was explored by reconstituting them with Gα and receptors or effectors in the adenyl cyclase cascade. The ability of the five βγ dimers to support receptor-αβγ interactions was examined using membranes expressing the β1-adrenergic or A2a adenosine receptors. These receptors discriminated among the defined heterotrimers based solely on the β isoform. The β4γ2 dimer demonstrated the highest coupling efficiency to either receptor. The β3γ2 dimer coupled poorly to each receptor, with EC50 values 40–200-fold higher than those observed with β1γ2. Strikingly, whereas the EC50 of the β1γ2 dimer at the β1-adrenergic receptor was similar to β2γ2, its EC50 was 20-fold higher at the A2a adenosine receptor. Inhibition of adenyl cyclase type I (AC1) and stimulation of type II (AC2) by the βγ dimers were measured. βγ dimers containing Gβ1–4 were able to stimulate AC2 similarly, and Gβ3–2γ2 was much less potent. β1γ2, β2γ2, and β3γ2 inhibited AC1 equally; β4γ2 was 10-fold less effective, and β5γ2 had no effect. These data argue that the β isoform in the βγ dimer can determine the specificity of signaling at both receptors and effectors.

Signal transduction involving heterotrimeric G proteins1 is a universal mechanism for the integration of extracellular stimuli such as hormones, neurotransmitters, odorants, and light (1, 2). The components involved in this signaling cascade are diverse, including a large number of receptors, G protein α and βγ subunits and effectors. Even though the diversity of the proteins in this system could potentially account for the known specificity of signaling in differentiated cells, the mechanisms for determining specificity are not completely defined. The β1-adrenergic receptor is one of the most well characterized seven transmembrane spanning receptors, and provides an excellent example of selective coupling to a particular α subunit, Gs. When activated, Gs can stimulate all nine adenyl cyclase isoforms (3, 4). The G protein βγ dimer, when released after receptor activation, is also able to regulate adenyl cyclase (5). However, the regulation of the various isoforms of adenyl cyclase by the βγ dimer is much more selective; apparently, only AC2, AC4 (6, 7), and AC7 (8) are stimulated by βγ, whereas the neuronal-specific AC1 (4) and possibly AC5 and AC6 are inhibited by the dimer (9). Moreover, there is evidence that AC2 does not respond well to dimers composed of certain β and γ subunits (10) or to dimers containing the phosphorylated γ2 subunit (11). Thus, to understand fully the regulation of adenyl cyclase by a Gs-coupled receptor, one needs to know which βγ dimers are most likely to support receptor G protein coupling and the effects of βγ dimers on the various isoforms of adenyl cyclase.

The number of functionally distinct βγ dimers is potentially very large, with seven G protein β isoforms (including two splice variants) and 12 γ isoforms characterized to date (12–14). Most in vitro studies involving coupling of receptors to Gs α or regulation of adenyl cyclases by distinct βγ dimers have used dimers containing the β1, β2, or β3 subunits (15, 16). The ubiquitous cellular and tissue distribution of Gs α provides the potential for interaction with all five β isoforms and underscores the importance of understanding the role of the different β isoforms on signaling pathways involving Gs α. For example, the antisense studies of Kleuss et al. (17–19) suggest that specific isoforms of the heterotrimer couple to different receptors, and a number of in vitro studies imply that defined βγ dimers may be released upon receptor activation (16, 20, 21). In addition, isolation of G protein heterotrimers from a variety of tissues using chromatography or immunoprecipitation has shown that certain β and γ subunits preferentially associate with one another as well as with distinct αo isoforms (22, 23). These data suggest that specific combinations of G protein subunits do exist in vivo and may have specialized roles in various signaling cascades.

To examine the roles of the various β subunits in receptor-Gs coupling, and in regulating adenyl cyclase, recombinant Gs α and βγ dimers containing β1–5 complexed with γ2 were expressed in baculovirus-infected SF9 insect cells and purified. Proteins were then reconstituted into partially purified SF9 cell membranes overexpressing either the β1-adrenergic receptor, the A2a adenosine receptor, AC1 or AC2. The effects of the β1–γ2, β1–γ3, and β1–γ4 combinations were measured in four assays as follows: 1) the ability to couple the Gs α subunit to the β1-adrenergic receptor; 2) the ability to couple the Gs α subunit to the A2a adenosine receptor; 3) the ability to stimulate AC2; and 4) the ability to inhibit AC1. Clear differences were observed among the five βγ dimers in both receptor coupling and effector regulation, suggesting that the diversity of the β subunit contributes extensively to signaling specificity.

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1 The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; SF9 cells, Spodoptera frugiperda cells; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; AMP-PNP, 5′-adenylylimidodiphosphate; PMPSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; Genapol C-100, polyoxyethylene (10) dodecyl ether; GRR, G protein receptor kinase; BGS, regulator of G protein signaling; AC1 or AC2, adenyl cyclase type I or type II; PLC-β, phosphatidylinositol-specific phospholipase C-β isoform; GTPγS, guanosine 5′-3-O-(thio)triphosphate; Ni2+-NTA, Ni2+-nitritroacetate acid; PAGE, polyacrylamide gel electrophoresis.
EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses—Construction of baculovi-

cruses encoding the β1, β2, β3, γs, γs3, the Gs α and, Gs α subunits has been described (11, 24–26). The viruses encoding AC1 and AC2 were the kind gift of R. Iyengar (27, 28). Baculoviruses encoding the rat β1-adrenergic receptor and the human A2a adenosine receptor were gifts from E. Ross (University of Texas, Southwestern Medical Center) and D. Rosskopf (University of Virginia), respectively (29). The human β3 cDNA (30), a gift from S. R. Ikeda (Guthrie Institute), was excised from pcDNA3 with BamHI and XhoI. The human β,γ cDNA, a gift from D. Rosskopf (Institute for Pharmacology, Essen, Germany), is a truncated variant of the full-length β,γ cDNA, in which the β,γ protein product has a deletion of amino acids 108–208 (32); excision of the β,γ cDNA from pDHET was accomplished with BamHI and PstI. The existing restriction sites were used to ligate digestion products into the multiple cloning site immediately downstream of the polyhedrin promoter in the baculovirus transfor-

m vector, pVL1393. All clones were sequenced to confirm the fidelity of the cDNA in pVL1393. Recombinant baculoviruses for β1, β3, and β2 were prepared by co-transfection of linear wild type BaculoGold® viral DNA (PharMingen) with pVL1393 containing the specific β sequences into Sf9 cells as described (26) and purified with one round of plaque purification (33).

Expression and Purification of Recombinant G Protein α and β γ Dimers—Sf9 cells were infected with recombinant baculoviruses encoding the desired α and/or βγ dimer combinations at a multiplicity of infection of three and harvested 48–60 h after infection. βγ dimer containing β1,α, and β3,α were purified by G s affinity chromatography as described (34). The dimer containing β1,α was expressed with a γs subunit engineered to have a hexahistidine and FLAG tag (26) at the N terminus (γs3), and purified from isoframe Sf9 cell membranes by FLAG affinity and Ni2+ affinity chromatography, followed by anion exchange chromatography (16). Mass spectrometry was used to examine post-translational processing of the purified βγ dimers. Purified βγ dimers were analyzed by matrix-assisted laser desorption ionization-mass spectrometry to obtain masses of the γ subunits as described in Linderor et al. (35). For βγ dimers with a protein concentration of less than 150 mg/ml, acetone precipitation was used to concentrate the protein before mass analysis (36). Post-translational processing of the γ3 isofrom includes cleavage of the N-terminal methionine, acetylation of the resulting N-terminal alanine, geranylation at the cysteine four residues from the C terminus, cleavage of three C-terminal residues, and carboxymethyla-

tion of the resulting C-terminal geranylated cysteine. These post-translational modifications have been observed in γ3 subunits isolated from bovine brain (37) and in Sf9 cells (35). The predicted mass of the properly processed γ3 isoform is 7750 Da; insertion of a His-Flag (HF) tag at the N terminus increases the predicted mass to 10,013 Da. Mass spectrometry of purified βγ isoforms containing the HF tag demonstrated that the major mass in each spectrum was consistent with these predicted masses within the accuracy of the instrument (35). For example, in one set of purified βγ dimers, the experimental masses of the γs subunits were as follows: β1,γ3, 7755 Da; β2,γ3, 7764 Da; β3,γ3, 7760 Da; β1,γs3, 7759 Da; and β2,γs3, 10,020 Da.

Attempts were made to purify β,γ3 combined with various γ s subunits. A protein with the appropriate molecular weight was expressed in Sf9 cells as judged by immunoblotting with a β-common antibody (PerkinElmer Life Sciences 808); however, the major barrier to purification was that it was not possible to solubilize the protein from the Sf9 cell pellet. For example, soluble extracts of whole cell pellets prepared using 1% (w/v) Genapol, 1% (w/v) CHAPS, or 1% (w/v) Cholate contained little if any protein in supernatant fractions that could be detected by the β-common antibody. Expression of various α and the γ3,γs3, γs, γs3 subunits with β,γ in Sf9 cells did not affect the solubility of the protein (data not shown), and thus characterization of this protein was not pursued.

Gs α was overexpressed with a β3 subunit engineered to have a hexahistidine and FLAG tag (11) at the N terminus (β3,γs3), along with the modified Gs α subunit. A modification of the method of Kosaza and Gilman (38). Briefly, harvested cells were resuspended in half the infection volume with cell lysis buffer (20 mM Tris, pH 8.0, 10 μM GDP, 17 μg/ml PMSF, and 2 μg/ml pepstatin, leupeptin, and aprotinin). After resuspension, cells were lysed by nitrogen cavitation (25), and membranes were collected by centrifugation at 28,000 × g for 20 min at 4 °C. A Potter-Elvehjem homogenizer was used to resuspend the pellets in a quarter of the original resuspension volume (~65 ml) of cell lysis buffer containing 10 μg/ml DNase. After a 15-min incubation on ice, membranes were collected again by centrifugation at 28,000 × g for 20 min at 4 °C and resuspended with a Potter-Elvehjem homogenizer in a volume of extraction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 0.5% (w/v) Genapol, 1 mM β-mercaptoethanol, 10 μM GDP, 5 mM imidazole, 17 μg/ml PMSF, and 2 μg/ml pepstatin, leupeptin, and aprotinin) and loaded onto a Ni2+-NTA Superflow column at 2 ml/min. Unless otherwise noted, all steps were performed at 4 °C. The volume of the eluate was ~5% of the volume of the Genapol extract. The column was washed with 6 volumes column volumes of Ni2+ column buffer, 6 volumes column volumes of Ni2+ column buffer containing 300 mM NaCl, and 3 more column volumes of Ni2+ column buffer. At this point, the column and buffers were warmed to room temperature for 10–20 min, and Gs α was activated and eluted with 4 columns volumes of activation buffer (Ni2+ column buffer containing 50 mM MgCl2, 150 mM NaCl, and 1 mM AlCl3) also warmed to room temperature. Although the increased temperature facilitates activation of the α subunit, this step should be completed as quickly as possible, as functional activity of α decreases with prolonged elevation of temperature. Pilot experiments using SDS-

PAGE to identify the Gs α subunit indicated that the first 8 ml of eluate after the void volume contained the protein. Therefore, all fractions were collected on ice and pooled. The fractions containing Gs α were diluted 5-fold with 15Q buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM MgCl2, 0.1% (w/v) CHAPS, 1 mM DTT, 10 μM GDP) and loaded onto a 200-μl Ni2+ anion exchange column. This dilution facilitates adsorption of the protein to the column by reducing of the Cl− concentration to ~50 mM. In addition to concentrating the protein, the 15Q step is necessary to remove AlF4−, imidazole, and Genapol, which would itself be concen-

trated along with the protein in the next concentration step. After the protein was loaded, the column was washed with 15Q buffer containing 10 mM NaCl (15Q buffer A) for 20 min at 1 ml/min. Protein was then eluted with 15Q buffer containing 600 mM NaCl (15Q buffer B) in a linear gradient of 0–50% 15Q buffer B over 15 min. One-mL fractions were collected, and 12% SDS-PAGE followed by either immunoblotting or staining with purified Gs α as a standard was used to deter-

mine which fractions contained Gs α. Fractions from the 15Q column containing Gs α were pooled and concentrated with a Centricron 30 that had been passivated with a 1% BSA solution as described (16). The concentrated protein was diluted 10-fold with 15Q buffer containing 100 mM NaCl to reduce the high NaCl concentration that resulted from the elution from the 15Q column, then concentrated once more to a volume of ~0.5 ml, stored at ~80 °C. The yield of purified Gs α from 10 g of Sf9 cell pellet (wet weight) was typically 10–20 μg. All protein estimates were determined using scanning densitometry of silver-stained gels as described previously (26), with standard curves generated from ovalbumin standards.

Gs α was purified by a similar method with the following exceptions. The Ni2+ column buffer contained 20 mM Tris, pH 8.0, 150 mM NaCl, 0.2% (w/v) CHAPS, 1 mM β-mercaptoethanol, 10 μM GDP, 5 mM imi-
dazole, 17 μg/ml PMSF, and 2 μg/ml pepstatin, leupeptin, and aprotinin. Protease inhibitors and imidazole were removed from the elution step, and the Gs α was taken directly to a Centricron 30 where it was concentrated and diluted 10-fold with Ni2+ column buffer supplemented with 2 mM MgCl2. This step was repeated, and 100–200 μl of Gs α at 100–200 ng/ml were stored in aliquots at ~80 °C. As one criterion for the viability of the Gs α and Gs α subunits, the ability of the proteins to bind GTPγS to solution was measured. The stoichiometry of nucleotide binding of two preparations of Gs α averaged 0.9 mol/mol. The Gs α subunit bound GTPγS at a stoichiometry of ~0.3 mol/mol and also contained effects the modifications. G1 adenosine receptor in assays similar to the one shown in Fig. 2A (data not shown).

Preparation of Membranes Containing Recombinant βi-Adrenergic Receptors, A2A Adenosine Receptors, or Adenylyl Cyclases—Sf9 cells were infected with recombinant baculoviruses encoding either the rat βi-adrenergic receptor, the human A2a adenosine receptor, and either type I or type II adenylyl cyclase (27, 28). In the case of the βi-adrenergic receptor, harvested cells were resuspended in membrane
homogenization buffer (20 mM HEPES, pH 7.5, 2 mM MgCl2, 1 mM EDTA, 17 μg/ml PMSF, and 2 μg/ml leupeptin and aprotinin), and cells were lysed by nitrogen cavitation. The cell lysate was centrifuged at 750 × g to pellet unbroken cells and nuclei. Membranes were prepared from the supernatant of the low speed spin by centrifugation at 25 000 × g for 30 min at 4 °C. Resuspension of the membranes was by gentle agitation at 142,000 × g for 30 min at 4 °C. Membranes were washed twice with resuspension buffer, and homogenization buffer was used to resuspend the membranes, which were stored in aliquots at −80 °C.

Membranes containing the A2a adenosine receptor were prepared using essentially the same method, except that homogenization buffer consisting of 25 mM HEPES, pH 7.5, 100 mM NaCl, 1% (w/v) glycerol, 17 μg/ml PMSF, and 2 μg/ml leupeptin and aprotinin was used through the preparation. Radioligand binding experiments with [3H]dipyridamycin and from the supernatant of the low speed spin by centrifugation at 28 000 × g at room temperature at a protein concentration of 0.9 mg/ml were performed essentially as previously described (32). Membranes were resuspended from the supernatant or inactivated by incubation with urea. Membranes containing the β2-adrenergic receptor were homogenized with resuspension buffer (50 mM HEPES, pH 7.5, 3 mM MgSO4, 1 mM EDTA, 17 μg/ml PMSF, and 2 μg/ml leupeptin and aprotinin) containing 7 μg/ml and allowed to incubate for 30 min at 4 °C. Resuspension buffer was used to dilute the membranes to 4 μg/ml prior to centrifugation at 142,000 × g for 30 min at 4 °C. Membranes were washed twice with resuspension buffer, and homogenization buffer was used to resuspend the membranes, which were stored in aliquots at −80 °C.

Reagents for Sf9 cell culture and purification of G proteins—Reagents for Sf9 cell culture and purification of the β2-adrenergic and A2a adenosine receptors, respectively. Stripping membranes with urea did not greatly affect the pharmacological properties of these two receptors (data not shown). The GTP-S binding experiments were performed using the radioligand [35S]GTPγS (final concentration 0.25 μM and the indicated [35S]GTPγS concentration) containing membranes expressing either the β2-adrenergic or the A2a adenosine receptor. Membranes expressing AC1 or AC2 were prepared as described previously (28). Total membrane protein concentration was determined by BCA assay using bovine serum albumin as a standard, and aliquots of membranes were stored at −80 °C.

Measurement of Agonist-stimulated GTP S Binding to Gα after Reconstitution with βγ2 Membranes—Expressing either the β2-Adrenergic Receptor or the A2a Adenosine Receptor—Kinetic parameters of agonist-stimulated binding of [35S]GTPγS to Gα in the presence of different concentrations of βγ2 were established with time course experiments. Aliquots of Sf9 cell membranes containing the β2-adrenergic receptor were pelleted by centrifugation and resuspended in 100–400 μl of GTPγS binding buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 0.1% BSA, 0.5 μM GDP, and 1 μM AMP-PNP) with a 28-gauge needle. The membrane suspension was reconstituted with 5 nM Gα subunit such that the Gα receptor ratio was 26:1; different concentrations of βγ2 were then added and allowed to incubate for 30 min at 4 °C. The incubation temperature was increased to 25 °C for 10 min to equilibrate the reconstituted system to the reaction temperature; additions of 17 γ and isoproterenol (final concentration 1 μM) initiated the time course. The binding of [35S]GTPγS to receptor-activated Gα was measured at 1-min intervals by vacuum filtration. Increasing concentrations of βγ2 increased the rate of receptor-catalyzed exchange of GDP for GTPγS on Gα. The observed rates were relatively linear (see Fig. 2A), thus the effect of βγ2 was quantified by the amount of [35S]GTPγS binding to the receptor, calculated as percentage of maximal GTPγS binding as determined by the one-site binding curves generated by GraphPad Prism. After normalization, the data were averaged for each βγ2 isoform, and GraphPad Prism was used to obtain estimates of the EC50 values and statistical analysis of the binding curves. These data are presented in Table 1.

By using this protocol, 10 different βγ2 dimers were used to estimate EC50 values for the potential of Gα-stimulated AC2 activity by βγ2. At least three experiments using data from two different sets of βγ2 were analyzed, and average values were reported in Table 1. For AC1, GraphPad Prism was used to generate inhibition curves for each of the experiments with the different βγ2 dimers; the data were then normalized as percent inhibition of the estimated rate of cAMP production with 50 nM GTPγS-stimulated AC1 as 100%. Normalized data from three different experiments and two different sets of βγ2 dimers were averaged and analyzed by GraphPad Prism to obtain IC50 estimates (Table 1). Statistical significance for differences among binding curves for both receptors and AC1 and AC2 was determined using the F-statistic; this technique is able to discern small but significant differences between two binding curves (42). Signaling peptides for Sf9 cell culture and purification of βγ dimers has been described previously (16, 25, 26, 34). 125I-ZM-241385 was a kind gift from J. Linden, University of Virginia; baculovirus transfer vector was from Invitrogen; the BaculoGold kit was from Pharmingen; DNase, GDP, imidazole, isoproterenol, and HEPES were from Sigma; adenosine deaminase, CHAPS, and GTPγS were from Roche Molecular Biochemicals; P-8 desalting gel was from Bio-Rad; 10% Genapol C-100 was from Calbiochem; Ni2+-NTA Superflow resin was from Qiagen; [35S]GTPγS and [125I]dipyridamycin from PerkinElmer Life Sciences; Source 15Q anion exchange resin was from Amersham Pharmacia Biotech; type HA 0.45-μm nitrocellulose filters and Centricon 30 concentrators were from Millipore. All other materials were of the highest available purity.

RESULTS

Preparation of G Protein α and βγ Subunits—Fig. 1A presents a silver-stained gel showing the purity of the five βγ dimers used in this study. Fig. 1B presents a similar gel showing the purity of the Gα used. Significantly, both the βγ dimers and the Gα subunit were purified using biological affinity columns. The dimers containing βγ4 were purified with a Gα1-agarose column ensuring that the proteins bound to α subunits with high affinity and that the C terminus of the γ subunit was properly modified (see “Experimental Procedures”). Even so, a β doublet was occasionally observed in the
Fig. 1. Purity of G protein α and β subunits. A, the five isoforms of the β subunit were overexpressed in Sf9 insect cells with the γ2 or γ2HF subunit and purified by Gs α affinity chromatography (βsγ2γ1 or Ni2⁺-NTA affinity chromatography (βsγ2γ1). B, Gs α was overexpressed in Sf9 insect cells with a β1γ2 containing a hexahistidine tag. The heterotrimer was adsorbed to a Ni2⁺-NTA column, and Gs α was eluted specifically with 8 M urea. Purity of β1γ2 dimers (250 ng of each isoform) and Gs α (150 ng) was visualized by silver staining after separation by 12% SDS-PAGE; positions of molecular weight markers are indicated at the right. C, Sf9 cell membranes expressing AC2 were incubated with increasing concentrations of Gs α activated with GTPγS, and cAMP levels were determined using a radioimmunoassay; the calculated EC50 for the experiment shown is 1.9 nM. D, Sf9 cell membranes expressing AC1 were characterized as in C; the calculated EC50 for the experiment shown is 8.5 nM.

SDS-PAGE analysis of βsγ2: The reasons for this behavior are not understood. The βsγ2HF dimer was visualized by silver staining after separation by 12% SDS-PAGE; positions of molecular weight markers are indicated at the right. C, Sf9 cell membranes expressing AC2 were incubated with increasing concentrations of Gs α activated with GTPγS, and cAMP levels were determined using a radioimmunoassay; the calculated EC50 for the experiment shown is 1.9 nM. D, Sf9 cell membranes expressing AC1 were characterized as in C; the calculated EC50 for the experiment shown is 8.5 nM.

The Ability of Different γ Subunits to Support Coupling of Receptors to Gs α—A major goal of this study was to examine the possibility that different β subunits interact selectively with certain G protein-coupled receptors. Since exchange of GDP for GTP is the first step of G protein signaling subsequent to receptor activation, an agonist-dependent GTPγS-binding assay was used. Fig. 2A presents an experiment performed with membranes expressing the β1γ2-adrenergic receptor reconstituted with Gs α and two concentrations of the βγ dimer. Note that the rate of isoproterenol-stimulated GTPγS binding is nearly linear and highly dependent on the concentration of βγ dimer. The triangles in Fig. 2A represent a basal rate of GTPγS binding to membranes reconstituted with Gs α; this rate was observed in the absence of βγ (as illustrated in the figure) or with a fully reconstituted system in the absence of isoproterenol. Coupling of receptor to G protein is a composite of many biochemical interactions, the most important being the interactions of α-βγ and receptor-α-βγ. Receptor-βγ interactions were probed with a variation of the protocol designed to be poised on the concentration of the βγ dimer. To define precisely the ability of β1γ2 to support coupling of Gs α to the β1-adre-
Fig. 3. Comparison of ability of different βγ isoforms to couple Gα to the β1-adrenergic and A2a adenosine receptors. A, five β1,γ2 isoforms were reconstituted with 5 nM Gα and membranes containing the β1-adrenergic receptor and the efficiency of coupling measured as described under “Experimental Procedures.” Data from three experiments were normalized as a percent of maximal binding of [35S]GTPγS, and the averaged data plotted; error bars, most of which were within ±10%, were omitted for clarity. B, five β1,γ2 isoforms were tested as in A, but with the A2a adenosine receptor. Data from at least three experiments were normalized and plotted as in A. C–G, data from A and B were replotted to highlight differences in each particular β1,γ2 isoform between the β1-adrenergic receptor (β) and the A2a adenosine receptor (A2a). Dotted lines indicate βγ concentrations of 1 and 10 nM on the x axis.

To determine if the rank order of affinities determined with the β1-adrenergic receptor was the same with another Gα-linked receptor, the ability of the two Gα-linked receptors to couple to the five different βγ isoforms. Perhaps the most dramatic differences occurred with the β1,γ2 isoform, which coupled 15-fold more efficiently to the β1-adrenergic receptor than to the A2a adenosine receptor (Fig. 3C). Similarly, β3,γ2 demonstrated a 7-fold difference between the two receptors (Fig. 3E). Note that β4,γ2 was the most effective at coupling Gα to either receptor (Fig. 3F), and that β5,γ2 coupled poorly (Fig. 3G). In contrast, there are minimal differences in the ability of β3,γ2 to couple to either receptor (Fig. 3D). It is important to stress that the only differences in these five sets of experiments are the types of recombinant receptor expressed in the SF9 membranes. The G protein α and βγ subunits reconstituted into the membranes were identical in each case. Thus, the data clearly demonstrate that specific G protein β subunits exhibit distinct preferences for different receptors. Importantly, these preferences are a result of interactions of receptor with the type of β subunit in the dimer, since the Gα-βγ interactions are presumably identical for both receptors.

Activation of AC2 by βγ Isoforms—The dramatic differences in the ability of the panel of βγ dimers to couple to Gα-linked receptors imply that different βγ dimers might be released by receptor activation to act on downstream effectors. Since βγ is a known potentiator of Gα-stimulated AC2 activity, and differences have been observed in the ability of dimers containing the β1 or β5 subunits to activate AC2 (9, 16), the ability of all five β subunits to activate AC2 was compared. The role of βγ in the activation of AC2 is particularly interesting in that βγ can increase the rate of cAMP production approximately 5-fold over the maximal effect of Gα (Fig. 4), suggesting βγ can regulate cAMP levels in vivo. Ten nM GTPγS-activated Gα and increasing concentrations of the five purified βγ isoforms were reconstituted with SF9 membranes expressing AC2, and cAMP production was measured. A representative experiment is presented in Fig. 4. Dimers containing β1–4 were similar in their ability to potentiate AC2 activity in the presence of acti-
Experimental Procedures; bold numbers indicate EC\textsubscript{50} or IC\textsubscript{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.

| \(\beta\gamma\) isofrom | \(\beta_1\gamma_2\) | \(\beta_2\gamma_2\) | \(\beta_3\gamma_2\) | \(\beta_4\gamma_2\) | \(\beta_5\gamma_2\) |
|-------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| \(EC_{50}, \text{nM} \) | 1.0 (0.7–1.4)     | 15.7 (11.5–21.5)  | 3.5 (2.4–5.1)     | 12.9 (10.3–16.3)  | 12.2 (8.0–18.6)   |
| \(IC_{50}, \text{nM} \) |                  | 5.7 (3.8–8.5)    | 5.5 (3.6–8.5)    | 5.5 (3.6–8.5)    | 16.8 (10.3–27.4)  |

\(a\) Significantly different from \(EC_{50}\) values for other four \(\beta\gamma\) isofroms; \(p < 0.001\).  
\(b\) Only isofroms not significantly different from each other; \(p > 0.001\).  
\(c\) Significantly different from \(\beta_1\gamma_2\) and \(\beta_2\gamma_2\) and \(\beta_5\gamma_2\); \(p < 0.001\).  
\(d\) Significantly different from the other four \(\beta\gamma\) isofroms; \(p < 0.001\).

**DISCUSSION**

Although considerable research has focused on the role of the \(\alpha\) subunit in receptor signaling via \(G\) proteins, it is also clear that the \(\beta\gamma\) dimer is required for coupling the \(\alpha\) subunit to receptors (16, 39, 44, 45), and both the \(\beta\) and \(\gamma\) subunits appear to contribute to the interaction. Current evidence indicates that the C-terminal 10 amino acids of the \(\gamma\) subunit and the nature of the prenyl group (farnesyl versus geranylgeranyl) are very important determinants of the coupling of \(G\) proteins to receptors (20, 21, 39). Compared with \(\alpha\) and \(\gamma\), less is known about the important domains in the \(\beta\) subunit, but cross-linking experiments indicate that the C terminus of the \(\beta\) subunit is able to interact directly with the receptor (46). However, the regions close to the C terminus of the \(\beta\) subunit are likely to be quite discrete because mutations in amino acids His\textsuperscript{311}, Arg\textsuperscript{314}, and Trp\textsuperscript{333} have no effect on the ability of the dimers to support receptor coupling but cause a major disruption in the ability of the \(\beta\gamma\) dimer to activate PLC-\(\beta\) or AC2 (47).

Whereas the diversity of the \(\beta\) and \(\gamma\) subunits offers attractive possibilities for determining the specificity of cellular signaling, the functional significance of this heterogeneity has not been completely elucidated. Some insight has come from an elegant set of experiments in which antisense mRNAs to various \(G\) protein \(\alpha\) and \(\beta\gamma\) subunits were injected into the nuclei of GH3 cells, leading to the observation that specific receptors couple to distinct isofroms of the \(G\) protein heterotrimer. These experiments indicated that the M\textsubscript{4} muscarinic receptor preferred a heterotrimer composed of \(\alpha\textsubscript{1}\); \(\beta_3\gamma_4\), whereas the soma-
carnic receptors efficiently to the Gsα subunit (16), was particularly ineffective at coupling the Gsα subunit to either receptor. In contrast, the β2γ2 dimer was consistently highest in coupling efficiency for either receptor. Even more surprising was the finding that the β1γ2 dimer, which in most assays of βγ function is potent and efficacious, was very poor at coupling Gsα to the A2a adenosine receptor. These results lead to the following conclusions: 1) receptors can discriminate among the G protein heterotrimers based on the β isofrom alone; 2) all β1,4 isofoms can function in signaling cascades involving Gsα; and 3) dimers containing β3 are not likely to be released from Gs-coupled receptors. Finally, since different βγ dimers may be released upon receptor activation, the downstream effects of the distinct β isofoms may have different signaling properties.

One of the immediate downstream targets for βγ is the family of receptor kinases that phosphorylate G protein-coupled receptors upon recruitment to the membrane by the dimer (52). Experiments have examined the ability of defined βγ dimers to interact with the receptor kinases. For example, dimers containing β1 and β2 interact with GRK2, the kinase responsible for down-regulation of the β-adrenergic and A2a adenosine receptors (53) far better than dimers containing β4 (54). Another study examined the ability of a variety of defined βγ dimers to promote the phosphorylation of both the βγ-adrenergic receptor and rhodopsin by GRK2. These results indicate a significant difference in the ability of the various βγ dimers to promote phosphorylation of the βγ-adrenergic receptor or rhodopsin and suggest that the type of β subunit could determine selectivity between the two receptors (55). Thus, even though β1,4 are over 80% identical, the accumulating evidence suggests the type of β subunit in the dimer may have a larger role in determining signaling specificity than previously appreciated.

Another immediate downstream target for the βγ subunit is the effector adenyl cyclase (3). Results presented here demonstrate that dimers containing β1, β3, or β4 were able to regulate either type of adenyl cyclase effectively. In contrast, β2γ2 was not particularly effective at inhibiting AC1 in or stimulating AC2 (16). Intriguingly, β2γ2 was almost 10-fold weaker at inhibiting AC1 as compared with the β1,4 isofoms (Table I), whereas it was equally effective on AC2. These results suggest that upon stimulation of certain Gs-linked receptors, co-activation of AC2 by βγ is relatively nonspecific, whereas inhibition of AC1 by βγ is more selective and may be receptor-dependent. This specificity of interaction between AC1 and the different β isofoms suggests that dimers containing the β3 subunit have signaling roles distinct from those containing β1, β2, or β4.

The regions of the βγ dimer that are thought to interact with AC1 and AC2 have been examined using competition experiments with synthetic peptides and alanine mutagenesis. Peptides identical to residues 86–105 and 115–135 of the β1 subunit were able to inhibit stimulation of AC2 by the βγ dimer, implicating these residues of the β subunit (56), as well as others (57), as sites on the molecule that interact with AC2. Moreover, the QEHA peptide, which represents a sequence containing the β3 subunit (56) and interacts with AC2, was almost 10-fold weaker at inhibiting AC1 as compared with the β1,4 isofoms (Table I), whereas it was equally effective on AC2. These results suggest that upon stimulation of certain Gs-linked receptors, co-activation of AC2 by βγ is relatively nonspecific, whereas inhibition of AC1 by βγ is more selective and may be receptor-dependent. This specificity of interaction between AC1 and the different β isofoms suggests that dimers containing the β3 subunit have signaling roles distinct from those containing β1, β2, or β4.

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AC1, but seems to have little effect on activation of AC2 (60). The observation that ADP-ribosylation of Arg129 of β1, a residue also present in β2,4, prevents the inhibition of AC1 by the βγ dimer supports the argument that this is an important domain in the interaction of the dimer with AC1 (61).

Examination of the regions identified by the experiments discussed above in the β1,γ2 subunits shows minimal differences in the amino acid sequence. This is consistent with the observation that these four βγ dimers activated AC2 equally. A similar conclusion applies to the ability of three of the dimers to inhibit AC1; the intriguing exception was β2,γ2, which was far less effective. Unfortunately, there are no obvious differences in the amino acid sequence of the β2 subunit in these regions to explain the differences in activity, suggesting that some other region in the molecule is also involved in the interaction with AC1. There are, however, sequence variations that could explain the lack of activity of β2,γ2 on either isoform of cyclase. In contrast to the near identity of the β1,γ2 subunits in the regions outlined above that are thought to interact with AC2 and AC1, the β2 subunit has 13 amino acid differences in these regions when compared with the β1 subunit. Moreover, there is a two-amino acid insert in the region between residues 130–132, a site identified by ADP-ribosylation experiments as being important for the inhibition of AC1 (61). Although not definitive, these differences provide a reasonable starting point for future experiments.

Despite the homology in amino acid sequence among these β isoforms, differences in localization have been observed. For example, in contrast to the other β isoforms that were localized to the membrane, β2 was observed predominantly in cytoplasmic fractions in both heart (62) and retina (63). This property of β2 does not seem likely to be responsible for the observed differences in inhibition of AC1, since β2,γ2 was just as effective as the other βγ dimers at activating the membrane-bound protein AC2 and supporting coupling to the β1-adrenergic and A2A adenosine receptors. A more reasonable explanation is that certain residues unique to β2 impart specificity either through altering the contacts with an effector molecule such as AC1, or that unique residues slightly influence the conformation of the β subunit, thereby altering interactions with other components. Whatever the reason, one important conclusion is that differences in AC1 inhibition may result from the release of different βγ dimers by receptor activation.

This concept appears to apply especially well to dimers containing the β2 subunit. Even though the current data suggest that the β2,γ2 dimer is unlikely to be released from G-linked receptors, it clearly can be released by activation of G-linked receptors (16). However, accumulating evidence shows that the β2,γ2 dimer does not regulate a variety of effectors, including AC1, AC2, phosphatidylinositol 3-kinase, PLC-β and the mitogen-activated protein kinase pathway (this report and Refs. 64 and 65). Even though the β2,γ2 dimer did not regulate adenylyl cyclase in our experiments, transfection of the dimer into COS-7 cells caused an inhibition of both AC1 and AC2 (66). These conflicting data may be explained by other potential partners for β2, such as RGS 6, 7, 9, and 11 (67, 68), which may impinge upon the adenylyl cyclase cascade in vivo. This evidence of multiple partners for the β2 subunit suggests the β2 protein may have functions not normally associated with β subunits and makes the physiological role of β2 on effectors unclear. Especially interesting is the possibility that the β2 subunit may exist as a monomer and exchange between γ subunits and RGS proteins (69). Although the role of β2 in signaling is clearly complex, one conclusion from this information is that receptors that couple to and release dimers containing the β2 subunit are less likely to generate cross-talk between signaling systems because of the limited effect of β2 containing dimers on downstream effectors.

Brain is one tissue where all of the signaling components studied in this report are expressed at high levels (70–73). Thus, the differential effects of the β isoforms demonstrated in this report could have major effects on signaling cascades in the brain. Some experimental support for this concept comes from experiments showing that small amounts of βγ derived from Gα activation can inhibit the neuronal-specific AC1 in vivo (74). Further information needed to corroborate these proposed differences in βγ signaling includes cellular and subcellular localization of these molecular components. Once the subcellular architecture in these tissues is better understood with respect to G proteins, signaling models based on specific G protein β subunits can be refined with precision.

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βγ Isoforms and Gs α Signaling
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-Adrenergic and A2a Adenosine Receptors
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