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Reconstitution of Recombinant Bovine A_1 Adenosine Receptors in Sf9 Cell Membranes with Recombinant G Proteins of Defined Composition

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SUMMARY

We investigated the coupling of A_1 adenosine receptors to recombinant G proteins. Recombinant baculoviruses were used to express bovine A_1 adenosine receptors in Sf9 insect cells that lack endogenous adenosine receptors. Binding parameters for recombinant receptors expressed in Sf9 cell membranes using the antagonist radioligand [^{125}I]BW-A844U ([^{125}I]8-cyclopentyl-3-iodoaminophenethyl-1-propylxanthine) are B_{max} = 2–5 pmol/mg of protein and K_D = 0.53 ± 0.12 nM. In competition assays, the potency order of agonists is (*R*)-phenylisopropyladenosine > (*S*)-phenylisopropyladenosine > 5′-*N*-ethylcarboxamidoadenosine, properties characteristic of native bovine A_1 adenosine receptors. The agonist radioligand ^{125}I-N^6-4-aminobenzyladenosine binds to two affinity states of the recombinant A_1 adenosine receptors with K_D values of 0.09 and 10.4 nM. The high affinity binding site represents <10% of total sites and is increased 7-fold on reconstitution with both α and $\beta\gamma$ G protein subunits but not with either subunit alone; thus, exogenous α and $\beta\gamma$ subunits do not functionally interact with endogenous Sf9 $\beta\gamma$ and α subunits, respectively. Four different α subunits (αi1, αi2, αi3, and αo) and six different $\beta\gamma$ subunits ($\beta1\gamma1$, $\beta1\gamma2$, $\beta1\gamma3$, $\beta2\gamma2$, $\beta2\gamma3$, and bovine brain $\beta\gamma$)) increased GTP-sensitive, high affinity agonist binding. The results indicate that bovine A_1 adenosine receptors couple equally well to G protein αi and αo subunits in combination with $\beta\gamma$ subunits containing the $\beta1$ or $\beta2$ subunits and $\gamma2$ or $\gamma3$ subunits. G protein heterotrimers that contain the $\beta1\gamma1$ dimer couple with similar potency but reduced efficacy to A_1 adenosine receptors.

A_1 adenosine receptors are members of a large family of cell surface receptors that use G proteins as transducers. Based on a large body of experimental evidence, A_1 adenosine receptors are believed to couple to multiple pertussis toxin-sensitive G protein α subunits. For example, with the use of GTP-sensitive high affinity agonist binding to assess coupling in heart membranes, two populations of high affinity binding sites were found to be differentially sensitive to inhibition by GTP analogs, suggesting that the A_1 receptors couple to different G proteins (1). With affinity chromatography, bovine A_1 adenosine receptors were found to copurify with $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha o}$ as detected by selective G protein α subunit antisera (2). Furthermore, purified A_1 adenosine receptors reconstituted into platelet membranes couple selectively to endogenous pertussis toxin-sensitive G proteins, probably G_i or G_o (3). A_1 adenosine receptors purified from bovine brain can interact with G_i or G_o isolated from bovine brain or with recombinant G proteins expressed in *Escherichia coli* to reconstitute high affinity agonist binding (4, 5). Taken together, these studies indicate that A_1 adenosine receptors can couple to multiple G protein α subunits in the G_i/G_o family. The conclusions of these studies are limited because the composition of the α and $\beta\gamma$ subunits that interact with the receptor were not precisely defined. The G protein family is very diverse, consisting of 21 α (6), 5 β (7), and at least 11 γ subunits (8–10) based on identification of distinct cDNA species for each subunit. Recombinant G proteins with defined subunit composition have an advantage over tissue-derived G protein subunits, which are usually composed of a mixture of G protein isoforms. In previous studies in which recombinant G proteins were used, the proteins were purified from *E. coli* overexpression systems and thus did not contain all of the lipid modifications of their mammalian counterparts (11). The use of such unmodified pro-

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R.A.F. and S.G.G. contributed equally to this work; their names are listed alphabetically.

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ABA, 3-aminobenzamide; PIA, N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

teins may affect the results because these post-translational modifications of receptors and G protein α- and βγ subunits are required for optimal interaction of these proteins (12–15).

To address these limitations, we used recombinant G protein α and βγ subunits purified from baculovirus-infected Sf9 cells to reconstitute high affinity agonist binding to recombinant A_1 adenosine receptors expressed in Sf9 cell membranes. Major advantages of this system are that (a) Sf9 cells do not express A_1 adenosine receptors, providing a clear background for studying the expressed receptors; and (b) receptors and G proteins expressed in Sf9 cells have been demonstrated to undergo post-translational modifications characteristic of mammalian cells (16–19). Here, we show that only a small fraction of recombinant A_1 adenosine receptors expressed in Sf9 cell membranes are coupled to endogenous Sf9 cell G proteins, making it possible to reconstitute high affinity agonist binding in these membranes by adding stoichiometric amounts of purified recombinant G protein α and βγ subunits. All of the α and βγ subunits tested were able to establish the high affinity agonist binding conformation of the receptor, but β1γ1 was far less effective than the other βγ dimers.

Materials and Methods

Construction of the transfer vector used to make recombinant baculovirus coding for the A_1 adenosine receptor. The cDNA for the bovine A_1 adenosine receptor was obtained from A. Tucker (University of Virginia, Charlottesville, VA) (20). To minimize the length of the construct 5′ to the start codon, an A_1 adenosine receptor construct was created using the polymerase chain reaction with oligonucleotide primers engineered to introduce convenient restriction sites. The forward primer introduced a *Sma*I site immediately 5′ to the initiation codon. The reverse primer was complementary to a 29-bp sequence in the 3′ noncoding region and introduced an *Xba*I site. The resulting product was subcloned into pGEM7. To eliminate the possibility of polymerase errors in the largest portion of the clone, an internal *Stu*I/*Bgl*II fragment (789 bp) was cut from the pGEM7 construct and replaced with the *Stu*I/*Bgl*II fragment cut from the parent clone. The resulting insert was subcloned into the multiple cloning polylinker of the baculovirus transfer vector, pVL1393 (InVitrogen, San Diego, CA), using the 5′-*Sma*I and 3′ *Xba*I sites. To ensure fidelity, the completed construct was sequenced in the forward and reverse directions across the *Stu*I and *Bgl*II boundaries using primers designed to hybridize to polyhedrin gene sequences lying immediately 5′ and 3′ to the pVL1393 polylinker.

Recombinant baculoviruses were produced by standard techniques (21) and isolated by four sequential rounds of plaque purification. First-round selections were made by visual identification of the occ$^-$ phenotype. Second-round plaques were screened by Southern blotting for the expression of A_1 adenosine receptor DNA. Third- and fourth-round plaques were tested for expression of the A_1 adenosine receptor by radioligand binding of the selective antagonist [^{125}I]BW-A844U([^{125}I]8-cyclopentyl-3-iodoaminophenethyl-1-propylxanthine) (22). After the fourth round of purification, the viral titers were calculated according to the end-point dilution method (21).

Expression of the recombinant A_1 adenosine receptor. Sf9 cells were maintained in culture as described previously (16). To prepare membranes, harvested cells were thawed in 15 × their wet weight of ice-cold homogenization buffer (25 mM HEPES, 100 mM NaCl, 1 μM adenosine, 100 μM phenylmethylsulfonyl fluoride, 20 μg/ml benzamidine, and 2 μg/ml concentration each of aprotinin, leupeptin, and pepstatin A) and burst by N_2 cavitation (600 p.s.i., 20 min). Cavitated cells were centrifuged at 4° for 10 min at 750 × *g* to remove the unbroken nuclei and cell debris. The supernatant from the low-speed spin was centrifuged at 4° for 30 min at 28,000 × *g*. The supernatant was discarded, and the pellets were resuspended, pooled, washed twice in homogenization buffer, resuspended at a concentration of 5 mg of protein/ml, snap-frozen in liquid nitrogen, and stored at −70°. Bovine brain plasma membranes were prepared as described previously (22).

Expression and purification of recombinant G protein α and βγ subunits. The α subunits of the G proteins, G_{i1}, G_{i2}, G_{i3}, and G_o, were expressed using the baculovirus/Sf9 insect cell system and purified to homogeneity using DEAE, hydroxyapatite, and Mono P chromatography as described previously (16). The procedures used for the construction of the β and γ baculoviruses and coexpression of the β1γ1, β1γ2, β1γ3, β2γ2, and β2γ3 dimers have been described previously (17). The βγ subunits were purified to homogeneity by chromatography on DEAE followed by affinity chromatography on $G_{\alpha i2}$-agarose as described (23). The mixed bovine brain βγ isoforms used as a control in certain experiments were the kind gift of Dr. Paul Sternweis (Southwestern Medical Center, Dallas, TX).

Reconstitution of recombinant receptors with G proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge at 10,000 × *g* for 10 min at 4°, and resuspended at 1–2 mg of protein/ml in a reconstitution buffer consisting of 5 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 500 nM GDP, and 0.04% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (w/v), pH 7.5. G protein α and βγ subunits were mixed and diluted in reconstitution buffer containing 0.1 mg/ml bovine serum albumin such that the desired quantity was contained in a 2-10-μl volume. In some experiments, anti-G_α common antibodies (1:200, New England Nuclear, Boston, MA) were added to the incubation during reconstitution.

Typically, a 2–10-μl volume containing the desired quantity of G protein α and βγ subunits was added to a 40-μl volume of membrane suspension containing ~100 fmol of recombinant A_1 adenosine receptors (measured as [^{125}I]BW-A844U binding sites). The mixture was incubated at 30° for 15 min and kept on ice until the start of the agonist radioligand ^{125}I-ABA binding assay. Just before the start of the binding assay, the reconstitution mixture was diluted 5-fold with binding assay buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) so that ~20 fmol of A_1 adenosine receptors were contained in a 25-μl volume.

Radioligand binding. ^{125}I-ABA binding and [^{125}I]BW-A844U binding to recombinant A_1 adenosine receptors with various amounts of reconstituted G protein α and βγ subunits were determined in a radioligand binding buffer containing 10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 5 units/ml adenosine deaminase in the presence of various additional components in a final volume of 100 μl. Usually, 50 μl of radioligand in binding buffer was added to tubes containing a total of 50 μl of a combination of membranes, reconstituted G proteins, and various compounds. Binding assays were incubated to equilibrium for 3 hr at room temperature (25°). The binding assays were terminated by filtration over Whatman GF/C glass-fiber filters. The filters were rinsed three times with 4 ml of ice-cold 10 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$ at 4° and counted in a Wallac (Gaithersberg, MD) 1470 Wizard automatic gamma counter at an efficiency of 75%. For each reconstitution, binding was measured in triplicate in the absence and presence of 100 μM GTPγS, and nonspecific binding was measured in the presence of 10 μM (*R*)-PIA. Free radioligand was calculated as the difference between the total radioligand added and the total bound.

Results

Sf9 cells infected with the appropriate baculovirus expressed recombinant A_1 adenosine receptors in the range of 2–5 pmol/mg of membrane protein. Uninfected cells or cells infected with wild-type *Autographica californica* nuclear polyhedrosis virus did not express A_1 adenosine receptors as detected by radioligand binding. Brain membranes, which

are considered to be a rich source of A_1 adenosine receptors, contain fewer receptors (0.5–1 pmol/mg of protein) than do membranes from recombinant Sf9 cells infected with the recombinant A_1 adenosine receptor virus. The relative expression levels of representative Sf9 cell and bovine brain membrane preparations, as measured using 1 nM [^{125}I]BW-A844 (an antagonist), are shown in Fig. 1. The K_D value calculated based on equilibrium binding isotherms for three preparations of membranes derived from infected Sf9 cells was 0.53 ± 0.12 nM with a B_{max} of 2–5 pmol/mg of protein (average, 3.6 pmol/mg). This high affinity is characteristic of the bovine species of A_1 adenosine receptors for 8-cyclopentylxanthine radioligands (20, 22, 24).

Fig. 2A presents saturation isotherms for the binding of the agonist, ^{125}I-ABA, to membranes prepared from recombinant bovine A_1 adenosine receptor virus-infected Sf9 cells. Agonist binding was performed in the absence and presence of GTPγS, and the data were fit to two- and one-site binding equations, respectively. The two-site fit was optimal; the parameters obtained for each site are summarized in Table 1. The K_D value for the high affinity binding site (0.09 ± 0.05 nM) is in agreement with values reported in the literature for the native A_1 adenosine receptor in bovine brain membranes (0.09 ± 0.02 nM) (22). Low affinity A_1 adenosine receptor agonist-binding sites usually cannot be accurately measured directly using agonist radioligands. However, because of the high levels of receptor expression, the high affinity of the bovine species of A_1 adenosine receptors for ^{125}I-ABA, and the absence of "background" in Sf9 cell membranes, we were able to precisely measure low affinity agonist radioligand binding to A_1 adenosine receptors. The K_D value for the low affinity site was 10.4 ± 1.9 nM. The conversion of the A_1 receptor from a mixture of high and low affinity sites to a single site on the addition of GTPγS is clearly visible in the Scatchard plots in Fig. 2A (*inset*). Analysis of binding in the presence and absence of GTPγS indicates that <10% of the recombinant A_1 adenosine receptors couple to endogenous G proteins in the Sf9 cell membranes (Table 1). This compares to values of >70% observed in brain membranes as determined by the ratio of high affinity agonist binding to antagonist binding or by competition of agonists for antagonist binding sites (20, 22). Thus, expression of recombinant bovine A_1 adenosine receptors in Sf9 cells results in membranes containing a large number of receptors that can be precisely measured by both agonist and antagonist radioligands with

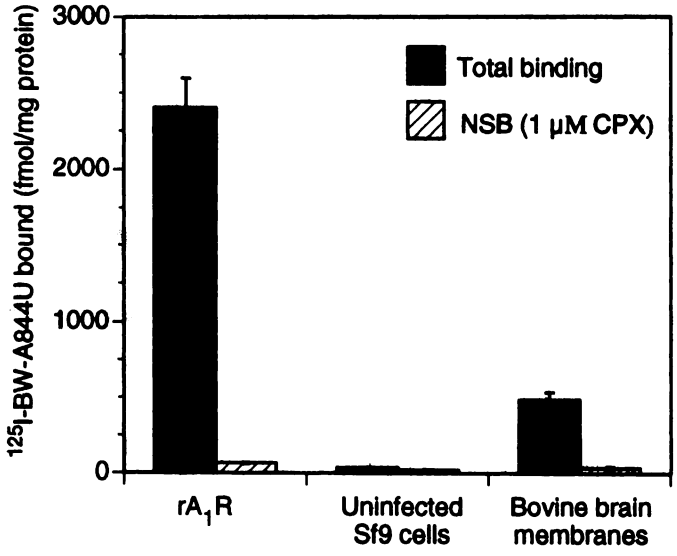
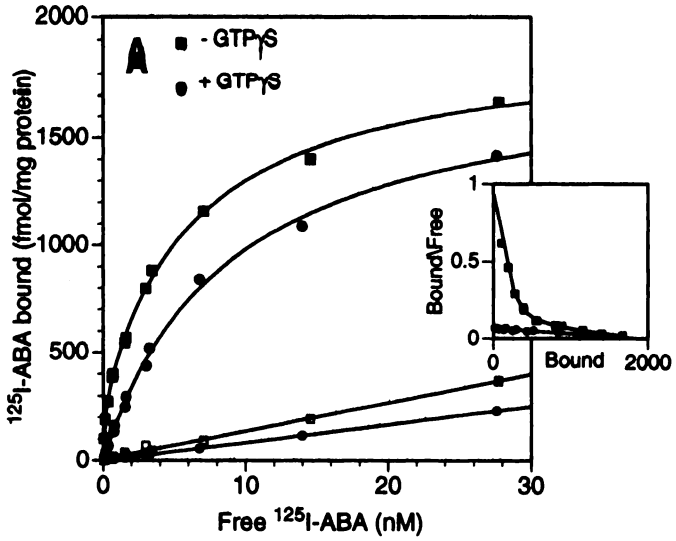


Fig. 1. Binding of the A_1-selective antagonist [^{125}I]BW-A844U to recombinant bovine A_1 adenosine receptors (*rA$_1$R*) expressed in Sf9 cell membranes. Sf9 cell or bovine brain membranes (25 μg) were incubated with 1 nM [^{125}I]BW-A844U (2× the K_D), and the amount of ligand bound was determined as described in Materials and Methods. Nonspecific binding (*NSB*) was determined by the addition of 1 μM 8-cyclopentyl-1,3-dipropylxanthine (*CPX*). Data are the mean ± standard error of triplicate determinations from a representative experiment repeated three times.



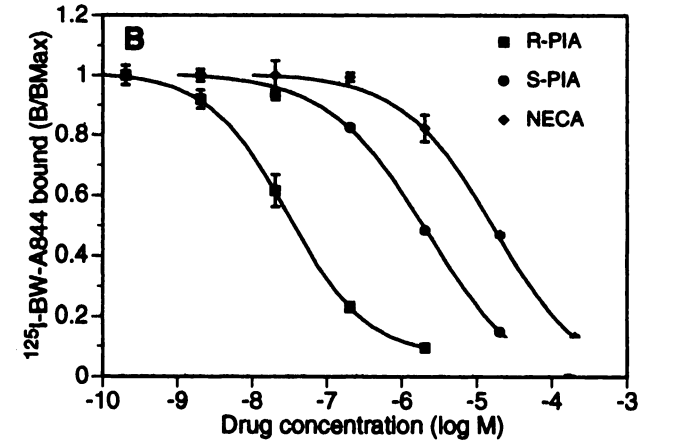


Fig. 2. Characterization of recombinant A_1 adenosine receptors expressed in Sf9 cell membranes. A, Saturation isotherms of ^{125}I-ABA binding to membranes from Sf9 cells expressing the recombinant A_1 adenosine receptor. Binding was measured in the (■) absence and (●) presence of 50 μM GTPγS. *Closed symbols*, specific binding. *Open symbols*, nonspecific binding as measured by the addition of 10 μM (*R*)-PIA. The B_{max} and K_d values were derived using nonlinear least-squares analysis (51). The binding data in the presence and absence of GTPγS were fit optimally to one- and two-site equations, respectively (52). The binding parameters determined from this analysis are reported in Table 1. *Inset*, Scatchard plot of specific binding. B, Competition by adenosine analogs for total [^{125}I]BW-A844U binding to Sf9 cell membranes expressing recombinant A_1 adenosine receptors. Membranes prepared from Sf9 cells were incubated in the presence of 1 nM [^{125}I]BW-A844U and various concentrations of competing compounds for 3 hr at 23°. Data are mean ± standard error of triplicate determinations from representative experiments repeated two or three times.

TABLE 1

Characterization of ^{125}I-ABA binding to membranes from Sf9 cells expressing recombinant bovine A_1 adenosine receptors

^{125}I-ABA binding to Sf9 cell membranes expressing recombinant receptors was measured as described in Materials and Methods. Binding isotherms were performed using radioligand (^{125}I-ABA) alone or with radioligand diluted with ^{125}I-ABA in 12 concentrations ranging from 0.05 to 30 nM. Data were fit to one- and two-site equations using Marquardt's nonlinear least-squares method (51). Two-site fits were judged superior to one-site fits using *F* tests evaluated at $p < 0.01$ (52). Parameters are the mean ± standard error from three binding isotherms.

^{125}I-ABA	B_{max} high affinity	K_D high affinity	B_{max} low affinity	K_D low affinity
Recombinant bovine A_1 adenosine receptor	*fmol/mg*	*nM*	*fmol/mg*	*nM*
−GTPγS	257 ± 46	0.09 ± 0.05	1808 ± 84	10.4 ± 1.9
+100 μM GTPγS			2420 ± 87	17.3 ± 1.2

low background binding and relatively little coupling to endogenous G proteins.

The pharmacology of the recombinant A_1 adenosine receptor expressed in Sf9 cell membranes was investigated by competition binding studies using the antagonist ligand [^{125}I]BW-A844U and various adenosine analogs (Fig. 2B). As summarized in Table 2, the order of potency observed [(*R*)-PIA > (*S*)-PIA > NECA] agrees with the known order of potency of the bovine brain A_1 adenosine receptor (20). The Hill coefficients for agonist competition curves were significantly <1, which is consistent with the existence of both high and low affinity binding sites, as shown in Fig. 2A. In addition, K_i values reported in the literature (22) for inhibition of specific [^{125}I]BW-A844U binding to the A_1 adenosine receptor in membranes by (*R*)-PIA (69.7 ± 11.4 nM) and NECA (9400 ± 3800 nM) agree well with those determined for recombinant bovine A_1 adenosine receptors in the current study (Table 2). The binding data suggest that recombinant bovine A_1 adenosine receptors are functionally similar to the native receptors in brain tissue.

Only a small fraction (10%) of recombinant bovine A_1 adenosine receptors bound ^{125}I-ABA with high affinity (K_D = 0.09 nM) and were sensitive to the addition of GTPγS (Fig. 2A), suggesting that these receptors were coupled to endogenous Sf9 cell G proteins. The majority (90%) of recombinant bovine A_1 adenosine receptors bound ^{125}I-ABA with low affinity (10 nM) and were insensitive to the addition of GTPγS, suggesting that these receptors were not coupled to endogenous G proteins. We exploited this experimental system to examine the nature of G protein subunits capable of interacting with the recombinant bovine A_1 adenosine receptors. To reconstitute high affinity agonist (^{125}I-ABA) binding, purified G protein α and βγ subunits were added to membranes from Sf9 cells expressing recombinant A_1 adenosine receptors. When a molar excess of purified, recombinant heterotrimeric G proteins were mixed with Sf9 cell membranes expressing recombinant A_1 receptors, there was a 7-fold increase in the GTPγS-sensitive component of ^{125}I-ABA binding, indicative of receptors converted from the low affinity to the high affinity agonist binding conformation (Fig. 3). For these experiments, ^{125}I-ABA was added at 0.4 nM, ~4.5-fold above the K_D value of its high affinity site and 25-fold below the K_D value for its low affinity site. Under these conditions, the reconstitution of high affinity binding is optimally detected. The increase in agonist binding is abolished by GTPγS, reflecting the fact that the added guanine nucleotide completely uncouples G proteins reconstituted with the receptor. The residual GTPγS-insensitive binding corresponds to the GTPγS-insensitive level determined in the absence of added G proteins and thus probably represents a low level of radioligand binding to uncoupled receptors. Note that the increased high affinity binding observed when G protein subunits are added is dependent on the addition of both α and βγ subunits to the system (i.e., no increase occurs when either subunit alone is reconstituted with the recombinant A_1 receptor or when the G protein α subunit is preincubated with G_α-common antisera) (data not shown). In one respect, this observation is consistent with those of previous studies using purified components which established that both α and βγ subunits are required for receptor coupling (25–27). However, these results are intriguing because they indicate that mammalian α

TABLE 2

Affinities of recombinant bovine A_1 adenosine receptors for adenosine agonists

For each adenosine ligand, competitive inhibition experiments were performed as described in Materials and Methods. IC_{50} and Hill coefficient values were obtained from nonlinear regression analysis (51). The IC_{50} values were converted to K_i values according to Linden (53). K_i values are mean ± standard error for two or three determinations. N = 2–3.

Ligand	Recombinant bovine A_1 adenosine receptor		
	K_i	pK_i	Hill coefficient
	nM		
(*R*)-PIA	19.9 ± 2.0	7.7	0.91
(*S*)-PIA	846 ± 55	6.07	0.68
NECA	8670 ± 944	5.06	0.86

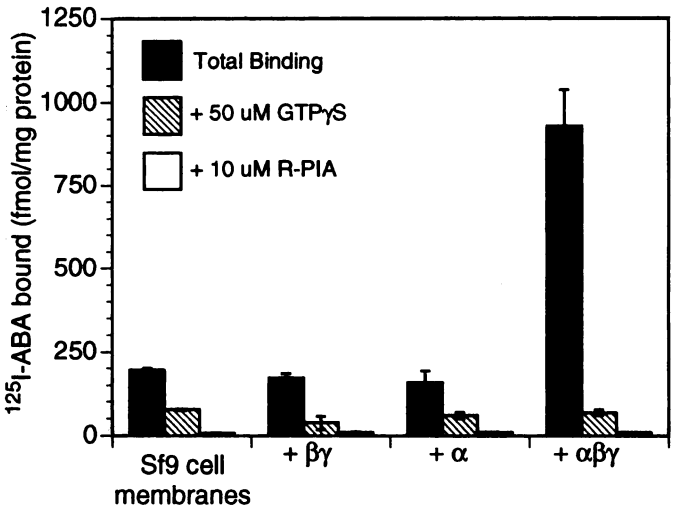


Fig. 3. Reconstitution of high affinity ^{125}I-ABA binding in membranes from Sf9 cells expressing recombinant A_1 adenosine receptors. Reconstitution of recombinant $G_{\alpha i2}$ and mixed bovine βγ isoforms into Sf9 cell membranes containing recombinant A_1 adenosine receptors was performed as described in Materials and Methods. Each tube in the binding assay contained 24 fmol of receptor. The molar ratio of receptor/α/βγ was ~1:70:40. For reasons described in the text, the concentration of ^{125}I-ABA in the assay was 0.4 nM. Data are the mean ± standard deviation of triplicate determinations from a representative assay repeated four times.

or βγ subunits added individually to Sf9 cell membranes cannot interact with the endogenous Sf9 cell G proteins (17, 28) to form competent heterotrimers. The calculated B_{max} value for high affinity ^{125}I-ABA binding sites, estimated from the concentration of radioligand added (1 nM) and the high affinity K_D value (0.09 nM), is ~1.5 pmol/mg of protein, accounting for ≥40% of the total receptor population in the membrane as determined by antagonist binding using [^{125}I]BW-A844U. Apparently some of the recombinant receptors are not accessible for reconstitution with G proteins.

The availability of recombinant mammalian G protein α and βγ subunits purified from Sf9 cells permits the assembly of heterotrimers of defined composition. The stoichiometry of recombinant α subunits needed to reconstitute high affinity ^{125}I-ABA binding was determined using various $G_{\alpha i}$ and $G_{\alpha o}$ subunits and a 100-fold molar excess of mixed bovine brain βγ isoforms over receptors, as shown in Fig. 4. The reconstitution of maximal high affinity binding required only a 5–10-fold excess of G protein α subunit over receptor and reached a plateau (Fig. 4A). Statistical analysis of the data showed no difference among the various G_α subunits with respect to their ability to reconstitute high affinity binding (see legend to Fig. 4). Increasing the α/receptor ratio to as high as 60:1 did not significantly increase maximal binding (Fig. 4B). The stoichiometry of various recombinant G protein βγ subunits needed to reconstitute high affinity agonist binding was examined using fixed concentrations of receptor and a 100-fold molar excess of $G_{\alpha i2}$ over receptors (Fig. 5). As was observed in the experiments in which the α subunits were titrated, maximal high affinity ^{125}I-ABA binding occurred with a 5–10-fold molar excess of various βγs over the receptor. Using a $G_{\alpha i2}$/receptor ratio of 100:1, various recombinant βγ subunits (β1γ2, β1γ3, β2γ2, β2γ3) were found to be equally potent and efficacious in reconstituting high affinity binding using the same mathematical analysis as was applied to the data in Fig. 4 (see legends to Fig. 4 or 5). In all of these reconstitution experiments, high affinity agonist binding was completely abolished by inclusion of 50 μM GTPγS (e.g., Fig. 5, ○).

The experiments shown in Figs. 4 and 5 determined the maximally effective concentrations of α and βγ subunits that re-established high affinity agonist binding. Next, we assessed the relative abilities of the various combinations of α and βγ subunits to reconstitute high affinity agonist binding. The ability of four α subunits and four different βγ dimers to reconstitute high affinity agonist binding was compared in the same experiment using submaximal concentrations (A_1 adenosine receptor/α/βγ ratio of 1:2.5:4). These stoichiometries were chosen from the curves shown in Figs. 4 and 5 to maximize the possibility of detecting differences in the affinities for the interactions between the reconstituted components. As shown in Fig. 6, all combinations of α with the various βγ subunits were equally effective in reconstituting high affinity agonist binding with the exception of the $G_{\alpha o}$ subunit, which was slightly less effective than the three $G_{\alpha i}$ subunits. The recombinant βγ subunits tested (β1γ2, β1γ3, β2γ2) and a preparation of mixed βγ isoforms purified from bovine brain were also similar in their ability to reconstitute high affinity agonist binding when paired with any of the α subunits.

Because the particular βγ subunits examined in Fig. 6 did not distinguish among the α subunits tested, two other recombinant βγ dimers that have been reported to be less effective than β1γ2 in other assays of βγ function were reconstituted with the $G_{\alpha i2}$ subunit. The β2γ3 dimer is reported to be approximately half as efficacious as other βγ dimers at stimulating phospholipase Cβ (23), and the β1γ1 dimer is reported to be less potent than other dimers in various reconstitution assays with effectors (29). Fig. 7A compares the activity of these two βγ dimers with the three other dimers shown in Fig. 6. Note that β2γ3 is equally effective with β1γ2 at reconstituting high affinity agonist binding and that β1γ1 is less effective than the other dimers. In an attempt to explain the reduced effectiveness of β1γ1, we compared the ability of various concentrations of β1γ1 and β1γ2 to reconstitute high affinity binding (Fig. 7B). Note that the data

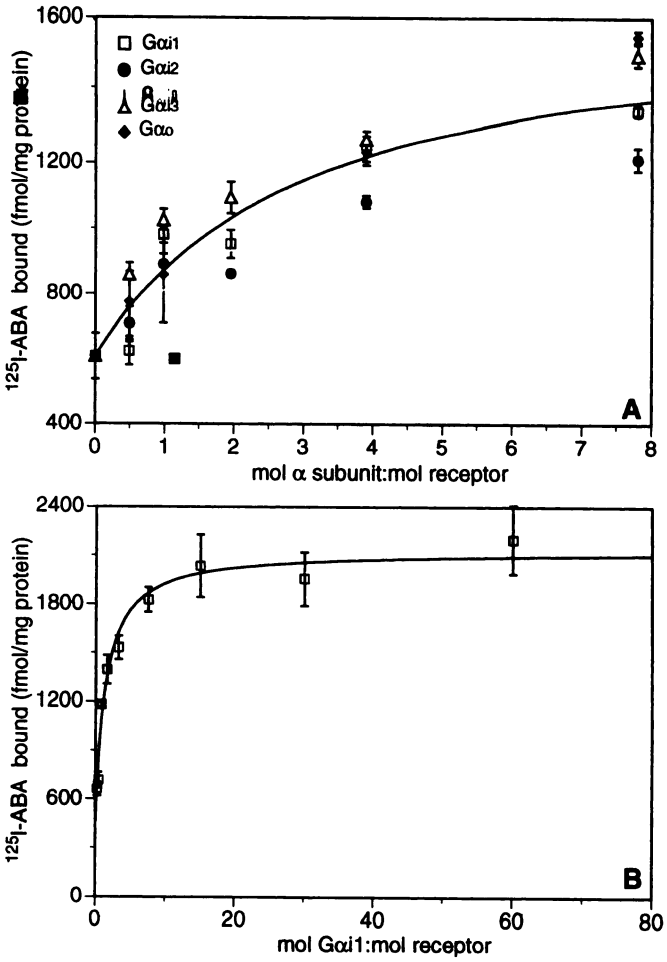


Fig. 4. Concentrations of $G_{\alpha i1}$ subunit required for high affinity agonist binding to recombinant A_1 adenosine receptors in Sf9 cell membranes. Membranes were reconstituted with purified mixed bovine brain βγ isoforms at a molar ratio of 100:1 βγ/receptor and the indicated amounts of recombinant G_α subunits. A, Each tube in the binding assay contained 24 fmol of receptor, and the ^{125}I-ABA concentration was 0.5 nM. Data are the mean ± standard deviation of triplicate determinations of specific ^{125}I-ABA binding from a representative experiment repeated three times. Data for each α subunit were fit to a rectangular hyperbola using Marquardt's method (51). There were no significant differences among individual data sets. *Line*, rectangular hyperbola fit to the pooled data for all of the α subunits. The B_{max} (fmol/mg of protein) and EC_{50} (mol of α/mol of receptor) values for the individual α subunits were calculated as αi1, 1708 fmol/mg of protein and 3.32 mol of α/mol of receptor; αi2, 1437 and 3.07; αi3, 1647 and 1.82; αo, 2187 and 5.5; and pooled data; 1707 and 3.14, respectively. B, Total ^{125}I-ABA binding measured in the presence of a wide range of $G_{\alpha i1}$/receptor.

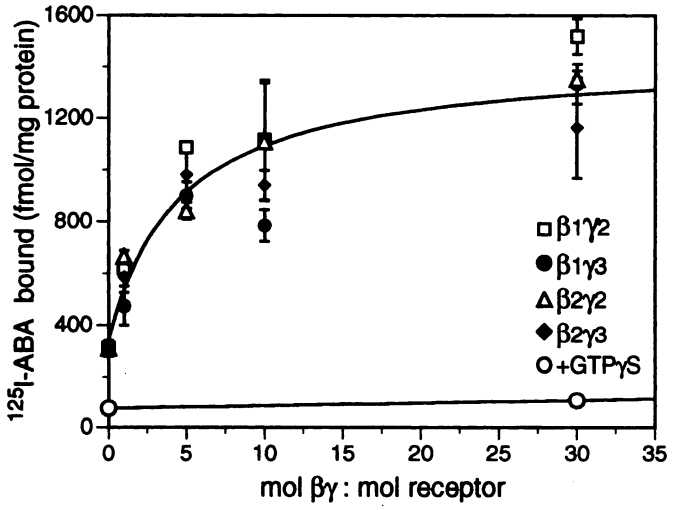


Fig. 5. Concentrations of βγ subunit required for high affinity agonist binding to recombinant A_1 adenosine receptors in Sf9 cell membranes. Various concentrations of different βγ dimers were reconstituted with recombinant $G_{\alpha i2}$ at a 100:1 molar ratio of α subunit/receptor. Each tube in the binding assay contained 24 fmol of receptor, and the ^{125}I-ABA concentration was 0.5 nM. ^{125}I-ABA binding was measured in the absence and presence (○) of 50 μM GTPγS. Data are the mean ± standard deviation of triplicate determinations from a representative experiment repeated twice. Data for each βγ subunit were fit to a rectangular hyperbola using Marquardt's method (51). There were no significant differences between individual data sets. *Line*, rectangular hyperbola fit to the pooled data for all the βγ subunits. The B_{max} (fmol/mg of protein) and EC_{50} (mol of βγ/mol of receptor) values for the individual βγ subunits were calculated as β1γ2, 1621 fmol/mg of protein and 4.2 mol of βγ/mol of receptor; β1γ3, 1649 and 11.4; β2γ2, 1511 and 5.52; β2γ3, 1181 and 2.08; and pooled data, 1438 and 4.58, respectively.

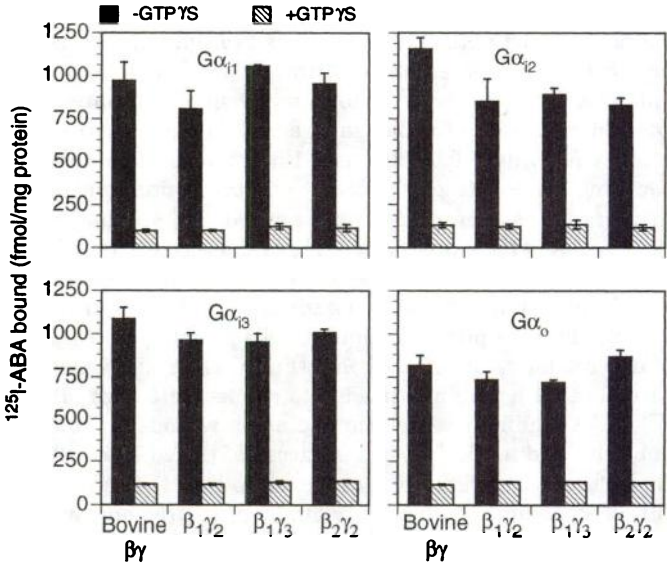


Fig. 6. Comparison of the abilities of recombinant α and βγ subunits to reconstitute high affinity ^{125}I-ABA binding in membranes from Sf9 cells expressing recombinant A_1 adenosine receptors. Sf9 cell membranes containing recombinant A_1 adenosine receptors were reconstituted with the indicated combinations of recombinant α and βγ subunits as described in Materials and Methods. Each tube in the binding assay contained 20 fmol of receptor. The molar ratio of receptor/α/βγ was ~1:2.5:4. The concentration of ^{125}I-ABA in the assay was 0.5 nM. Specific ^{125}I-ABA binding was measured in the (*solid bars*) absence and (*hatched bars*) presence of 50 μM GTPγS. Data are mean ± standard deviation of triplicate determinations from a representative experiment repeated three times.

suggest a 4-fold lower maximal efficacy of the β1γ1 dimer, with little or no difference in EC_{50} (see legend to Fig. 7). The combined results of the experiments shown in Figs. 4–7 indicate that there is no major difference in either the potency or the efficacy of the various α subunits and that most of the βγ subunits tested in their ability to reconstitute high affinity agonist binding to the A_1 adenosine receptor. An exception is the β1γ1 dimer, which is much less effective than the other βγ dimers.

Discussion

We used recombinant baculoviruses to infect Sf9 cells with bovine A_1 adenosine receptors or various G protein α or βγ subunits. The two most significant findings are discussed in detail below. First, we demonstrate that Sf9 cell membranes containing recombinant A_1 adenosine receptors can be used as a vehicle for the reconstitution of purified G protein subunits without interference from endogenous Sf9 cell G proteins. Second, we demonstrate that the addition of the β1γ1 G protein dimer produces less reconstitution of G protein coupling to A_1 adenosine receptors than do other βγ dimers. The poor reconstitution produced by β1γ1 can be attributed to reduced efficacy rather than to lower potency of reconstitution.

Recombinant bovine A_1 adenosine receptors expressed in Sf9 cells display binding affinities and a pharmacological profile identical to the properties of both native bovine A_1 adenosine receptors and recombinant bovine A_1 adenosine receptors expressed in mammalian cells (2, 20, 22, 24). In binding assays, A_1 adenosine receptors from the bovine species can be distinguished from A_1 receptors of other species by their unusually high affinities (subnanomolar K_D values) for the radioligands ^{125}I-ABA and [^{125}I]BW-A844U and by the unusual potency order of the three agonists [(*R*)-PIA > (*S*)-PIA > NECA]. Other species bind radioligands with lower affinities and bind agonists with a potency order of (*R*)-PIA ≥ NECA > (*S*)-PIA. The recombinant bovine A_1 adenosine receptor expressed in Sf9 cells retains these characteristics. In addition to expressing receptors with correct pharmacological properties, Sf9 cells express much higher levels of A_1 adenosine receptors, ~3-6 pmol/mg of membrane protein. Due to a combination of the high affinity of bovine A_1 adenosine receptors for ^{125}I-ABA and high expression levels, it was possible in this study to measure for the first time the binding of an agonist radioligand to the low affinity uncoupled conformation of the receptor (Fig. 2). In previous studies, the low affinity conformation of receptors could be demonstrated only in competition binding assays in which agonists were used to compete for the binding of antagonist radioligands (22).

The results of radioligand binding assays are consistent with those of others that Sf9 cells are capable of making post-translational modifications and inserting receptors into their cell membranes in a manner similar to that observed in mammalian cells (18, 30, 31). For example, recombinant 5-hydroxytryptamine, β-adrenergic, and muscarinic receptors (18, 32) have been expressed at levels of up to 30 pmol/mg of membrane protein.

Another advantage of Sf9 cell membranes as the source of recombinant A_1 adenosine receptors for this study is that

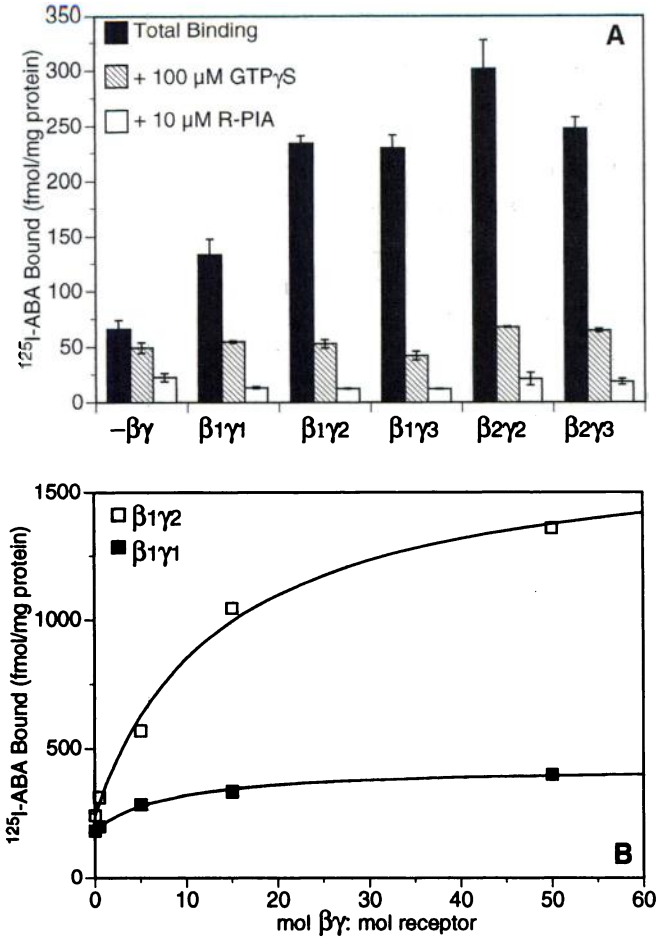


Fig. 7. Comparison of the abilities of five different βγ combinations to reconstitute high affinity binding of ^{125}I-ABA to recombinant A_1 adenosine receptors in Sf9 cell membranes. A, Sf9 cell membranes containing recombinant A_1 adenosine receptors were reconstituted with the indicated recombinant G protein heterotrimers as described in Materials and Methods. Each tube in the binding assay contained 22 fmol of receptor, and the molar ratio of receptor/αi2/βγ in the assay was 1:15:10. The concentration of ^{125}I-ABA was 0.3 nM. Data are mean ± standard deviation of triplicate determinations from a representative experiment repeated three times. The response to β1γ1 was significantly lower ($p < 0.01$) than that to other βγ combinations. B, Sf9 cell membranes containing recombinant A_1 adenosine receptors were reconstituted with the indicated concentrations of recombinant βγ-dimers as described in Materials and Methods. Each tube in the binding assay contained 20 fmol of receptor, 3 nM $G_{αi2}$, and 0.4 nM ^{125}I-ABA. *Error bars* are smaller than the symbols. Data were fit to rectangular hyperbola. For β1γ2, B_{max} = 1693 fmol/mg of protein and EC_{50} = 13.9 mol of βγ/mol of receptor. For β1γ1, B_{max} = 426 fmol/mg of protein and EC_{50} = 7.8 mol of βγ/mol of receptor.

based on the absence of radioligand binding, they do not seem to express any endogenous adenosine receptors. In addition, although Sf9 cells do express an endogenous G_o-like protein (28), it does not seem to couple efficiently to the expressed A_1 adenosine receptors because the coupling of the recombinant receptor to endogenous G proteins is <10% (Fig. 2). The data in Figs. 3–7 demonstrate that it is possible to use Sf9 cell membranes containing recombinant A_1 adenosine receptors as a system for monitoring receptor coupling to G proteins in reconstitution assays. A useful and intriguing finding of this study is that both α and βγ subunits must be reconstituted to elicit an increase in receptor/G coupling as assessed by high affinity agonist binding. This result is somewhat surprising because although it is known that both α and βγ subunits of G proteins are required for coupling to receptors (25–27), our data indicate that reconstituted exogenous α or βγ subunits cannot couple to endogenous Sf9 cell βγ or α subunits, respectively. This result implies that (a) endogenous Sf9 G proteins are fully coupled before G protein reconstitution because they are present in a very low stoichiometry compared with the overexpressed receptors or (b) endogenous Sf9 cell G protein α and βγ subunits are unable to interact effectively with reconstituted mammalian receptors or G proteins. Regardless of the reason, this outcome permits the study of recombinant receptors in a native cell membrane without interference from endogenous G proteins. Furthermore, a careful analysis of the interactions between the receptors and G protein α and βγ subunits can be made without the need to solubilize and purify receptors from the cell membranes, a process that may result in a loss in the ability of receptors to bind radioligands.

In assessing the ability of G protein α subunits to reconstitute high affinity agonist binding to A_1 adenosine receptors in the presence of a fixed concentration of βγ subunits, we found little difference among the αo or αi subunits. This result is similar to the outcomes observed in reconstitution assays using purified brain A_1 adenosine receptors and G protein α subunits purified from bovine brain (2, 5). Freissmuth *et al.* (4, 33) examined the interactions between bovine and human A_1 adenosine receptors and recombinant α subunits purified from *E. coli*. In these studies, a mixed population of bovine brain βγ subunits was used to reconstitute high affinity binding. In contrast to the data in our previous study and the current study, Freissmuth *et al.* found some preference of the A_1 adenosine receptor for the $G_{αi3}$ subunit over the other members of the $G_{αi}$ and $G_{αo}$ family. One potential reason for these differences could be that the G protein α subunits used in the current study were derived from Sf9 insect cells as opposed to *E. coli* (4, 33).

In all of the previous reconstitution studies with the A_1 adenosine receptor, a mixed population of βγ subunits was used to reconstitute high affinity binding (2, 4, 5, 33). Exhaustive mass spectrometric analysis of bovine brain βγ preparations have revealed a complex mixture of isoforms, including β1, β2, γ2, γ3, γ5, and γ7 (34, 35). Therefore, differences in the observed potencies of the reconstituted α subunits may be due to the nature of the available βγ subunits. We evaluated the reconstitution of high affinity binding using βγ subunits of defined composition. Our results indicate that none of the four α subunits tested distinguish among β1γ2, β1γ3, β2γ2, β2γ3, or mixed bovine brain βγ isoforms. It is possible that the functional similarities observed reflect the structural similarities of the α and βγ subunits examined. For example, the β1 and β2 subunits are 90% identical at the amino acid level, and based on the crystal structure of the heterotrimer, the amino acids contacting the α subunit are identical in these two proteins (36). Furthermore, the γ2 and γ3 subunits share an overall 77% identity and 89% homology at the amino acid level. In contrast, the γ1 amino acid sequence is only 39% and 36% identical to the γ2 and γ3 sequences, respectively, and β1γ1 was less effective than the other dimers in reconstituting A_1 adenosine receptor coupling to G proteins (Fig. 7, A and B). The data in Fig. 7B indicate than even at high concentrations, the β1γ1 subunit elicits less maximal coupling than the

other βγ subunits. Interestingly, Butkerait *et al.* (37) examined the interaction between 5-hydroxytryptamine$_{1A}$ receptors and coexpressed G protein α and βγ subunits in Sf9 cell membranes and also noted that β1γ1 was less effective than other βγ subunits in promoting receptor/G protein coupling. These findings with 5-hydroxytryptamine$_{1A}$ receptors are qualitatively similar to our findings with A_1 adenosine receptors. The difference in the maximal response between β1γ1 and other βγ subunits suggests that the result cannot be attributed to any possible difference in the efficiency with which the various βγ subunits are reconstituted into Sf9 cell membranes. Rather, the difference in activity may be due to the fact that the amino acid sequence of the γ1 subunit diverges substantially from those of the other γ subunits tested (γ2 and γ3) and/or that the lipid at the carboxyl terminus of the γ1 subunit is a farnesyl as opposed to a geranylgeranyl group (38–40). Preliminary results from ongoing studies with γ1 and γ2 prenyl mutants demonstrate that the nature of the lipid group contributes to the observed differences (41). It is important to note that the lower activity of the β1γ1 dimer is not universal. It is more effective at coupling rhodopsin to αt (42) and is approximately equally effective at coupling the αi and αo subunits to the 5-hydroxytryptamine$_{1Dβ}$ receptor.[2] Thus, with the notable exception of the γ1 subunit, the particular combinations of α and βγ subunits tested couple equally well to bovine A_1 adenosine receptors. Furthermore, with the exception of γ1, these particular β and γ subunits are highly expressed in brain and might be expected to couple well to the A_1 adenosine receptor, which is also widely distributed in brain (2). In summary, these findings with α and βγ subunits of defined composition are significant in that they provide clear evidence that protein/protein interactions between A_1 receptors and the various G protein α and βγ subunits tested in this study may be very similar.

Evidence based on antisense methods suggests that, although activation of a single receptor has the potential to activate multiple pathways, the number of signals actually produced is relatively small (43–45). In intact cells, such selectivity may reside at the level of G protein/effector coupling as well as at the level of receptor/G protein coupling. There also is evidence that in mammalian cells, certain βγ combinations are preferentially formed over other combinations (46–48). A plethora of additional mechanisms apparently contribute to the specificity of coupling seen in intact cells. Gudermann *et al.* (49) recently reviewed accumulating evidence that suggests that heterotrimeric G protein signaling is modulated at the level of receptors, G proteins, and effectors by other signaling cascades primarily described for growth factors and cytokines. There also is increasing evidence that the distribution and mobility of G proteins and receptors in cells are restricted to a surprising degree by segregation (e.g., in noncoated pits) (50). Thus, several factors apparently contribute to the fact that the selectivity observed by studying protein/protein interactions in reconstitution assays represents only a subset of the mechanisms used for producing selectivity of receptor effector coupling in the intact cell. The continued use of several different experimental techniques, including reconstitution, site-directed mutagenesis, and antisense knock-out strategies, will likely be required to further elucidate all of the factors that contribute to receptor/effector coupling selectivity.

[2] H. M. Clawges, E. M. Parker, and S. G. Graber, Human 5-HT$_2$ receptor subtypes exhibit distinct G protein coupling behavior in membranes from Sf9 cells, submitted for publication.

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