Receptor−G Protein γ Specificity: γ11 Shows Unique Potency for A_1 Adenosine and 5-HT_{1A} Receptors[†]

William K. Lim,[‡] Chang-Seon Myung,[§,||] James C. Garrison,[§] and Richard R. Neubig*[,‡,⊥]

Departments of Pharmacology and Internal Medicine/Hypertension, The University of Michigan, Ann Arbor, Michigan 48109-0632, and Department of Pharmacology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Received May 9, 2001; Revised Manuscript Received June 22, 2001

ABSTRACT: G protein coupled receptors activate signal transducing guanine nucleotide-binding proteins (G proteins), which consist of an α subunit and a $\beta\gamma$ dimer. Whole cell studies have reported that receptors signal through specific $\beta\gamma$ subtypes. Membrane reconstitution studies with the adenosine A_1 and α_{2A} adrenergic receptors have reached a similar conclusion. We aimed to test the generality of this finding by comparing the γ subtype specificity for four G_i-coupled receptors: α_{2A} adrenergic; A1 adenosine (A_1-R); 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}-R); mu opioid. Membranes were reconstituted with Gα_{i1} and five γ subtypes (dimerized to β1). Using a sensitive α-$\beta\gamma$ binding assay, we show that all recombinant $\beta\gamma$ (except $\beta1\gamma1$) had comparable affinity for α_{i1}. Using high affinity agonist binding as a measure of receptor−G protein coupling, $\beta\gamma$-containing γ11 was the most potent for A_1-R and 5-HT_{1A}-R ($p < 0.05$, one way ANOVA) while γ7 was most potent for the other two receptors. γ11 was 3−8-fold more potent for the A_1-R than were the other γ subtypes. Also, γ11 was 2−8-fold more potent for A_1-R than at the other receptors, suggesting a unique coupling specificity of the A_1-R for γ11. In contrast, the discrimination by receptors for the other $\beta\gamma$ subtypes (β1 and γ1, γ2, γ7, and γ10) was limited (2−3-fold). Thus the exquisite $\beta\gamma$ specificity of individual receptors reported in whole cell studies may depend on in vivo mechanisms beyond direct receptor recognition of $\beta\gamma$ subtypes.

Transmembrane G protein coupled receptors convey signals from the extracellular milieu into the interior of the cell (*1*).[1] They activate signal transducing G proteins, which consist of the α subunit and a $\beta\gamma$ dimer (*2*). To date, more than 17 α (*3*), 5 β (*4*), and 12 γ (*5*) isoforms have been identified. The large number of members in the G protein coupled receptor family compared to the more limited repertoire of G proteins suggests that each receptor signals through specific G protein partners. While receptors have selectivity for one or more of the four α subunit families, it is less clear if there is a specific $\beta\gamma$ partner for each receptor. There is 80−90% amino acid identity between the $\beta1-\beta4$ isoforms which bind γ. We (*6*) and others (*7*, *8*) have provided evidence that the β subunit directly contacts the receptor, and there is emerging evidence for receptor−β coupling specificity (*9*−*11*). There is substantially less homology among γ isoforms (only 40−60%), and they have been classified into four classes by sequence homology (*12*). Hence the γ subunit may be more significant as a determinant for $\beta\gamma$ signaling diversity.

Data from certain whole cell studies have provided evidence that each receptor signals via a specific G protein heterotrimer (*13*). Using an antisense approach, it was reported that the M4 muscarinic receptor inhibits voltage-sensitive Ca^{2+} channels through a G protein heterotrimer composed of $\alpha_{01}/\beta3/\gamma4$, while the somatostatin receptor was coupled through $\alpha_{02}/\beta1/\gamma3$ (*14*). This approach has been extended to other receptors (M1 muscarinic, angiotensin AT_{1A}, and galannin) and effector systems (*15*−*18*). A ribozyme approach has identified $\beta1\gamma7$ to be involved in β adrenergic receptor stimulation of adenylyl cyclase (*19*). However, neither of these approaches clearly distinguishes between $\beta\gamma$ specificity for receptor or effector or possibly both. Although β-subtype specificity has been reported for modulation of adenylyl cyclase (*10*), several effector systems showed no specificity for $\beta\gamma$ dimers formed from various β and γ combinations (*20*, *21*) (except for a lower potency of

[†] Supported by NIH Grants HL-46417 (R.R.N.) and DK-19952 (J.C.G.), a postdoctoral fellowship from the AHA (C.-S.M.), and a training grant from the University of Malaysia at Sarawak (W.K.L.).

* To whom correspondence should be addressed. Phone: 734-763-3650. Fax: 734-763-4450. E-mail: Rneubig@umich.edu.

[‡] Department of Pharmacology, University of Michigan.

[§] Department of Pharmacology, University of Virginia Health Sciences Center.

[||] Current address: Department of Pharmacology, Kwandong University College of Medicine, Kangwon-do 210-701, Korea.

[⊥] Department of Internal Medicine/Hypertension, University of Michigan.

[1] Abbreviations: G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; Sf9, *Spodoptera frugiperda*; PIC, *p*-iodoclonidine; YOH, yohimbine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; ABA, aminobenzyladenosine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; MPPF, 4-(2′-methoxy)phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]thylpiperazine; NLX, naloxone; R-PIA, *R*-phenylisopropyladenosine; GTPγS, guanosine 5′-(3-*o*-thiotriphosphate); CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; buffer A, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM EDTA; *E. coli*, *Escherichia coli*; FITC, F-α, fluorescein isothiocynate-labeled α_{i1}; b-$\beta\gamma$, biotinylated bovine brain $\beta\gamma$; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; ANOVA, analysis of variance; PDZ, PSD-95, Dlg, ZO-1 homology.

10.1021/bi010950c CCC: $20.00
Published on Web 08/07/2001

$\beta 1\gamma 1$ (*22, 23*)). It is thus possible that specificity is determined at the interface between receptor and $\beta\gamma$.

If the mechanism for this specificity is preferential coupling of receptor to its cognate $\beta\gamma$ subtype, then it should be demonstrable using in vitro reconstitution of receptor and G protein. Such data has been published for single receptor studies in the rhodopsin (*24*), A_1-R (*25*), α_{2A}-R (*9*), and M2/M4 muscarinic receptors (*26*). $\gamma 1$ was best at supporting rhodopsin-stimulated GTPγS binding (*24*) but was least effective for support of agonist binding in the A_1-R (*25*) and α_{2A}-R (*9*). In particular, γ subtypes dimerized to $\beta 1$ supported maximal levels of agonist binding to the α_{2A}-R coupled in the order $\gamma 2 = 3 = 4 = 7 > 5 = 10 > 1$ (*9*).

As the above cited studies were carried out on single receptor types, we aimed to test the generality of these findings by co-reconstituting α_{i1} and $\beta 1\gamma$ subtypes from all four gamma classes to four G_i coupled receptors. Using a flow cytometry-based assay to measure the affinity of interaction of FITC-α for $\beta\gamma$, we observed comparable affinity for α_{i1} among the $\beta\gamma$ subtypes. However, the potency of these subtypes in supporting high-affinity agonist binding to receptor differed. $\gamma 11$ was significantly more potent at the A_1-R and 5-HT_{1A}-R receptors, while $\gamma 7$ was most potent at the α_{2a}-R and μ-R. Surprisingly, all the $\beta 1\gamma$ dimers were able to stabilize nearly equivalent maximum agonist binding and only for A_1-R was $\gamma 1$ significantly less potent than the other γ subunits. This modest degree of $\beta\gamma$ specificity observed here suggests that results reported in whole cell studies of a unique $\beta\gamma$ dimer for each receptor probably involve mechanisms beyond direct receptor−G protein recognition.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]-*p*-Iodoclonidine (PIC, 2200 Ci/mmol), [^{3}H]yohimbine (YOH, 80 Ci/mmol), [^{3}H]-2-chloro-N^6-cyclopentyladenosine (CCPA, 55 Ci/mmol), [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine (CPX, 112 Ci/mmol), [^{3}H]-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 125 Ci/mmol), [^{3}H]-4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethylpiperazine (MPPF, 78 Ci/mmol), [^{3}H]-DAMGO (55 Ci/mmol), and [^{3}H]naloxone (NLX, 55 Ci/mmol) were from DuPont-New England Nuclear (Wilmington, DE). [^{125}I]Aminobenzyladenosine (ABA, 2200 Ci/mmole) was synthesized as described (*26*). UK14,304 was from Pfizer (Sandwich, England), CPX was from RBI (Natick, MA), and urea was from Fisher Scientific (Fair Lawn, NJ) while YOH, 5HT, DAMGO, and Gpp(NH)p were from Sigma (St. Louis, MO). All other reagents were of reagent grade or better.

Construction of Stable Cell Lines and Recombinant Baculoviruses. The porcine α_{2A}-R with an amino terminal HA tag was derived from the pCMV4-TAG α_{2A}-R construct, a gift from Dr. Lee Limbird (Vanderbilt University). Silent HindIII/NheI restriction sites were introduced, and the resulting pTAGα_{2A}-AR HN plasmid was stably transfected in CHO-K1 cells to derive clone TagL1 as described (*27*). C_6 glioma cells stably transfected with the rat μ-R were a gift of Dr. John Traynor (University of Michigan) (*28*).

For preparation of recombinant α_{2A}-R baculovirus, a 1579 bp KpnI restriction fragment from pTAGα_{2A}-R HN containing the HA-α_{2A}-R coding sequence was subcloned into the KpnI site in the pFastBac baculovirus transfer vector (Gibco BRL, Gaithersburg, MD) and the orientation checked by restriction mapping. Virus stocks were prepared according to manufacturer's protocol. Recombinant baculovirus encoding the bovine A_1-R receptor was prepared as described (*29*), while that encoding the human 5-HT_{1A}-R was provided by Dr. David Manning (University of Pennsylvania) (*30*). Viruses were amplified by infecting ∼100 mL of Sf9 cells at 2×10^6 cells/mL with a multiplicity of infection (MOI) of 0.1, and viral supernatants were collected after 50 h.

Cell Culture and Receptor Expression. CHO cells expressing the α_{2A}-R were maintained in Ham's F-12 medium (Gibco BRL) with 10% fetal bovine serum. C_6 glioma cells were grown in DME High Glucose HEPES (Gibco BRL) with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine. Both mammalian cell lines were maintained at 37 °C with 5% CO_2.

Sf9 insect cells from American Type Culture Collection (ATCC CRL-1711) were grown in suspension with Sf-900 II SFM serum-free media (Gibco BRL) and maintained at 28 °C with agitation at 136 rpm in an orbital shaker (Forma Scientific, Inc., Marietta, OH). For protein expression, cells (2×10^6/mL) were infected with the appropriate baculovirus at a MOI ratio of 1 and harvested 50 h later.

Membrane Preparation. Confluent monolayers of CHO and C_6 glioma cells from 25 tissue culture dishes (150 mm) were washed with PBS and lysed (at 4 °C for 15 min) in 1 mM Tris-HCl, pH 7.4, with protease inhibitors (10 μg/mL leupeptin, 2 μg/mL aprotinin, 20 μg/mL benzamidine, 0.1 mM PMSF). Cells were scraped from dishes and homogenized by 10 strokes of a glass−Teflon homogenizer and then sedimented at 1000*g* for 10 min. The postnuclear supernatant was centrifuged at 40 000*g* for 30 min, and the pellet was resuspended in buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.6) and rehomogenized. Protein was determined by the Bradford method (*31*). Aliquots were snap frozen in liquid nitrogen and stored at −80 °C for up to 12 months before use.

Sf9 cell pellets from 500 to 800 mL of cell suspension were harvested by 2 washes in PBS and resuspending in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% glycerol, and protease inhibitors, pH 8) at 4 °C. Cells were burst by N_2 cavitation (Parr bomb, 600 psi, 20 min) followed by sedimentation of nuclei (750*g*, 10 min). Membranes were obtained by pelleting the postnuclear supernatant (28 000*g*, 30 min) and redispersing in buffer A and protease inhibitors. These were homogenized by 10 strokes of a glass−Teflon homogenizer and stored as described for mammalian cell membranes.

Urea Treatment. Urea stock solutions were freshly made by dissolution of urea crystals in buffer A at room temperature and then kept on ice before use. Membranes were pelleted (10 000*g* for 10 min) and resuspended to ∼0.75 mg/mL in 7 M urea solution (*32*). After a 30 min incubation on ice, membranes were centrifuged at 140 000*g* for 30 min. The pellet was redispersed in (urea-free) resuspension buffer and then incubated and centrifuged as before. The final pellet (∼1 mg/mL) was resuspended in buffer A, homogenized, and stored as described above.

G protein Purification. Myristoylated α_{i1} was purified from *Escherichia coli* (BL21/DE3) following the method of Mumby and Linder (*33*). Sf9 cell-expressed α_{i1} (co-expressed

with his_6-β1 and his_6-γ2) was purified as described by Kozasa and Gilman (*34*). Protein was determined by the Bradford assay, and the specific activity was ~15 nmol/mg as measured by [^{35}S]GTPγS binding (*35*). Bovine brain $\beta\gamma$ was isolated from cortex synaptosomal membranes (a gift of Dr. Tetsufumi Ueda, University of Michigan) essentially by the method of Sternweis and Robishaw (*35*), with modifications as described (*27*). The concentration was estimated by Amido Black staining. Purified recombinant $\beta\gamma$ subtypes expressed in Sf9 cells were isolated using DEAE chromatography followed by affinity chromatography on a α_{i1}-agarose column as described (*36*). The protein concentration of the $\beta\gamma$ subunits was determined by SDS−PAGE (10% Laemmli gel) with ~1 μg of $\beta\gamma$ dimer followed by densitometry of the β subunit band stained with a Coomassie-based dye (Gelcode Blue Stain, Pierce, Rockford, IL). Bovine brain $\beta\gamma$ was used as a standard. For resolution of β and γ subunits, Tricine/SDS−PAGE was performed with ~0.5 μg of $\beta\gamma$ using a separating gel (16% acrylamide) containing 10% glycerol followed by staining with silver nitrate. The prenyl lipid modification of expressed γ subunits was shown to be farnesyl for γ1 and γ11 and geranylgeranyl for γ2, γ7, and γ10 by mass spectroscopy (*3*).

Determination of Affinity of $\beta\gamma$ for α_{i1}. Preparation of fluorescein-labeled myristoylated α_{i1} (F-α) and biotinylated bovine brain $\beta\gamma$ (b-$\beta\gamma$) subunits and flow cytometric analysis were carried out as described previously (*37*). For measurement of the affinity of recombinant $\beta\gamma$ dimers for F-α by flow cytometry, 1 nM b-$\beta\gamma$ was prebound to strepavidin-coated polystyrene beads (SVP-60-5, Spherotech Inc., Libertyville, IL) and incubated with 1 nM F-α and varying amounts of recombinant $\beta\gamma$ in buffer B (20 mM Hepes, pH 8, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1.2 mM $MgCl_2$, 0.1% Lubrol, 10 μM GDP). After a 30 min incubation at room temperature, samples were analyzed with a Becton Dickinson FACScan capturing events on the forward scatter, side scatter, and fluorescein (FL-1) channels. Histograms of FL-1 fluorescence from singlet bead populations were obtained and mean channel numbers calculated using LYSIS II software (BD, San Jose, CA).

Reconstitution of Receptor with G Proteins. Membranes expressing receptors were pelleted with an Eppendorf Centrifuge 5415 (12 000 rpm, 10 min at 4 °C) and resuspended in reconstitution buffer (buffer A supplemented with sodium cholate 0.1%) in a 1.5 mL Eppendorf tube at ~0.03−0.13 mg/mL protein. After addition of the indicated amounts of G proteins, the reaction mix was incubated on ice for 30 min and then diluted 10-fold into the binding assay mixture.

For radioligand saturation binding analysis of A1 adenosine receptors with different amounts of G protein, reconstitution was carried out with the indicated equimolar amounts of Sf9-expressed α_{i1} and $\beta\gamma$ subunits as described by Yasuda et al. (*10*).

Radioligand Binding. Binding assays were conducted in 100 μL buffer A in a 96-well plate with the appropriate radioligand: α_{2A}-R (10 nM [^{3}H]YOH and 1 nM [^{125}I]PIC), A_1-R (2 nM [^{3}H]CCPA and 0.5−10 nM [^{3}H]CPX), 5-HT_{1A}-R (2 nM [^{3}H]-8-OH-DPAT and 6 nM [^{3}H]MPPF), and μ-R (5 nM [^{3}H]DAMGO and 5 nM [^{3}H]NLX). Adenosine deaminase (0.03% w/v) was added for A_1-R receptor binding experiments. After incubation at room temperature for 1 h (or 1.5 h for 8-OH-DPAT, 2 h for CPX, and 3 h for CCPA), samples were filtered over Brandel GF/C filters using a Brandel cell harvester. After two 2 mL washes (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.6, at 4 °C), filters were air-dried and scintillation counting performed with 4 mL Scintiverse cocktail (Fisher Scientific, Fair Lawn, NJ) in a Beckman LS 5801 liquid scintillation system. Receptor density of the α_{2A}-R, A_1-R, 5-HT_{1A}-R, and μ-R was estimated by single point binding with the respective [^{3}H]-labeled antagonists YOH, CPX, MPPF, and NLX assuming K_d values of 5, 3, 3, and 5 nM, respectively. Nonspecific binding to these 4 receptors was determined in the presence of an excess of the respective unlabeled ligands YOH (10 μM), CPX (2 μM), 5HT (2 μM), and DAMGO (5 μM). Saturation binding analysis of A1 adenosine receptors with [^{125}I]ABA was done as described by Yasuda et al. (*10*).

Data Analysis. Curve fitting used the nonlinear least-squares method in the computer program Prism (GraphPad Software, San Diego, CA). Data points are graphed as mean ±SEM (or SD for $n = 2$).

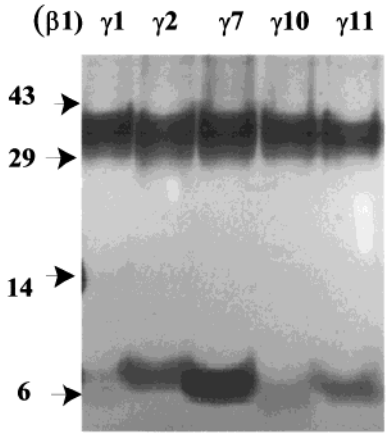

FIGURE 1: Purity of recombinant G protein $\beta\gamma$ subunits. A 0.5 μg amount of each $\beta\gamma$ subunit was loaded onto a 16% acrylamide, 10% v/v glycerol SDS−Tricine gel and stained with silver after electrophoresis: lane 1, β1γ1; lane 2, β1γ2; lane 3, β1γ7; lane 4, β1γ10; lane 5, β1γ11. Molecular size markers are indicated on the left.

RESULTS

We aimed to compare the coupling of different γ subtypes (dimerized with β1) to receptor by their ability to support high-affinity agonist binding after membrane reconstitution. The subtypes were synthesized in Sf9 cells and purified to homogeneity by anion exchange followed by α subunit affinity chromatography as previously described (*36*). Figure 1 shows the purity of the recombinant $\beta\gamma$ subtypes as examined by Tricine−SDS gel electrophoresis.

We wanted to monitor the activity of each purified $\beta\gamma$ preparation, to ensure that equally active protein was utilized in subtype comparison experiments.[2] For this purpose, we used a previously reported fluorescence flow cytometry assay for quantitative measurements of high-affinity α and $\beta\gamma$ interactions in detergent (*37*). A fluorescence signal is

[2] In preliminary experiments, a small number of aliquots of $\beta\gamma$ were inactive or had low activity in reconsitution experiments. When these were tested for FITC-α_{i1} binding, they were inactive in those assays as well. This indicated a critical need for quality control of $\beta\gamma$ subunits used for receptor reconstitution studies.

Table 1: IC_{50} Values for Purified βγ Subtypes Binding to Fluorescein-Labeled α_{i1} in Competition with Bovine Brain βγ Bound to Polystyrene Beads[a]

		γ subtype				
	brain βγ	γ1	γ2	γ7	γ10	γ11
IC_{50} (nM)	1.1 ± 0.2	4.4 ± 0.8[b]	1.6 ± 0.2	0.9 ± 0.02	0.9 ± 0.02	1.9 ± 0.4

[a] IC_{50} values were determined from nonlinear regression analysis of competition binding curves (Figure 3) using a one-site competition formula (Graphpad Prism). Data represent mean ± SE of at least three experiments per group. Comparisons were made using one-way ANOVA with Tukey post test (Graphpad Prism). [b] $p < 0.05$ compared with all other subtypes.

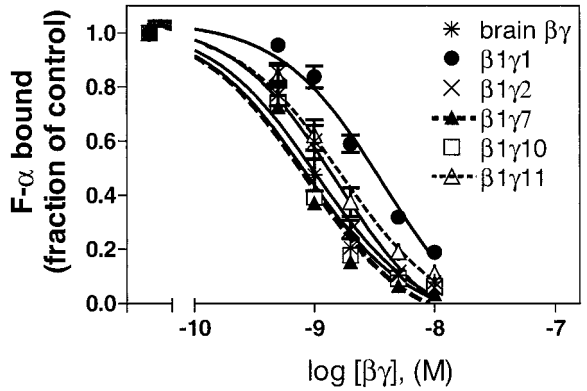

FIGURE 2: Binding of b-βγ to F-α in competition with 5 purified recombinant βγ subtypes and bovine brain βγ. b-βγ was prebound to strepavidin-coated polystyrene beads in HEDNML and incubated in the same buffer at 23 °C with 1 nM F-α and 0−10 nM of β1γ1 (●), β1γ2 (×), β1γ7 (▲), β1γ10 (□), β1γ11 (△), and bovine brain βγ (∗). After 30 min, binding of F-α to b-βγ was assayed as described in Experimental Procedures. Data are specific binding normalized to initial fluorescence and represent the mean ± SE of 3−4 independent experiments done in duplicate. The curve represent nonweighted nonlinear least-squares fitting of the data using a one site competition function (Graphpad Prism). Average IC_{50} values determined from this analysis are shown in Table 1.

detected by the flow cytometer when fluorescein-labeled bacterially expressed α_{i1} (F-α) is bound to biotinylated bovine brain βγ (b-βγ) linked to strepavidin-coated polystyrene beads. Addition of active unlabeled βγ will lead to loss of fluorescence via competition with b-βγ for binding to F-α. Thus, we monitored the activity of purified Sf9-expressed βγ subtypes by measuring the IC_{50} for binding F-α for each βγ prep (Figure 2). With 1 nM F-α and b-βγ, the IC_{50} for bovine brain βγ is 1.1 nM (Table 1). The five recombinant βγ preps of β1 dimerized to γ2, -7, -10, and -11 had IC_{50}s which differed from this value by less than 2-fold. Only γ1 had a higher IC_{50} of 4.4 nM (Table 1). Using one-way ANOVA, the IC_{50} for β1γ1 was statistically significantly greater than that of the other βγ subtypes ($p < 0.05$), a finding in agreement with another report (*38*). Aliquots of purified βγ were routinely assayed for activity so that, aside from β1γ1, only those with IC_{50}s within 2-fold of that for bovine brain βγ were used in receptor reconstitution. Hence the α subunit interaction measurements are a sensitive assay for activity of the recombinant βγ preparations and permitted us to eliminate inactive βγ samples.[2]

We compared the ability of these 5 βγ subtypes (representing 4 classes of γ) to support high-affinity agonist binding to four G_i-coupled receptors. We have previously shown that α_{2A}-R expressed in Sf9 membranes exhibited significant binding of radiolabeled agonist (∼10% of total receptor sites) (*32*). This is apparently the result of coupling to endogenous insect cell G proteins since the binding is abolished with Gpp(NH)p. However, 7 M urea-washing of membranes abolished basal agonist binding and subsequent reconstitution with purified G proteins resulted in up to a 20-fold increase in agonist binding (*32*). Hence, we pretreated the membranes in this study with 7 M urea so that the observed high-affinity agonist binding reflects coupling of receptor solely to the reconstituted G proteins. Receptor density was estimated by single point binding with the respective antagonists (see Experimental Procedures). Reconstitution of 5 nM (bacterially expressed) α_{i1} and 0−20 nM βγ to α_{2A}-R (∼8 pmol/mg), A_1-R (∼20 pmol/mg), and 5-HT_{1A}-R (∼5 pmol/mg), all in Sf9 membranes, and μ-R (∼2.5 pmol/mg) in C6 glioma membranes led to a 4−20-fold increase in binding of the respective agonists. At maximum βγ concentrations, the agonist occupied up to 50% of total receptor sites.

Our results show that all five βγ subtypes supported comparable maximum high-affinity agonist binding for all four receptors (Figure 3). The EC_{50} for the five βγ subtypes to support high affinity agonist binding varied by a maximum of 3−8-fold between the most potent and the least potent subtypes at any given receptor. Thus while magnitude differences in βγ potency are seen, no βγ dimers exhibit an absolute specificity. In that context, γ11 was the most potent subtype for the A_1-R and 5-HT_{1A}-R ($p < 0.05$, one way ANOVA; Table 2). Although not statistically significant, γ7 was the most potent subtype observed for the α_{2A}-R and μ-R. β1γ1 was the least potent for the A_1-R consistent in a qualitative but not quantitative manner with a previous report (*25*). Surprisingly, β1γ2 was least potent at the 5-HT_{1A}-R ($p < 0.05$) and it was not significantly more potent than β1γ1 at the α_{2A}-R. Overall, the high potency of β1γ11 at A_1-R and 5-HT_{1A}-R is striking and novel.

Previous studies of the A_1-R (*39*) and α_{2A}-R (*9*) reported strikingly lower maximum agonist binding after reconstitution with β1γ1 compared to other subtypes, notably γ2. Our results show γ1 to be less potent than γ2 for these two receptors (2.5-fold for A_1-R and only a nonsignificant 20% for α_{2A}-R), but maximal levels of agonist binding did not differ significantly between γ1 and γ2. To identify possible reason(s) for the smaller differences seen here, we tested β1γ1 and β1γ2 under different conditions to better replicate the published reports (*9, 25*). Specifically, we compared the effects of urea treatment of membranes, full versus partial α_{2A}-R agonist, and bacterial versus Sf9 expressed α_{i1}. (i) Urea pretreatment of membranes: Urea treatment was utilized as we found that the α_{2A}-R expressed in CHO or Sf9 cells supported high affinity agonist binding to 10−20% of total receptor sites in the absence of added G protein (*32*), a finding corroborated by a report that the α_{2A}-R fused to an α_{i1} subunit (overexpressed in fibroblasts) coupled to endogenous G_i-like G proteins such that ∼70% of agonist-

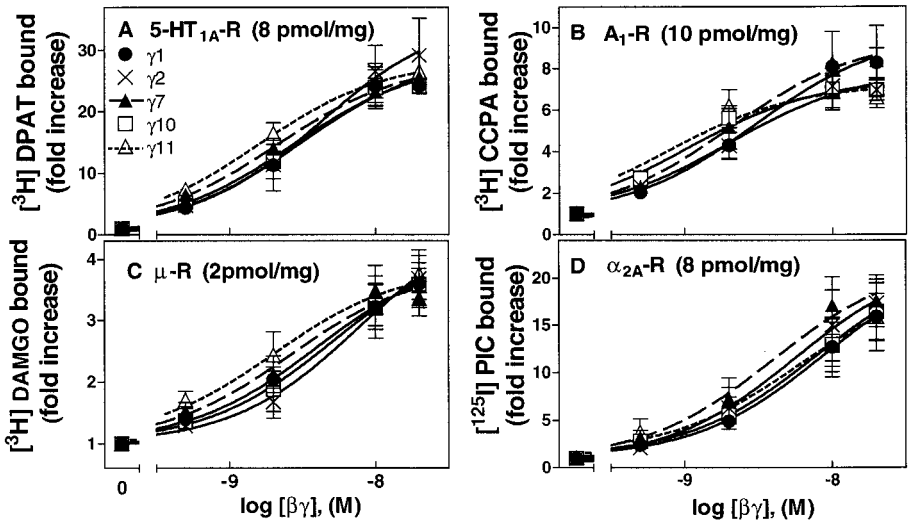

FIGURE 3: Formation of high-affinity agonist binding to 4 receptors by 5 $\beta\gamma$ subtypes. Sf9 membranes expressing $5HT_{1A}$-R (A), A_1-R (B), and α_{2A}-R (D) or C6 glioma membranes expressing μ-R (C) were extracted with 7 M urea and reconstituted with 5 nM α_{i1} and 0−20 nM $\beta1\gamma1$ (●), $\beta1\gamma2$ (×), $\beta1\gamma7$ (▲), $\beta1\gamma10$ (□), or $\beta1\gamma11$ (△). After a 30 min incubation on ice, membranes were assayed as described in Experimental Procedures for binding of the respective agonist [^{125}I]PIC, [^{3}H]CCPA, [^{3}H]-8-OH-DPAT, or [^{3}H]DAMGO. Each tube contained ~1 μg (α_{2A}-R), 8 μg (A_1-R, $5\text{-}HT_{1A}$-R) or 15 μg (μ-R) of membrane protein representing a receptor concentration of 0.1−1 nM. Specific binding data are mean ± SEM of triplicate determinations from 3 experiments. The curves are nonweighted nonlinear least-squares fit of the data to a one site competition function (GraphPad Prism). The resultant EC_{50} values are detailed in Table 2.

Table 2: EC_{50} (nM) of Different γ Subtypes in Supporting High-Affinity Agonist to Receptor in Sf9 Membranes Co-reconstituted with α_{i1}[a]

	EC_{50} for γ subtypes				
receptor	$\gamma1$	$\gamma2$	$\gamma7$	$\gamma10$	$\gamma11$
α_{2A}-R	12.0 ± 3.1	10 ± 5.1	**4.1 ± 1.1**	7.9 ± 3.8	6.7 ± 2.4
A_1-R	6.0 ± 2.1[b]	2.4 ± 0.6	2.3 ± 0.5	2.2 ± 1.0	**0.8 ± 0.1**[b]
$5HT_{1A}$-R	3.8 ± 1.1	5.0 ± 1[b]	2.1 ± 0.2	2.8 ± 0.15	**1.7 ± 0.33**[b]
μ-R	4.1 ± 0.6	9.0 ± 2.5	**3.3 ± 1.3**	8.3 ± 5.2	4.1 ± 2.7

[a] 5 nM α_{i1} and 0−20 nM of $\beta1\gamma$ subtypes were reconstituted with receptor in Sf9 membranes and assayed for high-affinity agonist binding. EC_{50} was estimated by nonlinear regression analysis (Graphpad Prism). Data are the mean ± SE of three experiments done in triplicate. Comparisons of potency of γ's in each receptor was made using one-way ANOVA analysis (Graphpad Prism). The most potent subtype for each receptor is highlighted in bold. [b] Indicates a significant difference from the other $\beta\gamma$ subunits at this receptor, $p < 0.05$ by one way ANOVA with Tukey's multiple comparison test.

stimulated GTPase activity was produced via endogenous G proteins (*40*). In contrast the A_1-R coupled less well to endogenous Sf9 G proteins. As is evident in Figure 4A,B, $\beta1\gamma2$ was not markedly more active than $\beta1\gamma1$ for supporting agonist binding to the A_1-R in non-urea-treated membranes compared to urea-treated membranes. (ii) Full versus partial agonist: We (*41*) and others (*42*) have reported that different agonists may preferentially activate a specific Gα subtype. Richardson and Robishaw utilized the full α_{2A}-R agonist [^{3}H]-UK 14,304 (*9*) while our data in Figure 3 are with the partial agonist [^{125}I]PIC. Figure 4C,D shows that both agonists generated similar results with respect to $\beta1\gamma1$ and $\beta1\gamma2$ reconstitution. (iii) Bacterially or Sf9 cell synthesized α subunits: We utilized purified α_{i1} subunits expressed in *E. coli* with coexpression of myristoyl-CoA:protein *N*-myristoyl-transferase to obtain myristoylated α subunits as previously described (*43*). As previous reports with the α_{2A}-R (*9*) and A_1-R (*25, 44*) utilized α subunits synthesized in Sf9 cells, we compared these two α subunits in A_1-R reconstitution. The EC_{50}s for $\beta1\gamma1$ and $\beta1\gamma2$ with bacterially expressed myristoylated α_{i1} were 0.3 and 0.26 nM, respectively, while with the Sf9-expressed α_{i1} they were 0.22 and 0.20 nM (data not shown). Both $\beta1\gamma1$ and $\beta1\gamma2$ supported comparable levels of agonist binding with both sources of α_{i1}. Thus the major differences in procedures or reagents did not account for our less striking differences for $\beta1\gamma1$ and $\beta1\gamma2$ at the α_{2A}-R and A_1-R.

To further examine the lack of difference in level of agonist binding supported by $\beta1\gamma1$ versus $\beta1\gamma2$ for the A_1-R, we determined full saturation isotherms for the binding of the A_1-R agonist [^{125}I]ABA in the presence of different amounts of Sf9 cell-expressed α_{i1} and $\beta\gamma$ subunits (Figure 5). As expected, both $\alpha_{i1}/\beta1\gamma1$ and $\alpha_{i1}/\beta1\gamma2$ showed significant enhancements in the affinity of [^{125}I]ABA as shown in the Scatchard transformations of the binding isotherms (Figure 5). Interestingly, at high concentrations (40 nM) of the heterotrimer, both α_{i1} with $\beta1\gamma1$ and with $\beta1\gamma2$ produced nearly identical enhancements of agonist binding. Consistent with other results in this article but at odds with previous conclusions, the main difference was in the potency with which this occurred. For $\alpha_{i1}/\beta1\gamma2$, there was a significant upward shift in the Scatchard curves even at 2 and 4 nM while 8 nM $\alpha_{i1}/\beta1\gamma1$ was required to cause a significant enhancement of [^{125}I]ABA binding. For 20 nM $\alpha_{i1}/\beta1\gamma2$ the effect was nearly complete while there was some additional increase from 20 to 40 nM $\alpha_{i1}/\beta1\gamma1$. The likely explanation of the discrepancy in interpretation relates to the use of higher concentrations of $\beta1\gamma1$-containing heterotrimer (in the present study) which gave an effect near to the maximum. Additionally, we show that the full

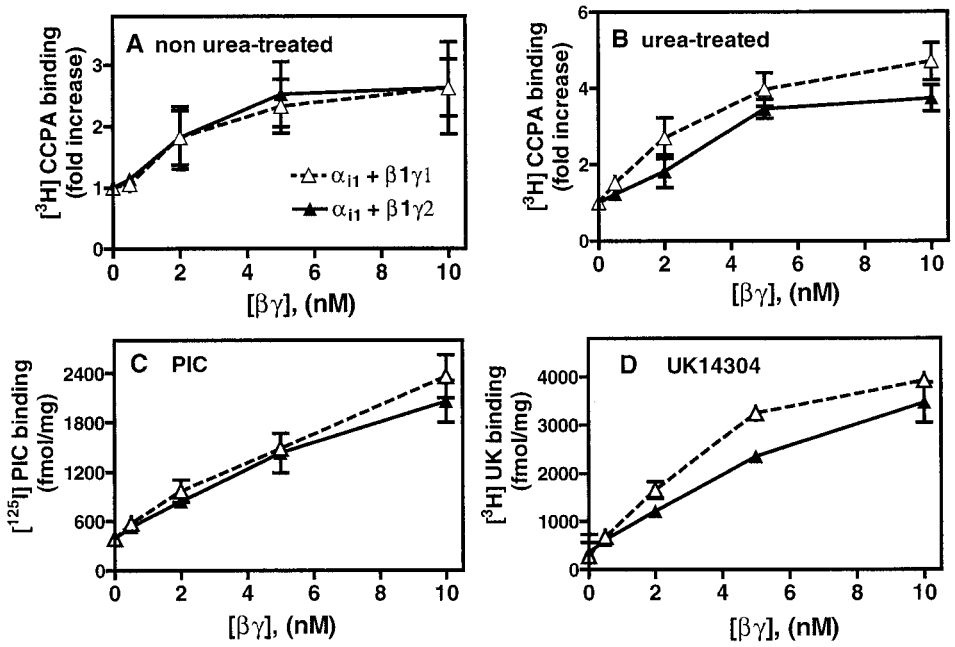

FIGURE 4: Comparison of high-affinity agonist binding supported by $\beta1\gamma1$ and $\beta1\gamma2$ with differing membrane treatment and agonist. Sf9 membranes expressing the α_{2A}-R (C, D) and A_1-R (A, B) were washed with 7 M urea (except B) and reconstituted with 5 nM α_{i1} and 0−10 nM $\beta1\gamma1$ (dotted line) or $\beta1\gamma2$ (solid line). Each tube contained 4 μg (C, D) or 2 μg (A, B) of membrane protein representing 0.3 or 0.7 nM receptor, respectively. After a 20 min incubation on ice, bindings of the agonist [^{3}H]CCPA (A, B), [^{125}I]PIC (C), or [^{3}H]UK14,304 (D) were assayed as described in the Experimental Procedures. Data are the mean ± SD of specific binding done in triplicate or duplicate from 2 to 3 experiments.

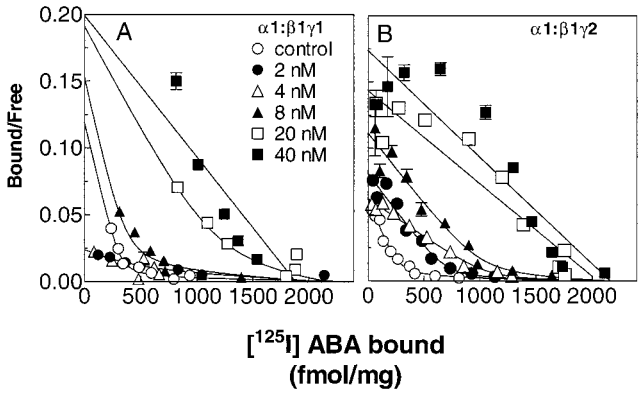

FIGURE 5: Agonist dependence of high-affinity agonist binding supported by $\beta1\gamma1$ and $\beta1\gamma2$. Sf9 membranes expressing the A_1-R were reconstituted with the indicated concentrations of Sf9-expressed α_{i1}, $\beta1\gamma1$ (A), or $\beta1\gamma2$ (B). Saturation binding curves were determined for [^{125}I]ABA (0.3−21 nM) with nonspecific binding determined in the presence of 10 μM R-PIA. The number of A_1-R binding sites in this membrane preparation was 2270 fmol/mg as determined by Scatchard plots with the antagonist [^{3}H]CPX. Data are means of 2 experiments each performed in duplicate). Curved lines were determined by nonlinear least-squares fitting of the specifically bound [^{125}I]ABA results to a 1- or 2-site hyperbolic function as described in Experimental Procedures.

complement of receptors (2270 fmol/mg of antagonist [^{3}H]-CPX binding) is completely converted to a high affinity agonist binding state by $\alpha_{i1}/\beta1\gamma2$ while the high-affinity component reaches at least 70−80% of this B_{max} value with $\alpha_{i1}/\beta1\gamma1$. Thus, all of the receptor is capable of forming a high-affinity complex with agonist and G protein. Last, even when G protein is in excess of receptor (8 nM vs 0.4 nM), there is a substantially curved Scatchard plot. This is not consistent with all of the heterotrimer having equal and unrestricted access to the receptors in the membranes. As previously suggested (*49*), there may be other cellular components modulating the receptor−G protein interactions.

DISCUSSION

G protein coupled receptors are classified into four major classes (G_i, G_s, $G_{q/11}$, or $G_{12/13}$) on the basis of the Gα subtype(s) that they selectively activate. Less is known about receptor selectivity for $\beta\gamma$ subtypes. The $\beta\gamma$ subunit is absolutely required in G protein signal transduction (*45*, *46*). $\beta\gamma$ was originally thought to be solely a negative regulator for α but was subsequently shown to modulate effectors ranging from ion channels to enzymes (*47*) and to serve as a scaffold for targeting β-adrenergic receptor kinase to the membrane (*48*). Data from intact cell studies have suggested a remarkable selectivity of receptor for $\beta\gamma$ subtypes. Such specificity could be mediated by "in vivo" mechanisms such as compartmentation of signaling components or region-specific distribution of signaling proteins (*49*). Alternatively, receptors may preferentially couple to specific $\beta\gamma$ subtypes. The latter mechanism is testable using in vitro reconstitution of receptor with G proteins. Such in vitro data for $\beta\gamma$ subtypes have been published for several receptors: rhodopsin (*24*); adenosine A_1 (*25*, *44*); α_{2A} adrenergic (*9*); adenosine $_{2A}/\beta_1$ adrenergic receptors (*10*); M2 muscarinic (*50*). They suggest that receptors have direct coupling specificity for $\beta\gamma$ subtypes. We tested the generality of this finding, by reconstituting α_{i1} and $\beta1$ dimerized with γ subunits from all four classes, to four representative G_i coupled receptors: α_{2A} adrenergic (α_{2A}-R); adenosine A1 (A_1-R); 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}-R); the mu opioid receptor (μ-R). This report is the first to compare receptor-γ specificity across multiple G_i-coupled receptors. We show that $\gamma7$ and $\gamma11$ are the most potent γ subtypes for support of high-affinity agonist binding to the four receptors. The potency differences

for a given receptor were generally modest. However, for the A_1-R, γ11 was up to 8 times more potent than the other γ subtypes. These results suggest that for G_i-coupled receptors, there is some specificity for $\beta\gamma$ subunits but no unique $\beta\gamma$ pair, so that the exquisite receptor−$\beta\gamma$ selectivity reported in whole cells may be mediated by in vivo mechanisms rather than simple receptor−$\beta\gamma$ contact.

We found γ11 to be the most potent subtype for reconstitution of A_1-R and 5-HT_{1A}-R ($p < 0.05$, one-way ANOVA). The high potency of γ11 for G_i-coupled receptors has not been previously reported. This subunit is widely expressed in many tissues (*51*−*53*). Although it is not detected in the brain, γ11 has been noted to be particularly abundant in platelets and leukocytes (*51*). G_i-coupled receptors expressed on platelets include the α_{2A}-R (*54*), thrombin (*55*), and ADP (P2Y12) receptors (*56*) while examples of those on leukocytes are the C−C chemokine (*57*) and formyl−methionyl peptide (*58*) receptors. The platelet activating factor receptor is expressed in both tissues (*59*). Expression of γ11 (*51, 53*) and A_1-R overlaps in adipose tissue, testis (*60*), heart, aorta, and kidney and bladder (*61*), while there is coexpression of γ11 and $5HT_{1A}$R in the spleen (*62*), pancreas and stomach (*63*), and kidney (*64*). γ7 was the most potent γ subtype for reconstitution of the α_{2A}-R and μ-R (and second most potent for the 5-HT_{1A}-R). The α_{2A}-R overlaps with γ7 in brain (*54*), spleen (*65*), and heart, kidney, and lung (*66*) while the μ-R is in the same tissues as γ7 in the kidney, lung, spleen, and liver (*67*). Hence the γ subtypes we have found to be uniquely potent with certain receptors are possible in vivo coupling partners of these receptors.

The reliability of the results of $\beta\gamma$ subtype comparison experiments is critically dependent on all of the purified subtypes being equally active. The most commonly used assay for confirming activity of purified $\beta\gamma$ subunits has been pertussis toxin catalyzed ADP-ribosylation of α subunits, as first proposed by Neer et al. (*68*). This reaction is greatly enhanced by addition of purified $\beta\gamma$ subunits, but only substoichiometric amounts are required (*69*). This suggests that $\beta\gamma$ subunits act catalytically, and thus, the ribosylation assay may not be a direct measure of affinity for α, as previously assumed (*9*). Rather, $\beta\gamma$ enhancement of ADP-ribosylation has been proposed to be a measure of the ability of $\beta\gamma$ to recycle between α subunits such that a high rate of ADP-ribosylation may be reflective of a higher rate of $\beta\gamma$ dissociation from α (*38*). We have utilized a direct assay of $\beta\gamma$ affinity for α subunits, using a fluorescence flow cytometry approach as described previously (*37*). We monitored $\beta\gamma$ preparations for activity by examining the affinity of the $\beta\gamma$ for fluorescein-labeled α_{i1} (F-α_{i1}) subunits in competition with biotinylated bovine brain $\beta\gamma$ (b-$\beta\gamma$) at low nanomolar concentrations (Figure 3). β1 (70) and γ2 (71) have been reported to be the major $\beta\gamma$ subtypes in bovine brain. We found that all active subtypes had an IC_{50} within 2-fold of the value for bovine brain $\beta\gamma$ (Table 2) except that $\beta1\gamma1$ was 3−4-fold less potent than the other $\beta\gamma$ subtypes in binding F-α_{i1}, a difference that is statistically significant (one way ANOVA, $p < 0.05$). This is the first report detailing relative affinities of different $\beta1\gamma$ subtypes for α_{i1}. The reduced affinity of $\beta1\gamma1$ for α_{i1} compared with other γ subtypes is consistent with published literature showing $\beta1\gamma1$ to be least effective compared to $\beta1\gamma2/7/12$, for stabilizing α_{i1} in the inactive conformation (*38*).

The similarities between γ1 and γ11, which share 76% homology, have been reviewed (*53*). Both are farnesylated, are not expressed in brain, do not dimerize with β2, and possess a highly similar amino terminal sequence compared to other γ subtypes. In addition, we now report that (β1)γ1 and γ11, which form one γ subclass, have the lowest affinity for α_{i1} compared to representative members of the other three γ subclasses. This suggests a lower affinity of the C15 farnesyl moiety for the acyl group on the α subunit. While $\beta1\gamma1$ has both lower affinity for α_{i1} and lower potency for receptor reconstitution, the overall rank order of $\beta\gamma$ subtype affinity for α_{i1} does not correspond to the order of potency in support of high-affinity agonist binding (Table 2). This was especially striking for γ11 which has the second lowest affinity for α_{i1} but was the most potent subtype for the A_1-R and 5-HT_{1A}-R. Hence the determinants for $\beta\gamma$ coupling to receptor are independent of its affinity for the α subunit. Furthermore, the farnesylated γ1 and γ11, which had the lowest affinities for α, showed vastly different potencies for receptor coupling (with the exception of μ-R where γ1 was second most potent). For example, with the A_1-R, the EC_{50}s of γ1 and γ11 were 6.0 and 0.8 nM, respectively. Hence the γ subunit peptide sequence (apart from the isoprenoid group) is an important determinant in receptor coupling as previously indicated by Gautam and colleagues (*72*). Two possible mechanisms for this would be (i) the γ peptide is in contact with the receptor, as has been suggested from peptide studies (*73*), and (ii) the γ peptide induces a conformational change in the β subunit, which is in contact with the receptor.

Our data for the α_{2A}-R and A_1-R are in broad agreement with published results. The potency of γ2 and γ10 for supporting high affinity binding to the A_1-R (as well as the maximum increase in binding) has been reported to be comparable (*39*), as observed here. Of the four receptors, $\beta\gamma$ subtype potency data has only been reported in the A_1-R (*25, 44, 74*), and our results are consistent with the latter report (*74*). For the α_{2A}-R, our results concur with the finding that the levels of agonist binding supported by γ2, γ7, and γ11 are comparable (*9*). However, we did not observe the lower amount of agonist binding for γ10 and γ5 was not tested in our study.

Our data most directly address the potency of $\beta\gamma$ subtypes for receptor coupling, which, aside from the A_1-R, has not been previously described. We found that γ1 (for α_{2A}-R and A_1-R) and γ2 (for the 5-HT_{1A}-R and μ-R) are the least potent $\beta\gamma$ subtypes. γ2 was one of the earliest γ subtypes to be identified (*75*), and (β1)γ2 has been widely used experimentally as a generic $\beta\gamma$. Its low potency for these G_i coupled receptors has not been previously reported. Additionally, we observed a comparatively lower potency of $\beta1\gamma1$ compared to the other γ subtypes. For example, in the A_1-R, EC_{50} was 6 nM for γ1 versus <2.4 nM for the other γ's, probably in part due to a weaker affinity of the $\beta1\gamma1$ for α_{i1} as discussed below. This corroborates many reports showing that this retinal-expressed subtype, aside from support of signal transduction by the photoreceptor rhodopsin (*24*), is the least effective in signaling as exemplified by support of G_i-receptor coupling (*9, 25*) and effector modulation (*23*).

In terms of maximal levels of agonist binding, we observed that all five $\beta\gamma$ subtypes supported comparable levels of binding for all four receptors. This represents the first such report for the μ-R. For the 5-HT_{1A}-R, Manning and colleagues reported that agonist binding was lower by a factor of 2 when $\beta1\gamma1$ was coexpressed with receptor, as compared to $\beta1\gamma2$ (*30*). However, this methodology does not allow the quantitative assessment of the level of protein expression or activity. Importantly, it was reported for the α_{2A}-R (*9*) and A_1-R (*25*) that $\gamma1$ supported a lower level of agonist binding than other γ subtypes, notably $\gamma2$. Hence our data differ from those reports mainly in levels of agonist binding supported by $\gamma1$ versus $\gamma2$. To address this discrepancy, we investigated three possible reasons for the difference: urea pretreatment of membranes; type of agonist; source of α subunits. Our results suggested that none of these reasons influenced γ subtype discrimination by receptor. The hexahistidine-tagged α_{i1} used in the α_{2A}-R study (*9*) was not tested. That study also utilized the human α_{2A}-R while we have used the porcine α_{2A}-R. However, we also observed differences in results between this and the A_1-R study (*25*) despite using the same bovine A_1-R construct.

Using full saturation isotherms of [^{125}I]ABA binding to the A_1-R, we are able to explain, at least in part, the reason for the similar levels of agonist binding supported by $\beta1\gamma1$ versus $\beta1\gamma2$. At high G protein concentrations (which exceed the K_d of $\beta1\gamma1$ and $\beta1\gamma2$ for α_{i1} as measured in the fluorescence flow cytometry assay, Table 1) the maximum level of binding supported by the two $\beta\gamma$ subtypes are similar. Hence the more comprehensive data in this paper, which includes higher G protein concentrations, shows that it is the affinity of receptor for heterotrimer containing $\alpha_{i1}/\beta1\gamma1$ versus $\alpha_{i1}/\beta1\gamma2$ which differs rather than increased maximum effects of $\alpha_{i1}/\beta1\gamma2$ on agonist binding to the A_1-R (Figure 5).

We have observed modest differences among $\beta\gamma$ subtypes for four G_i coupled receptors but not the absolute specificity reported in intact cell systems whereby individual receptors (even within the same subclass) appear to signal via specific $\beta\gamma$ subtypes (*14*, *15*). Those data in whole cells may be explained by mechanisms other than direct receptor−$\beta\gamma$ subtype recognition. First, specificity could arise from targeting of specific $\beta\gamma$ subtypes to distinct locations. It has been reported that rod and cone photoreceptors had different β and γ subunits in their outer segments (*76*). $\gamma8_{olf}$ ($\gamma9$) is specifically expressed in the olfactory and vomeronasal neurons (*77*) while $\gamma13$ is only expressed in taste receptor cells (*5*). Within the brain (*78*−*80*), heart (*81*), and inner ear (*82*), distinct cellular and regional pattern of distribution of β and/or γ subtypes have been observed. e.g. there is a striking overlap of $\beta4$ and $\gamma2$ mRNA in a specific layer of the rat occipital cortex, suggesting region-specific (rather than receptor specific) $\beta\gamma$ subtypes mediate signal transduction in the brain (*78*). Moreover, differential subcellular localization of $\beta\gamma$ subtypes is a possibility, as $\gamma5$ has been found to be enriched at focal adhesions (*83*). Another possible in vivo mechanism for receptor−$\beta\gamma$ specificity is selective up- or down-regulation of specific G protein subtypes. Chronic opioid receptor activation in guinea pig ileum has led to downregulation of α_s and upregulation of α_i, α_0, and particularly β subunits, although the subtype(s) was undefined (*84*). This variation in receptor and $\beta\gamma$-subtype stoichiometry can drive distinct $\beta\gamma$ partners for receptors. This phenomenon may be time dependent, as it has been observed that the degree of expression and distribution of β and γ subtypes between different regions of the heart changed with development (*81*). Last, various multiprotein complexes have been reported to be assembled with other transmembrane receptors, such as the receptor tyrosine kinase (*85*). An increasing number of proteins have been reported to bind to G protein coupled receptors via a PDZ domain interaction. These includes the Na^+/H^+ exchanger regulatory factor (NHERF) (*86*), nitric oxide synthase (*87*), and the Shank family of scaffold proteins (*88*). The $\beta\gamma$ subunit is able to facilitate the formation of multiprotein complexes as evident by its role in bringing the β-adrenergic receptor kinase to the receptor (*48*). The formation of a receptor−G protein signaling network via a scaffolding protein that brings together specific receptors with their cognate $\beta\gamma$ subtypes is an exciting and testable possibility.

ACKNOWLEDGMENT

The authors thank Dr. Lee Limbird for the α_{2A}-R construct, Dr. David Manning for baculovirus encoding the 5-HT_{1A}-R, Dr. John Traynor for C_6 glioma cells expressing the μ-R, Dr. Tetsufumi Ueda for bovine cortex synaptosomal membranes, and Dr. Joel Linden for assistance with Scatchard transformation analysis.

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BI010950C