Fusion of β_2-Adrenergic Receptor to $G_{\alpha s}$ in Mammalian Cells: Identification of a Specific Signal Transduction Species Not Characteristic of Constitutive Activation or Precoupling†

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ABSTRACT: The forward and antegrade interactions that comprise the agonist receptor−G protein complex were studied in Chinese hamster fibroblasts transfected to express the β_2-adrenergic receptor (β_2AR), the β_2AR and the α-subunit of its cognate G protein (G_s), and a protein consisting of the β_2AR fused at its carboxy terminus with $G_{\alpha s}$ (β_2AR−G_s). Expression levels were matched at ∼600 fmol/mg. Basal adenylyl cyclase activities were increased with the fusion receptor membranes compared to coexpressed receptor plus $G_{\alpha s}$, and to wild-type β_2AR (20.5 ± 1.8 vs 9.0 ± 0.88 vs 8.7 ± 0.93 pmol min^{-1} mg^{-1}), confirming in mammalian cells that the fusion of β_2AR and $G_{\alpha s}$ results in a state not attained by expression of unfused components. However, agonist-stimulated activities were not increased proportionally, such that the stimulation over basal of the β_2AR−G_s fusion protein (1.5-fold) was less than wild-type β_2AR (2.1-fold). Agonist competition studies performed in the absence of guanine nucleotide exhibited high-affinity binding sites with a lower K_H (1.75 vs 8.47 nM) and greater $\%R_H$ (51% vs 44%) for β_2AR−G_s, but GppNHp failed to convert most of these to the low-affinity state. Functional studies with the inverse agonist ICI 118551 did not show enhanced efficacy or potency with the fusion protein. Adenylyl cyclase studies with three partial agonists with diverse structures (dobutamine, ritodrine, and phenylephrine) showed no enhancement of efficacy with β_2AR−G_s and a minor trend toward enhanced potency. Taken together, these results indicate that the tethering of $G_{\alpha s}$ to the β_2AR causes a conformational change in the receptor that stabilizes a species "trapped" between the non-guanine nucleotide-bound state and the GTP-bound form. Functionally the receptor is not characterized by a consistent pattern of properties ascribed to other states such as constitutive activation or precoupling, but rather represents a unique state in the transition from high- to low-affinity forms.

The β_2-adrenergic receptor (β_2AR)[1] signals to the cell interior via coupling to the stimulatory guanine nucleotide binding protein G_s, which in turn activates the effector adenylyl cyclase. The mechanisms by which the receptor binds and activates the G protein have been investigated using multiple approaches. Recently, the fusion of the β_2AR with G_s has provided a useful tool for investigating this interaction (*1*, *2*). The first report of a β_2AR−$G_{\alpha s}$ fusion protein was by Bertin et al. (*2*). In these studies, the cyc- variant S49 lymphoma cell, which lacks a functional G_s and is unresponsive to β-agonists, was transfected with a construct consisting of the β_2AR coding region followed by the $G_{\alpha s}$ open reading frame. Cells expressing this protein were compared to wild-type S49 lymphoma cells. Cells transfected with the fusion receptor not only showed cAMP responsiveness to agonist, but also the maximal response was higher than that of the wild-type S49 cell. It was concluded that the fusion of receptor to G protein created a complex which enhanced signaling over that of wild-type receptor and a competent G_s. However, the comparisons made in this study were between wild-type S49 cells (expressing ∼120 fmol/mg of β_2AR) and the cyc-transfected cells (expressing ∼800 fmol/mg of the fusion receptor). And thus it is not clear whether some portion of the enhanced activity that was observed was due to the overexpression of the fusion receptor as compared to wild-type S49 endogenous receptor expression. It was also not clear whether transfection of the β_2AR−G_s fusion receptor itself, or cotransfection of receptor and $G_{\alpha s}$, would have resulted in the same signaling phenotype. And, finally, basal levels of cAMP were not found to be elevated in cells expressing the fusion receptor, a result inconsistent with the model of the spontaneous transition of β_2AR to the active conformation in the absence of agonist (*3*, *4*).

Subsequent work by others utilized Sf9 insect cells and baculovirus infection resulting in profound overexpression (up to 100 μmol/mg) of the β_2AR or various receptor−$G_{\alpha s}$ fusion proteins (*1*, *5*−*7*). These studies have suggested that the β_2AR−$G_{\alpha s}$ (long form) fusion protein acts as a consti-

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; $G_{\alpha s}$, α-subunit of the stimulatory guanine nucleotide binding protein; CHW, Chinese hamster fibroblasts; K_L, K_H, low- and high-affinity binding constants, respectively; $\%R_H$, percentage of receptors in the high-affinity state; ^{125}I-CYP, ^{125}I-cyanopindolol; ICI 118551, *erythro*-DL-1-(7-methylindan-4,1-oxy)-3-isopropylaminobutan-2-ol.

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tutively activated mutant receptor. The use of insect cells and the reliance on extensive overexpression may make these results not applicable to mammalian cellular signal transduction. Also, the significant alteration in the stoichiometry of receptor/fusion protein to endogenous adenylyl cyclase and other potential components of the signaling pathway may have introduced additional variables that affect the phenotype. In the current paper, we define constitutive activation as enhanced functional signaling due to a mutation-induced conformation of the receptor that mimics the agonist-bound state. Based on current models, the agonist-stimulated function of a constitutively activated receptor is also increased (*3, 4*). Precoupling is a condition by which a wild-type receptor undergoes spontaneous transition to the activated state, to a degree that results in measurable increases in basal (i.e., nonagonist dependent) signaling. A number of G protein coupled receptors, expressed recombinantly or endogenously, display precoupling (*8*−*11*). This property is the basis for inverse agonist-promoted decreases in signaling due to these agents' stabilization of the nonactivated state. Precoupling alters basal signaling but does not influence the maximal agonist-stimulated response.

In the current study, we utilized Chinese hamster fibroblasts as a host cell, transfecting β_2AR, β_2AR with $G_{\alpha s}$, and a β_2AR−$G_{\alpha s}$ fusion protein to equivalent levels at the upper end of the physiologic range of receptor expression. Under these conditions, the β_2AR−$G_{\alpha s}$ fusion protein displayed a unique phenotype not characteristic of a constitutively activated or a precoupled receptor. These results suggest several new features of a complex model of G protein activation by β_2AR.

MATERIALS AND METHODS

Constructs. The β_2AR construct consisted of the human cDNA (*12*) with 40 bp of 5′ UTR and 580 bp of 3′ UTR subcloned into the expression vector pBC12BI. The $G_{\alpha s}$ construct consisted of the long form of the rat $G_{\alpha s}$ cDNA (*13*) with 143 bp of 5′ UTR and 184 bp of 3′ UTR subcloned into the expression vector pcDNA1 (Invitrogen). The β_2AR−$G_{\alpha s}$ fusion receptor construct was in pCDNA1 and consisted of the above β_2AR cDNA with the stop codon mutated to encode alanine and ligated in-frame at an NCO1 site immediately preceding the initiator methionine codon of the $G_{s\alpha}$ long-form cDNA.

Tissue Culture. CHW cells were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were permanently transfected using a calcium phosphate precipitation technique as described (*14*). Positive clones were selected based on resistance to 300 μg/mL G418 and maintained in the above medium with 80 μg/mL G418. For experiments using protein kinase A inhibitors, cells were grown to confluency, cultured in medium lacking fetal calf serum for 16 h, and then incubated an additional 2 h in the presence of 100 μM adenosine 3′,5′-cyclic monophosphorothioate, R_p isomer (R_p-cAMP), or in 10 μM of the cell-permeable myristoylated protein kinase A inhibitory peptide (*15*) (Calbiochem).

Radioligand Binding. Cells were washed twice with phosphate-buffered saline, detached with a rubber policeman in 5 mM Tris, pH 7.4, 2 mM EDTA buffer at 4 °C. Particulates were centrifuged at 30000*g* for 10 min and resuspended in the appropriate buffer for radioligand binding or adenylyl cyclase assays. Pellets were resuspended in 75 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA buffer for radioligand binding studies. Saturation binding experiments were carried out by incubating membranes with varying concentrations of ^{125}I-CYP in the absence (total binding) and presence (nonspecific binding) of 1 μM propranolol as described (*16*). Reactions were carried out for 1.5 h at 37 °C and terminated by dilution and rapid filtration over glass fiber filters. Specific binding was defined as the difference between total and nonspecific binding, normalized to protein, and expressed as femtomoles per milligram. One-site agonist competition binding studies were carried out with 40 pM ^{125}I-CYP, 100 μM GppNHp, and varying concentrations of isoproterenol as indicated for 1.5 h at 37 °C. To assess high- and low-affinity agonist receptor binding, membranes were prepared as described above, except two additional centrifugations were performed to ensure the removal of endogenous GTP. Membranes were incubated with 40 pM ^{125}I-CYP and 18 concentrations of isoproterenol without guanine nucleotide for 1.5 h at 37 °C. Competition data were fit to one-site and two-site models by an iterative least-squares technique as described previously (*17*) using the curve-fitting software Prizm. A two-site model was considered valid if by F-test the fit was statistically better ($p < 0.05$) than that obtained with a one-site model (*18*).

Immunoprecipitation and Western Blots. Western blots were carried out using whole cell lysates or immunoprecipitated protein essentially as previously described (*19*) except that proteins were fractionated by 10% SDS−PAGE and transferred without methanol in blots revealing the β_2AR−$G_{\alpha s}$ fusion protein. For immunoprecipitation experiments, β_2AR−$G_{\alpha s}$ constructs were transiently transfected into Cos-7 cells (*20*). After 48 h, whole cell lysates were immunoprecipitated with 1 μg of polyclonal antiserum $G_{\alpha s/olf}$ (Santa Cruz) as described (*21*). Immunoprecipitated $G_{\alpha s}$ protein was quantitated by Western blot using $G_{\alpha s/olf}$ at a titer of 1:200. Subsequently, Western analysis was performed on immunoprecipitated protein, normalized for $G_{\alpha s}$ content, using polyclonal antiserum G_β (Santa Cruz) at a titer of 1:200.

Adenylyl Cyclase Activity Measurements. Membranes were incubated with 30 mM Tris, pH 7.4, 2 mM $MgCl_2$, 0.8 mM EDTA, 120 μM ATP, 60 μM GTP, 2.8 mM phosphoenolpyruvate, 50 μg/mL myokinase, 100 μM cAMP, and 1 μCi of [α-^{32}P]ATP for 30 min at 37 °C as described (*16*). Reactions were carried out with varying concentrations of isoproterenol, the inverse agonist ICI 118551, and the indicated partial agonists. [^{32}P]cAMP was separated from [α-^{32}P]ATP by chromatography over alumina columns (*22*). A [^{3}H]cAMP standard included in the stop buffer accounted for individual column recovery.

Miscellaneous. Protein determinations were made by the copper bicinchoninic acid method (*23*). Results are presented as mean ± standard errors except when stated otherwise. Statistical comparisons were made by paired or unpaired two-way *t*-tests, as appropriate, with significance imparted for $p < 0.05$.

RESULTS AND DISCUSSION

As introduced earlier, our first goal was to determine if the enhanced signaling observed by Bertin et al. (*2*) was due

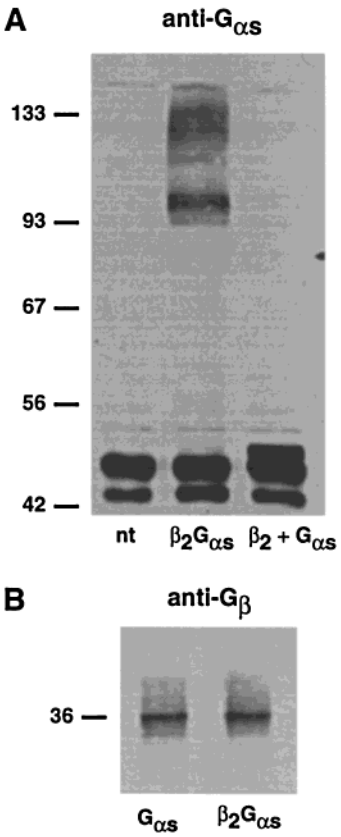

FIGURE 1: Immunological characterization of the β_2AR−G_s fusion protein and $G_{\alpha s}$ in CHW and COS-7 cells. (A) Western blot analysis of 75 μg of protein from permanent CHW cell lines was performed using the $G_{\alpha s/olf}$ antibody, reactive to the short and long forms of $G_{\alpha s}$, as described under Materials and Methods. The antisera identified the β_2AR−G_s fusion protein at the expected molecular mass as well as at a somewhat lower molecular mass. Nontransfected (nt) cells express the short form of $G_{\alpha s}$ but not the long form (molecular masses are 45 and 52 kDa, respectively). Cotransfection of the nonfused receptor and $G_{\alpha s}$ revealed the expected band for the latter. (B) Western blot analysis using G_β antisera of $G_{\alpha s/olf}$ immunoprecipitated protein (normalized for $G_{\alpha s}$ content) identifies approximately equal amounts of a ~36 kDa protein corresponding to G_β in COS-7 cells transiently transfected with $G_{\alpha s}$ and β_2AR−G_s (see Materials and Methods).

to the disparity in receptor expression between S49 wild-type and the cyc- cells transfected with the fusion receptor or could be accounted for by expression of $G_{\alpha s}$ without fusion to the receptor. CHW cells expressing β_2AR alone (569 ± 61 fmol/mg), $G_{\alpha s}$ and β_2AR expressed separately (710 ± 59 fmol/mg of β_2AR), and the β_2AR−G_s fusion receptor (690 ± 85 fmol/mg) were utilized. Figure 1A shows the results of Western blots using antisera against $G_{\alpha s}$. β_2AR expressed in CHW cells migrated as a broad band centered at ~70−80 kDa (*20*). Therefore, as expected, the β_2AR fusion receptor migrated at a higher molecular mass, with a band centered at ~120 kDa, consistent with its fusion to the long form of $G_{\alpha s}$ (52 kDa). Furthermore, Western analysis confirms expression of $G_{\alpha s}$ in cells cotransfected with the $G_{\alpha s}$ and the β_2AR constructs. (CHW cells do not express the long form of $G_{\alpha s}$, as indicated by a lack of a band at the appropriate molecular mass in the nontransfected cells.) In addition, we also investigated whether the β_2AR−$G_{\alpha s}$ fusion protein could interact with $G_{\beta\gamma}$ at levels similar to those of $G_{\alpha s}$ alone. In this series of experiments, transient transfections in COS-7 cells were performed to achieve high levels of

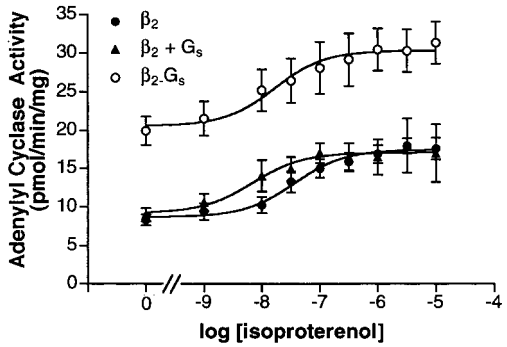

FIGURE 2: Adenylyl cyclase activities in CHW membranes expressing β_2AR, β_2AR with $G_{\alpha s}$, and β_2AR−G_s. Absolute adenylyl cyclase activities were determined in permanent cell lines with matched receptor expression as described under Materials and Methods. Shown are data from four independent experiments carried out with each line.

$G_{\alpha s}$ and β_2AR−G_s expression (relative to endogenous $G_{\alpha s}$ expression) so that in subsequent immunoprecipitation and Western blot analyses with $G_{\alpha s/olf}$ and G_β antisera the relative contribution of endogenous G protein complexes would be minimal. When normalized for the amount of immunoprecipitated $G_{\alpha s}$, Western blots of $G_{\alpha s}$ immunoprecipitated protein probed with G_β antisera showed similar amounts of a 36 kDa protein corresponding to G_β (Figure 1B), suggesting that β_2AR−G_α and $G_{\alpha s}$ appear to associate equally well with $G_{\beta\gamma}$ subunits. Our data are in agreement with Bertin et al. (*2*), who also showed that $G_{\beta\gamma}$ associates with the fusion receptor, with ADP-ribosylation by cholera toxin inducing significant increases in cAMP in cells expressing a β_2AR−G_s fusion receptor, a reaction requiring the presence of $\beta\gamma$ subunits. Despite this, however, it is not clear whether $\beta\gamma$ complexes are necessary for fusion protein function since ternary complex formation was not enhanced in Sf9 cells with the addition of $G_{\beta\gamma}$ (*1*).

Adenylyl cyclase activities from membranes derived from the three cell lines are shown in Figure 2. As can be seen, coexpression of $G_{\alpha s}$ with β_2AR does not increase receptor signaling over cells transfected to express the β_2AR to the same level. Thus, any enhancement observed with the β_2AR−G_s receptor is likely due to the direct fusion of these two elements rather than simply co-overexpression of the two components separately. However, as shown in Figure 2, basal adenylyl cyclase activities of the β_2AR−G_s fusion receptor are increased over wild-type β_2AR (20.5 ± 1.78 vs 8.7 ± 0.93 pmol min^{-1} mg^{-1}, $n = 4$, $p < 0.01$). Indeed, basal levels of the β_2AR−G_s fusion receptor are essentially the same as the maximal agonist-stimulated levels in membranes from the β_2AR-bearing cells (20.5 ± 1.78 vs 17.6 ± 1.19 pmol min^{-1} mg^{-1}, $n = 4$, $p =$ NS). This is in contrast to the studies by Bertin et al., who found no differences in basal levels of cAMP (*2*). Of note, we found no differences in expression of the dominant adenylyl cyclase isoforms, as assessed with Western blots, between the different cell lines (date not shown). The elevated basal activities of the current study are consistent with a precoupled receptor (*24*), and are partially consistent with models of constitutively activated receptors (*3*, *4*). With isoproterenol exposure, β_2AR−G_s adenylyl cyclase levels increased to 30.7 ± 2.49 pmol min^{-1} mg^{-1}. Interestingly, the fold stimulation over basal of the β_2AR−G_s fusion receptor was not enhanced,

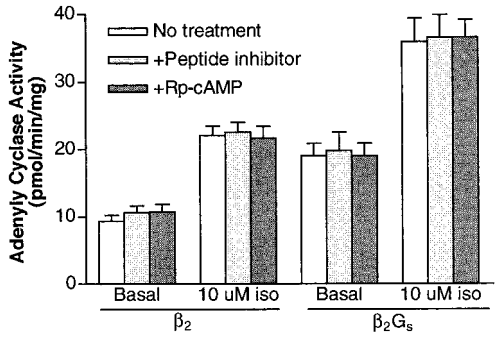

FIGURE 3: Adenylyl cyclase activities in CHW membranes expressing β_2AR and β_2AR−G_s pretreated with protein kinase A inhibitors. As described under Materials and Methods, pretreatment of permanent cell lines expressing β_2AR and β_2AR−G_s with either a cell-permeable peptide inhibitor or R_p-cAMP failed to show enhancement of adenylyl cyclase activity compared with untreated controls, suggesting that spontaneous desensitization of β_2AR−G_s does not occur.

and indeed tended to be *less* than that of the β_2AR (1.5 ± 0.05- vs 2.1 ± 0.23-fold, $p = 0.09$). The above characteristics of the two receptors were also maintained when assessed as a percentage of forskolin-stimulated adenylyl cyclase activity, with basal levels for the fusion protein increased over β_2AR (43.6 ± 3.96 vs 26.7 ± 4.03% of the forskolin response, $n = 4$, $p < 0.001$) and basal levels of the fusion protein similar to maximally stimulated levels of β_2AR (43.6 ± 3.96 vs 53.4 ± 5.66%, $n = 4$, $p =$ NS). These maximal agonist-stimulated activities with the fusion receptor were 67.9% of forskolin-stimulated levels. The results are in contrast to the findings of Kobilka and colleagues (*1*), where the fold stimulation induced by isoproterenol in a β_2AR−G_s fusion receptor expressed in Sf9 cells was found to be increased over that of the β_2AR and $G_{\alpha s}$ expressed to the same levels. Our findings of a lack of enhanced agonist stimulation over basal are not consistent with the accepted model of a constitutively activated β_2AR (*3*, *4*), where agonist-stimulated adenylyl cyclase activity is predicted to be enhanced.

Given the higher levels of basal adenylyl cyclase activity in clones expressing the β_2AR−$G_{\alpha s}$ fusion protein and the fact that this receptor has been shown to undergo agonist-promoted desensitization (*1*), we considered whether the reduced agonist-induced activation is the result of ongoing desensitization of the fusion protein due to elevated levels of cellular cAMP, activation of protein kinase A (PKA), and receptor phosphorylation. If this were true, we would expect enhanced adenylyl cyclase activity following treatment of β_2AR−$G_{\alpha s}$-expressing cells with PKA inhibitors. Treatment of β_2AR- and β_2AR−$G_{\alpha s}$-expressing cells with two different PKA inhibitors, however, had no effect on the adenylyl cyclase activities for either the wild-type or the β_2AR−$G_{\alpha s}$ fusion protein (Figure 3).

Ligand binding properties of the receptors from the cell lines expressing β_2AR or the β_2AR−G_s fusion protein are shown in Table 1 and Figure 4. The K_D for the radioligand ^{125}I-CYP, which is a weak partial agonist, was 2-fold lower for the β_2AR−G_s receptor compared to β_2AR. This enhanced affinity is consistent with the properties of a constitutively activated receptor (*4*). Isoproterenol competition studies revealed additional alterations in the interaction of agonist with the fusion receptor (Figure 4 and Table 1). In the absence of guanine nucleotide, data from both cell lines were best fit to a two-site model, with resolution of high- and low-affinity binding sites. The binding constants for these two states (K_H and K_L, respectively) were clearly different for the β_2AR−$G_{\alpha s}$ fusion receptor compared to wild-type β_2AR. As is shown, K_H for the fusion receptor was ∼1.75 nM compared to ∼8.47 nM for wild-type β_2AR. In addition, the fraction of receptors in the high-affinity state was increased (51 vs 44%). The K_L values were also lower for the fusion receptor. However, the K_L/K_H ratio, which has been proposed as one measure of the potential for free energy transfer upon activation (*25*), was in fact not different between the two receptors. This latter result is in agreement with our functional data, in that the fold stimulation of adenylyl cyclase over basal is not enhanced (indeed, slightly less) with the fusion receptor (Figure 2). Agonist competition studies performed in the presence of 100 μM of the nonhydrolyzable GTP analogue GppNHp revealed the expected rightward shift in the curves for wild-type β_2AR with a loss of the biphasic character. (Mean Hill slopes increased from −0.52 to −0.91; see Figure 4 and Table 1.) These data were best fit to a single (low-affinity)-site model with a mean K_i of ∼700 nM. Interestingly, these effects of GppNHp were not as apparent with the β_2AR−G_s fusion receptor. In the presence of GppNHp, the curves derived from the data with the fusion receptor tended to remain somewhat flat (mean Hill slopes changed from −0.49 to −0.64; see Figure 4 and Table 1), and in three of the four experiments, the data were best fit to two-site, instead of one-site, models. The K_H for this two-site fit in the presence of GppNHp for the fusion receptor was nevertheless higher (by ∼6-fold) than the analogous value in the absence of the nucleotide, and the % R_H was reduced to ∼38%. Taken together, then, the *differences* in agonist-mediated binding in the absence vs presence of GppNHp were less with the fusion protein compared to wild-type
β_2AR. This is consistent with the functional (adenylyl cyclase activation) data, where agonist stimulation over basal was less with the fusion protein compared to wild-type β_2AR. The increased basal adenylyl cyclase activity of the fusion protein correlates with the greater percentage of high-affinity receptors (and their higher affinity) compared to wild-type β_2AR, as would be predicted by the current model of a constitutively activated receptor (*3*). The relative insensitivity of the β_2AR−G_s receptor to GppNHp that we found is in contrast to what has been reported in Sf9 cells where guanine nucleotides caused a loss of two-site binding (*7*).

A characteristic property of functionally significant precoupling is the ability of agents that stabilize the inactive state (inverse agonists) to lower basal signaling (*24*). Constitutively activated receptors also display a decrease in basal signaling in response to inverse agonist binding, with an enhanced efficacy and/or potency compared to wild-type receptor (*26*). This property was examined in the current study with the inverse agonist ICI 118551. As shown in Figure 5, both the β_2AR and the β_2AR−G_s fusion protein displayed a decrease in adenylyl cyclase activities with this agent. The EC_{50} for this inhibition, however, was the same between the two receptors (2.35 ± 0.99 vs 2.36 ± 0.73 nM, $n = 5$, $p =$ NS). Also, the minimal activities observed were not different (6.1 ± 0.53 vs 7.7 ± 0.73 pmol min^{-1} mg^{-1}, $n = 5$, $p =$ NS). These results indicate that the fusion receptor is not structurally constrained in a manner that is unfavorable

Table 1: Binding Properties of β_2AR and β_2AR−G_s Fusion Protein Expressed in CHW Cells[a]

receptor	B_{max} (fmol/mg)	K_D (pM)	+Gpp(NH)p		−Gpp(NH)p				
			K_i (nM)	Hill slope	K_L (nM)	K_H (nM)	K_L/K_H	R_H (%)	Hill slope
β_2AR	569 ± 61.5	44.0 ± 2.78	878 ± 180	−0.91 ± 0.092	621 ± 87.1	8.47 ± 1.25	78.7 ± 15.3	44 ± 2.2	−0.52 ± 0.046
β_2AR−G_s	690 ± 85.0	22.3 ± 2.75[c]	*d*	−0.64 ± 0.082[c]	162 ± 56.3[c]	1.75 ± 0.464[b]	88.6 ± 8.82	51 ± 2.6[b]	−0.49 ± 0.0053

[a] ^{125}I-CYP binding was performed on membranes prepared from CHW cell lines stably expressing β_2AR and the β_2AR−G_s fusion protein as described under Materials and Methods. Isoproterenol competition binding experiments were analyzed by nonlinear regression for best fit to single-site or two-site binding. When data were best fit by two affinity states (by F-test), K_H and K_L designate the dissociation constants for the high- and low-affinity states of the receptor, respectively. % R_H indicates the percentage of high-affinity binding sites. When a single affinity state was detected, the dissociation constant is represented by K_i. The Hill slope was determined by nonlinear regression of data fitted to a sigmoidal dose−response curve (variable slope). Data shown represent the means ± SEM of four independent experiments. [b] $p < 0.05$. [c] $p < 0.01$. [d] Data for three of four experiments were best fit to two-site binding with mean K_H and K_L values of 13.7 ± 10.5 and 500 ± 17.2 nM, respectively, and a % R_H of 38 ± 10.

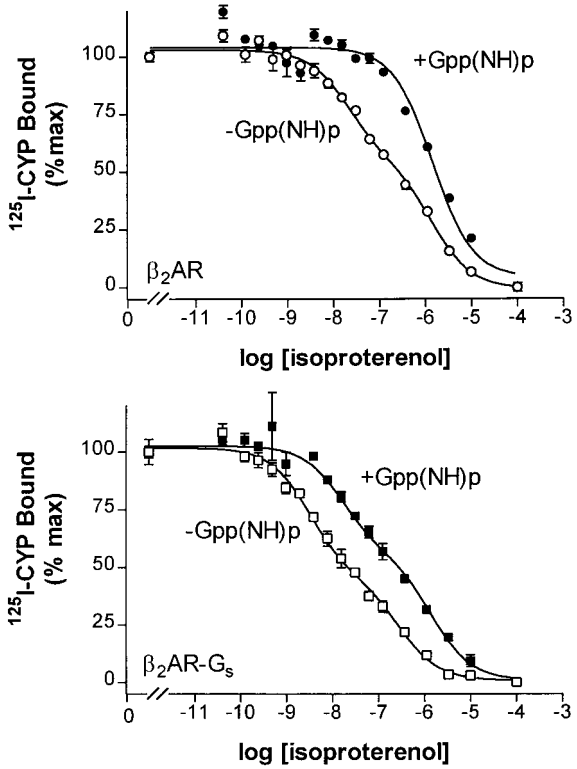

FIGURE 4: Agonist competition binding in CHW membranes expressing β_2AR and β_2AR−G_s. ^{125}I-CYP binding was performed as described under Materials and Methods in the presence or absence of Gpp(NH)p. In the absense of guanine nucleotide, both the β_2AR and β_2AR−G_s fusion protein data were best fit to a two-site model with high- and low-affinity components. With the addition of Gpp(NH)p, both curves were right-shifted, with data for β_2AR best fit to a one-site model with a single-affinity state and data for β_2AR−G_s best fit to a two-site model (see text). Shown are representative results from a single experiment performed in duplicate (mean ± SD). See Table 1 for mean results from four experiments.

for stabilization of the inactive conformation by inverse agonists. Of note, since the basal levels of adenylyl cyclase activity are higher with the β_2AR−G_s fusion receptor, and the minimal activities in response to ICI 118551 are the same, the "% inhibition" by this agent would be greater with the fusion receptor compared to wild-type β_2AR. When expressed in this way, the efficacy appears to be greater for the fusion receptor, but this is entirely due to the basal activities being higher. We contend, though, that the absolute level of activity resulting from inverse agonist binding is the pertinent value indicative of stabilization of the inactive state.

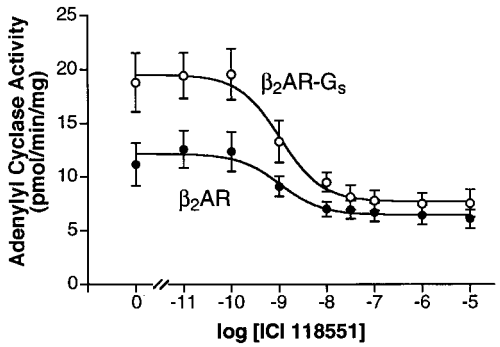

FIGURE 5: Inhibition of adenylyl cyclase activities by ICI 118551 in CHW cell membranes expressing β_2AR and β_2AR−G_s. Absolute adenylyl cyclase activities were determined in permanent cell lines with matched receptor expression as described under Materials and Methods. Shown are data from five independent experiments carried out with each line.

Table 2: Potencies and Intrinsic Activities of β_2AR Partial Agonists at β_2AR and β_2AR−G_s[a]

ligand	EC_{50} (nM)		intrinsic activity (%)	
	β_2AR	β_2AR−G_s	β_2AR	β_2AR−G_s
dobutamine	820 ± 5.57	393 ± 91.7[b]	55.3 ± 4.77	49.3 ± 3.83
ritodrine	1204 ± 191	718 ± 117	62.7 ± 6.62	65.1 ± 4.73
phenylepherine	3890 ± 622	2140 ± 790	74.9 ± 2.06	80.7 ± 5.47

[a] Adenylyl cyclase studies were performed in membranes prepared from CHW cell lines stably expressing β_2AR and β_2AR−G_s as described under Materials and Methods. EC_{50} values were calculated by nonlinear regression. "Intrinsic activity" indicates the ratio between the maximal adenylyl cyclase activity elicited by each drug and that obtained with 10 μM of the full agonist isoproterenol (basal levels subtracted) × 100%. Shown are means ± SEM of three experiments. [b] $p < 0.05$ vs value for β_2AR.

Another property of constitutively activated receptors is increased efficacy and/or potency of partial agonists compared to wild-type receptor (*3*, *26*). For these experiments, dobutamine-, ritodrine-, and phenylephrine-stimulated adenylyl cyclase activities were determined along with maximal isoproterenol-stimulated activities. The intrinsic activities of these three agonists (based on the isoproterenol responses) were not different between the β_2AR−G_s fusion and wild-type receptor (Table 2). Although these specific studies have not been reported in Sf9 cells, a comparison between fusion proteins consisting of the short and long forms of $G_{\alpha s}$ showed a greater efficacy for partial agonists for the long form (*5*). If we assume that the short-form $G_{\alpha s}$ fusion protein has an efficacy for partial agonists equal to or greater than β_2AR alone in Sf9 cells, our data are not in agreement with the notion that partial agonist efficacy is greater with fused

receptors. The lack of enhanced efficacy is not consistent with a constitutively activated receptor (*3*, *4*). A slight increase in potency (decrease in EC_{50} by ~2-fold) was observed for dobutamine, with a similar trend noted for the other two partial agonists.

These results indicate that fusion of the β_2AR to $G_{\alpha s}$ results in a unique receptor status. The signaling has some properties of precoupling, since basal activities are elevated and are inhibited by inverse agonists. However, a receptor acting purely in the precoupled state would not have greater absolute levels of agonist-stimulated adenylyl cyclase compared to wild-type receptor. β_2AR−G_s signaling also has some properties of a constitutively activated receptor, including the elevated basal activity and increased high-affinity binding. However, agonist-stimulated activities over basal would be expected to be higher with a constitutively activated receptor, and the potencies and efficacies of inverse agonists and partial agonists greater than wild-type β_2AR. The relative insensitivity of the high-affinity binding to GTP is also not consistent with constitutive activation. In CHO cells transfected with a β_2AR mutated in the carboxy terminus of the third loop, which displays constitutive activation, GppNHp caused a complete loss of high-affinity binding with agonist competition curves being best fit to a single site (*3*). The experimental conditions of these studies were not unlike those of the current ones, in that they were carried out in mammalian cells with receptor expressions similar to those we obtained in CHW cells. It is intriguing to consider how this minimal effect of guanine nucleotide is associated with higher absolute levels of agonist-stimulated adenylyl cyclase, given that the GTP-bound state of $G_{\alpha s}$ is the moiety that activates cyclase. Interestingly, a discrepancy between GTP binding and adenylyl cyclase activation with β_2AR−G_s fusion proteins with various tether lengths expressed in Sf9 cells has recently been shown to be due to altered GTPase activities (*6*). While GTPase activity or radiolabeled GTPγS binding cannot be measured in low-expressing mammalian cells, our data are consistent with these Sf9 cell results in this regard.

The current results support an extended cubic ternary complex model (*27*) of receptor−G protein interaction. The model has been useful in predicting many states that have been identified experimentally, although some species have yet to be found. The current work indicates that the fusion of β_2AR to $G_{\alpha s}$, with expression in mammalian cells at physiologic levels, results in a receptor with properties unlike any previously described. Key properties are the elevated basal activities, an agonist stimulation over basal that is less than that of wild-type β_2AR, increased high-affinity binding (both % R_H, K_H), a relative insensitivity of high-affinity binding to GTP, and the lack of significant differences in potencies or efficacies of inverse agonist or partial agonists.

Shown in Figure 6 is a representation of the cubic ternary complex model depicting various inactive−active transitions in the presence or absence of agonist binding and G protein interactions. The majority of the β_2AR−G_s fusion receptors appear to be in a quasi-stable transitional state represented by the shaded area. None of the four discrete states indicated at the corners of the region adequately depict this state, and thus the receptor is best localized to the indicated *plane* rather than a specific species. It would appear that this receptor is "trapped" in the transition from the high-affinity agonist/receptor/$G_{\alpha s}$ complex (without bound GTP) to the low-affinity GTP-bound state. Previously it has not been possible to study the former state in functional studies since it is highly unstable. The fact that it has less fold stimulation over basal activities and an unchanged K_L/K_H ratio (compared to wild-type) is consistent with the proposal that the GTP-bound $G_{\alpha s}$ complex is more efficient at stimulating adenylyl cyclase compared to the unbound state. This transitional state is not characterized by a consistent pattern of properties ascribed to other states such as constitutively activated or precoupled receptors, but represents a unique form of the ternary complex with specific characteristics.

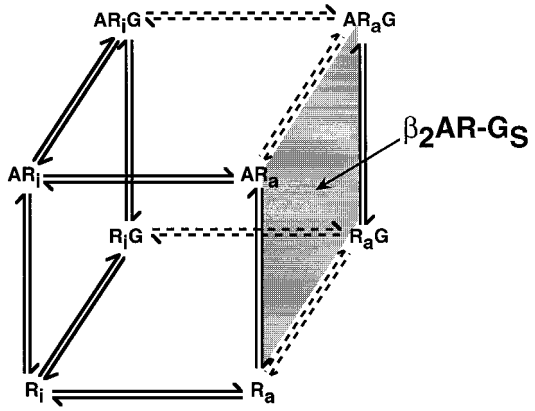

FIGURE 6: Representation of a cubic ternary complex model of β_2AR activation. The model is an extension of the extended ternary complex model (see ref *27*). The β_2AR−G_s fusion receptor is proposed to exist in a quasi-stable transitional state within the plane indicated by the shaded region. A = agonist, R_a = active receptor, R_i = inactive receptor, G = G protein. The rate constants are not shown for clarity.

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