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Different Effects of $G_s\alpha$ Splice Variants on β_2-Adrenoreceptor-mediated Signaling

THE β_2-ADRENORECEPTOR COUPLED TO THE LONG SPLICE VARIANT OF $G_s\alpha$ HAS PROPERTIES OF A CONSTITUTIVELY ACTIVE RECEPTOR*

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Roland Seifert‡§, Katharina Wenzel-Seifert‡§, Tae Weon Lee‡, Ulrik Gether‡¶, Elaine Sanders-Bush‡‖, and Brian K. Kobilka‡‡‡**

*From the ‡Howard Hughes Medical Institute, **Division of Cardiovascular Medicine, Stanford University Medical School, Stanford, California 94305-5428*

The β_2-adrenoreceptor (β_2AR) couples to the G-protein G_s to mediate adenylyl cyclase activation. The splice variants of $G_s\alpha$ differ by a 15-amino acid insert between the Ras-like domain and the α-helical domain. The long splice variant of $G_s\alpha$ ($G_s\alpha_L$) binds GDP with lower affinity than the short splice variant ($G_s\alpha_S$), but the impact of this difference on the interaction of $G_s\alpha$ with the β_2AR is not known. We studied the β_2AR/$G_s\alpha$ interaction using receptor/G-protein fusion proteins ($\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$) expressed in Sf9 cells. Fusion of the β_2AR to $G_s\alpha$ promotes efficient coupling as shown by high-affinity agonist binding and GTPase and adenylyl cyclase activation and ensures fixed stoichiometry between receptor and G-protein. Importantly, fusion does not change the fundamental properties of the β_2AR or $G_s\alpha$. The β_2AR in $\beta_2ARG_s\alpha_L$ showed hallmarks of constitutive activity (increased potency and intrinsic activity of partial agonists, increased efficacy of inverse agonists, and increased basal GTPase activity) compared with the β_2AR in $\beta_2ARG_s\alpha_S$. The apparent constitutive activity of the β_2AR in $\beta_2ARG_s\alpha_L$ may be due to the lower GDP affinity of $G_s\alpha_L$ compared with $G_s\alpha_S$, *i.e.* $G_s\alpha_L$ is more often nucleotide-free than $G_s\alpha_S$ and, therefore, more frequently available to stabilize the β_2AR in the active (R*) state. This study demonstrates that subtle structural differences between closely related G-protein α-subunits can have important consequences for the functional properties of a G-protein-coupled receptor.

Numerous hormones and neurotransmitters exert their effects through G-protein-coupled receptors (GPCRs)[1] (1–4). The β_2-adrenoreceptor (β_2AR), a prototypical GPCR, interacts with the G-protein G_s, causes GDP/GTP exchange at its α-subunit ($G_s\alpha$) and, thereby, leads to activation of adenylyl cyclase (AC). Recently, the ternary complex model of GPCR activation has been extended to explain the finding that GPCRs can activate G-proteins, even in the absence of agonist, and that certain receptor ligands, namely inverse agonists, can suppress the G-protein activation mediated by agonist-free GPCRs (5–14). The agonist-independent activity of a GPCR is referred to as constitutive activity. The extended ternary complex model (two-state model) assumes that agonists stabilize GPCRs in the active (R*) state, while inverse agonists stabilize the inactive (R) state. Although constitutive GPCR activity can be most easily observed when receptors are overexpressed (10–12) or mutated (7, 8, 13), it also occurs at physiological receptor expression levels (5, 6, 9, 14). Hallmarks of constitutive GPCR activity are increased potency and efficacy of partial agonists, increased efficacy of inverse agonists, and elevated basal G-protein activity (5–14). These properties of constitutive activity are generally associated with GPCR function, and little is known about the ability of different G-proteins to influence the efficacy and potency of ligands.

$G_s\alpha$ exists as a short ($G_s\alpha_S$) and a long ($G_s\alpha_L$) splice variant. Compared with $G_s\alpha_S$, $G_s\alpha_L$ contains additional 15 amino acids inserted at position 72 of the polypeptide chain, and there is an exchange of glutamate for aspartate at position 71 (15, 16) (Fig. 1*A*). Based on the α-carbon model of the α-subunit of the retinal G-protein tansducin (17), the sequence within which the 15-amino acid insert is localized in $G_s\alpha_L$ serves as a linker between the Ras-like domain and the α-helical domain (Fig. 1*B*). The guanine nucleotide-binding site is embedded between these two domains. Thus, a change in this linker sequence might be expected to influence the binding kinetics of guanine nucleotides. In fact, purified $G_s\alpha_L$ releases GDP more than twice as fast as $G_s\alpha_S$ (18).

The results of a previous study indicate that $G_s\alpha_S$ may be more effective than $G_s\alpha_L$ in activating AC (19), but with regard to β_2AR coupling, studies have not revealed significant differences between $G_s\alpha_S$ and $G_s\alpha_L$ (18, 20, 21). Studying differences

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§ Recipients of a research fellowship of the Deutsche Forschungsgemeinschaft.

¶ Present address: Dept. of Cellular Physiology, Institute of Medical Physiology 12.5, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2100 Copenhagen N, Denmark.

‖ Permanent address: Dept. of Pharmacology, Vanderbilt School of Medicine, Nashville, TN 37232-6600.

‡‡ To whom correspondence should be addressed: Howard Hughes Medical Institute, B-157, Beckman Center, Stanford University Medical School, Stanford, CA 94305-5428. Tel.: 650-723-7069; Fax: 650-498-5092; E-mail: kobilka@cmgm.stanford.edu.

[1] The abbreviations used are: GPCR(s), G-protein-coupled receptor(s); β_2AR, β_2-adrenoreceptor; β_2AR_{CAM}, constitutively active mutant of the β_2AR; $G_s\alpha$, α-subunit of the G-protein G_s; $G_s\alpha_L$, long splice variant of the α-subunit of G_s; $G_s\alpha_S$, short splice variant of the α-subunit of G_s; $\beta_2ARG_s\alpha_L$, fusion protein consisting of the β_2-adrenoreceptor and the long splice variant of $G_s\alpha$; $\beta_2ARG_s\alpha_S$, fusion protein of the β_2-adrenoreceptor and the short splice variant of $G_s\alpha$; DCI, dichloroisoproterenol; [^{3}H]DHA, [^{3}H]dihydroalprenolol; EPH, (−)-ephedrine; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GMPS, guanosine 5′-phosphorothioate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); (−)-ISO; (−)-isoproterenol; (+)-ISO, (+)-isoproterenol; ICI, ICI 118,551; SAL, salbutamol; DOB, dobutamine; ALP, (−)-alprenolol; AC, adenylyl cyclase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

in the interaction of structurally very similar G-proteins with a given GPCR is technically difficult, because functional interactions between receptors and G-proteins are strongly influenced by their relative expression levels (22). Specifically, defined receptor/G-protein stoichiometries have to be achieved to be able to detect subtle differences in GPCR/G-protein coupling.

To facilitate the examination of receptor/G-protein interactions we constructed fusion protein DNAs in which the C terminus of the β_2AR was linked to the N terminus of $G_s\alpha_S$ (β_2ARG$_s\alpha_S$) or $G_s\alpha_L$ (β_2ARG$_s\alpha_L$) (Fig. 1*A*) and expressed the fusion proteins in Sf9 cells. Fusion proteins have a fixed ratio of receptor to α-subunit (23, 24). Thus, ambiguities in data analysis because of varying stoichiometry of the signaling partners can be eliminated. Using the fusion protein approach, we observed that the β_2AR coupled to $G_s\alpha_L$ has properties of constitutively active GPCR.

EXPERIMENTAL PROCEDURES

Materials—Rat $G_s\alpha_L$ DNA was kindly provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD) (25). For generation of recombinant baculoviruses encoding for rat $G_s\alpha_L$, its DNA sequence was transferred into the baculovirus transfer vector pVL 1392 (11). [γ-^{32}P]GTP (6000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were from NEN Life Science Products. [^{3}H]Dihydroalprenolol ([^{3}H]DHA) (85–90 Ci/mmol) was from Amersham Corp. Anti-$G_s\alpha$ antibody was from Calbiochem. Guanosine 5′-phosphorothioate (GMPS) was from U. S. Biochemical Corp. All other nucleotides were from Boehringer Mannheim (Mannheim, Germany). Sources of other materials have been described elsewhere (11, 13, 26).

Construction of β_2ARG$_s\alpha_L$ and β_2ARG$_s\alpha_S$ DNAs—β_2ARG$_s\alpha_L$ DNA was generated by a two-step PCR protocol using *Pfu* polymerase. A set of fusion primers (sense and antisense), encoding 18 base pairs from the C terminus of the β_2AR, 18 base pairs encoding a hexahistidine tag, and 21 base pairs from the N terminus of $G_s\alpha_L$, were synthesized. In PCR 1A, the sequence between a primer 5′ of the *Eco*RV site of the human β_2AR and the antisense fusion primer was amplified using β_2AR DNA in pGEM-3Z as template. In this vector, referred to as pGEM-3Z-SF-β_2AR-6His, the β_2AR is tagged at the N terminus with the cleavable influenza-hemagglutinin signal sequence followed by the Flag epitope (IBI, New Haven, CT), and the C terminus of the β_2AR is tagged with a hexahistidine tail (Fig. 1*A*) (26). In PCR 1B, the sequence between the sense fusion primer and the antisense primer with an extra *Sal*I site 3′ of the stop codon of $G_s\alpha_L$ was amplified using rat $G_s\alpha_L$ DNA in pGEM-3Z as template. In PCR 2, the products of PCRs 1A and 1B were annealed and the sense primer 5′ of the *Eco*RV site in the β_2AR sequence and the antisense primer 3′ of the stop codon of $G_s\alpha_L$ were used. In this way, a fragment encoding the C terminus of the β_2AR, a hexahistidine tag, and $G_s\alpha_L$ was obtained. This fragment was digested with *Eco*RV and *Sal*I and cloned into pGEM-3Z-SF-β_2AR-6His digested with *Eco*RV and *Sal*I to obtain the full-length fusion protein DNA sequence (pGEM-3Z-SF-β_2AR-6His-$G_s\alpha_L$). For generation of β_2ARG$_s\alpha_S$ DNA, a set of deletion primers (sense and antisense) and an antisense primer 3′ of the *Eco*RI site of $G_s\alpha_L$ were synthesized. In PCR 3A, the sequence between a primer 5′ of the *Eco*RV site in the β_2AR and the antisense deletion primer was amplified using pGEM-3Z-SF-β_2AR-6His-$G_s\alpha_L$ as template. In PCR 3B, the sequence between the sense deletion primer and the antisense primer 3′ of the *Eco*RI site of $G_s\alpha_L$ was amplified using the same template as in PCR 3A. In PCR 4, the products of PCRs 3A and 3B were annealed, and the sense primer 5′ of the *Eco*RV site in the β_2AR and the antisense primer 3′ of the *Eco*RI site of $G_s\alpha_L$ were used. In this way, a DNA fragment encoding the C terminus of the β_2AR, a hexahistidine tail and the N-terminal portion of $G_s\alpha_S$, missing the sequence for amino acids 72–86 in $G_s\alpha_L$ and encoding the Glu-71 → Asp substitution (15), was created (Fig. 1*A*). This fragment was digested with *Eco*RV and *Eco*RI and cloned into pGEM-3Z-SF-β_2AR-6His-$G_s\alpha_L$ digested with *Eco*RV and *Eco*RI. PCR-generated DNA sequences were confirmed by enzymatic sequencing. Fusion protein DNAs were cloned into the baculovirus transfer vector pVL 1392 (11). Recombination of viruses was confirmed by reverse transcriptase PCR.

Cell Culture—Recombinant baculoviruses were generated and amplified as described (11). Sf9 cells were seeded at 3.0×10^6 cells/ml and infected with 1:50 or 1:500 dilutions of high titer virus stocks. Cells were cultured for 24–48 h to obtain various expression levels of fusion proteins and β_2AR. For co-expression studies, Sf9 cells were infected with a 1:10,000 dilution of a high titer β_2AR baculovirus stock and a 1:50 dilution of a high titer $G_s\alpha_L$ baculovirus stock to achieve a receptor to G-protein stoichiometry of ~1:100. Cells were cultured for 48 h. Membranes were prepared according to Gether *et al.* (11).

[^{3}H]DHA Binding–For determination of K_d and B_{max} values, Sf9 membranes (5 μg of protein) were suspended in 500 μl of buffer containing 75 mM Tris/HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1 mM EDTA, supplemented with 0.1–10 nM [^{3}H]DHA and 0.2% (w/v) bovine serum albumin. Nonspecific binding was assessed in the presence of 10 μM (−)-alprenolol (ALP). Incubations were performed for 90 min at 25 °C and shaking at 200 rpm. Competition binding experiments were carried out with 15–30 μg of membrane protein with 1 nM [^{3}H]DHA in the presence of unlabeled ligands at various concentrations without or with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (10 μM). In some experiments, tubes contained 1 nM [^{3}H]DHA, 1 μM salbutamol (SAL), and various nucleotides at increasing concentrations.

GTPase Activity—Assay tubes (100 μl) contained 10 μg of membrane protein, 0.1 μM [γ-^{32}P]GTP (0.1–0.5 μCi/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, and ligands at various concentrations. Reactions were conducted for 20 min at 25 °C and were terminated by the addition of 900 μl of a slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Reaction mixtures were centrifuged for 15 min at room temperature and $15,000 \times g$. Seven-hundred μl of the supernatant fluid of reaction mixtures were removed and [^{32}P]P_i was determined by liquid scintillation counting.

AC Activity—Assay tubes (50 μl) contained 15 μg of membrane protein, 1 μM GTP, 40 μM [α-^{32}P]ATP (2.5 μCi/tube), 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, 1 IU of myokinase, 0.1 mM cAMP, 5 mM $MgCl_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, and ligands at various concentrations. Reactions were conducted for 20 min at 37 °C. Separation of [^{32}P]cAMP from [α-^{32}P]ATP was performed as described (27).

Western Blot Analysis—Solubilized Sf9 membrane proteins (5–10 μg of protein/lane) were separated by SDS-PAGE (8% (w/v) acrylamide). Proteins were visualized using either M1 antibody or anti-$G_s\alpha$ antibody and the ECL Western blotting system (Amersham). $G_s\alpha_L$ expression in Sf9 membranes was quantitated by immunoblotting with anti-$G_s\alpha$ antibody using defined amounts of β_2ARG$_s\alpha$ fusion protein as standard.

Miscellaneous—Protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad). Data were analyzed by nonlinear regression, using the program Prism (GraphPad, Prism, San Diego, CA). Statistical comparisons between β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$ were done with the Wilcoxon test. Data are given as means ± S.D. of three to seven independent experiments performed in duplicate or triplicate.

RESULTS

Expression of β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$ in Sf9 Membranes—Expression of fusion proteins in Sf9 membranes was confirmed by SDS-PAGE using the M1 monoclonal antibody to detect the N-terminal Flag epitope of the β_2AR (Fig. 1*A*). The nonfused β_2AR expressed in Sf9 cells runs as a broad glycosylated 52-kDa protein in SDS-PAGE (11, 26). The apparent molecular masses of $G_s\alpha_S$ and $G_s\alpha_L$ are 45 and 52 kDa, respectively (16). Accordingly, the apparent molecular masses of β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$ were expected to be 97 and 104 kDa, respectively. The data obtained are in agreement with this expectation (Fig. 1*C*). Immunoblots with an anti-$G_s\alpha$ antibody confirmed the presence of $G_s\alpha$ in the fusion proteins and the difference in apparent molecular mass between β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$. In membranes from uninfected cells, no immunoreactive bands in the 97–104-kDa region were detected with the M1 and anti-$G_s\alpha$ antibody (data not shown).

Ligand Binding Properties of β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$, Comparison with the Nonfused β_2AR—The K_d values of [^{3}H]DHA for β_2ARG$_s\alpha_S$ and β_2ARG$_s\alpha_L$ were very similar (Table I). In competition experiments, we studied the effects of (−)-isoproterenol ((−)-ISO), (+)-isoproterenol ((+)-ISO), SAL, dobutamine (DOB), (−)-ephedrine (EPH), dichloroisoproterenol (DCI) and ICI 118,551 (ICI) on [^{3}H]DHA binding. (−)-ISO binds to βARs with higher affinity than (+)-ISO, but both stereoisomers are full agonists (28–30). SAL, DOB, EPH, and

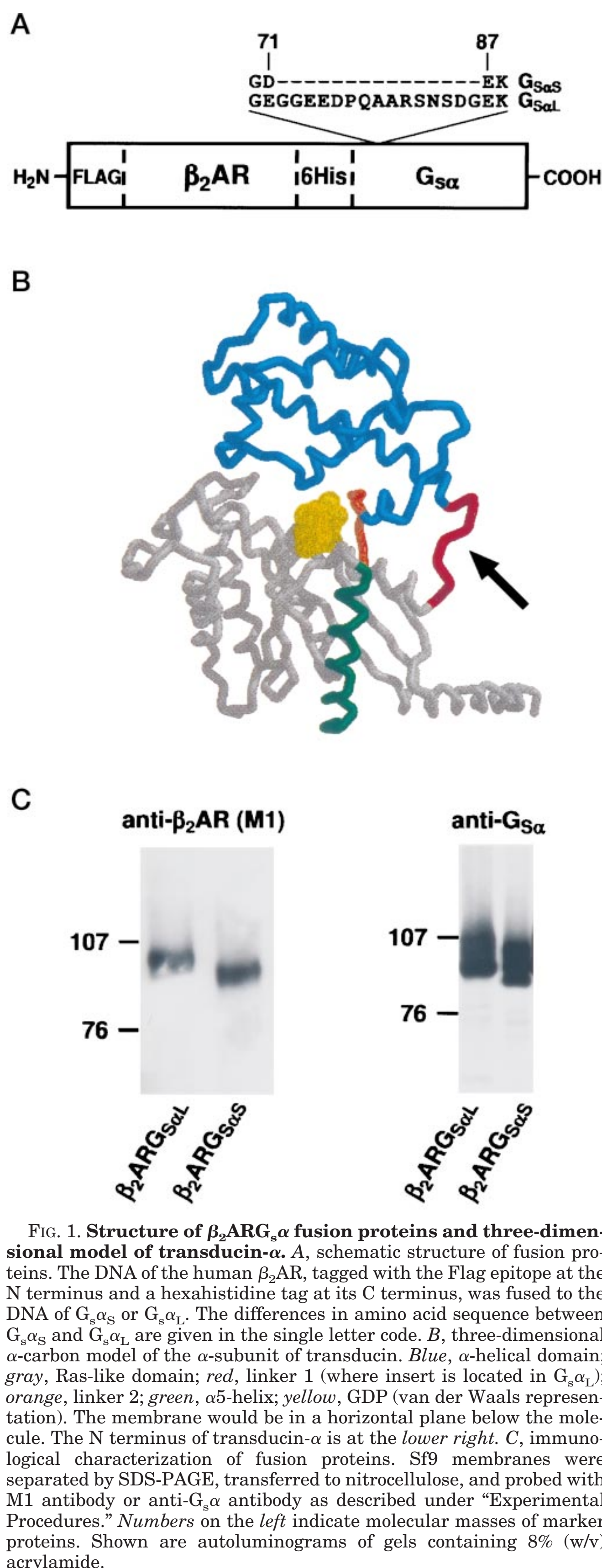

FIG. 1. **Structure of $\beta_2ARG_s\alpha$ fusion proteins and three-dimensional model of transducin-α.** *A*, schematic structure of fusion proteins. The DNA of the human β_2AR, tagged with the Flag epitope at the N terminus and a hexahistidine tag at its C terminus, was fused to the DNA of $G_s\alpha_S$ or $G_s\alpha_L$. The differences in amino acid sequence between $G_s\alpha_S$ and $G_s\alpha_L$ are given in the single letter code. *B*, three-dimensional α-carbon model of the α-subunit of transducin. *Blue*, α-helical domain; *gray*, Ras-like domain; *red*, linker 1 (where insert is located in $G_s\alpha_L$); *orange*, linker 2; *green*, α5-helix; *yellow*, GDP (van der Waals representation). The membrane would be in a horizontal plane below the molecule. The N terminus of transducin-α is at the *lower right*. *C*, immunological characterization of fusion proteins. Sf9 membranes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with M1 antibody or anti-$G_s\alpha$ antibody as described under "Experimental Procedures." *Numbers* on the *left* indicate molecular masses of marker proteins. Shown are autoluminograms of gels containing 8% (w/v) acrylamide.

DCI are partial β_2AR agonists (7, 10, 11), and ICI is an inverse agonist (8, 11–13). At both fusion proteins, full and strong partial agonists ((−)-ISO, (+)-ISO, SAL, and DOB) showed a high- and low-affinity binding component (Table I). The high-affinity agonist binding was abolished by GTPγS. For agonists with lower intrinsic activity (EPH and DCI), high- and low-affinity binding sites were not discriminated by curve fitting analysis, but GTPγS still reduced the affinity of these ligands to $\beta_2ARG_s\alpha$ fusion proteins. There were no significant differences in the low- and high-affinity K_i values of the agonists studied between $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$. There was a trend toward higher fractions of high-affinity agonist-binding sites for full agonists and strong partial agonists at $\beta_2ARG_s\alpha_S$ compared with $\beta_2ARG_s\alpha_L$, but this was significant only for (+)-ISO.

TABLE I
Binding properties of β_2AR ligands at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$

[^{3}H]DHA binding was determined as described under "Experimental Procedures" in membranes expressing $\beta_2ARG_s\alpha_S$ or $\beta_2ARG_s\alpha_L$ at 3.5–6.5 pmol/mg. Competition binding isotherms were analyzed by nonlinear regression for best fit to single-site or two-site binding. K_h and K_l designate the dissociation constants for the high- and low-affinity state of the β_2AR, respectively. $\%R_h$ indicates the percentage of high-affinity binding sites. When competition isotherms were best fit to a single-site model, the respective K_i values are listed under K_l. K_i (+GTPγS) indicates the K_i values obtained in the presence of 10 μM GTPγS. Data shown represent the means ± S.D. of five to seven independent experiments performed in duplicate or triplicate. K_i values are expressed in nanomolar.

	Ligand	K_h	K_l	$\%R_h$	K_i(+GTPγS)
$\beta_2ARG_s\alpha_S$	(−)-ISO	2.0 ± 1.3	130 ± 62	50.6 ± 4.5	210 ± 21
	(+)-ISO	30 ± 11	2200 ± 610	50.2 ± 2.8	6300 ± 1100
	SAL	44 ± 20	1700 ± 420	43.5 ± 2.6	2700 ± 390
	DOB	61 ± 12	3200 ± 940	43.3 ± 7.1	4000 ± 950
	EPH		5100 ± 1300		20400 ± 2900
	DCI		160 ± 49		268 ± 16
	ALP		0.27 ± 0.08[a]		ND[b]
	ICI		1.7 ± 0.4		1.4 ± 0.3
$\beta_2ARG_s\alpha_L$	(−)-ISO	3.0 ± 1.3	124 ± 19	42.5 ± 7.5	154 ± 28
	(+)-ISO	17 ± 9	2200 ± 890	39.1 ± 0.2[c]	4400 ± 1800
	SAL	24 ± 18	1900 ± 530	38.0 ± 4.5	1900 ± 530
	DOB	48 ± 24	2500 ± 410	40.6 ± 8.6	3500 ± 1700
	EPH		4400 ± 1800		16000 ± 4700
	DCI		93 ± 19		162 ± 46
	ALP		0.36 ± 0.03[a]		0.34 ± 0.05[a]
	ICI		1.7 ± 0.4		1.4 ± 0.1

[a] K_d values for [^{3}H]DHA.
[b] ND, not determined.
[c] $p < 0.05$ for comparison of $\beta_2ARG_s\alpha_S$ *versus* $\beta_2ARG_s\alpha_L$.

In Sf9 membranes expressing the nonfused Flag epitope- and hexahistidine-tagged β_2AR (11) at similar levels (4.0–6.5 pmol/mg) as $\beta_2ARG_s\alpha$ fusion proteins (3.5–6.5 pmol/mg), the K_d value for [^{3}H]DHA was 0.36 ± 0.03 nM. ICI inhibited [^{3}H]DHA binding with a K_i value of 1.2 ± 0.3 nM. (−)-ISO inhibited [^{3}H]DHA binding to the nonfused β_2AR according to a steep monophasic function (K_i, 200 ± 13 nM). The (−)-ISO competition curve was not affected by GTPγS (10 μM) (K_i, 201 ± 37 nM). The lack of high-affinity agonist binding was also observed of the avian βAR expressed in Sf9 cells (31). In membranes from uninfected Sf9 cells, no specific [^{3}H]DHA binding was detected (data not shown), indicative for the absence of endogenous β_2ARs. Collectively, these data show that in the $\beta_2ARG_s\alpha$ fusion proteins, the receptor productively interacts with the attached G-protein to induce high-affinity agonist binding, while the interaction of the β_2AR with endogenous G-proteins of Sf9 cells is not efficient enough to result in measurable high-affinity agonist binding. In addition, the antagonist and agonist binding properties of the β_2AR in $\beta_2ARG_s\alpha$ fusion proteins compare favorably with the ligand binding properties of nonfused β_2AR (Table I) (7, 8, 12, 28–30).

Regulation of GTPase Activity in $\beta_2AR_s\alpha$ Fusion Proteins, Comparison with a Co-expression System Consisting of β_2AR and $G_s\alpha$—Activation of the GTPase of G_s by agonist-occupied βARs can be studied with great sensitivity in reconstituted systems (32, 33), but in most plasma membrane systems, the

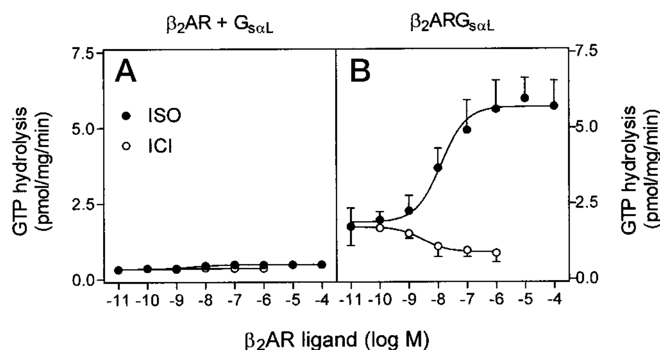

FIG. 2. **Comparison of the ligand regulation of GTPase activity in Sf9 membranes expressing the β_2AR and $G_s\alpha_L$ as separate proteins and in membranes expressing the $\beta_2ARG_s\alpha_L$ fusion protein.** GTP hydrolysis was determined with 100 nM [γ-^{32}P]GTP as substrate as described under "Experimental Procedures." Reaction mixtures contained (−)-ISO and ICI at the indicated concentrations and membranes expressing the β_2AR (1.4 pmol/mg) plus $G_s\alpha_L$ (~100 pmol/mg) (*A*) or membranes expressing $\beta_2ARG_s\alpha_L$ (5.0 pmol/mg) (*B*). Data shown are the means ± S.D. of three to five independent experiments performed in duplicate.

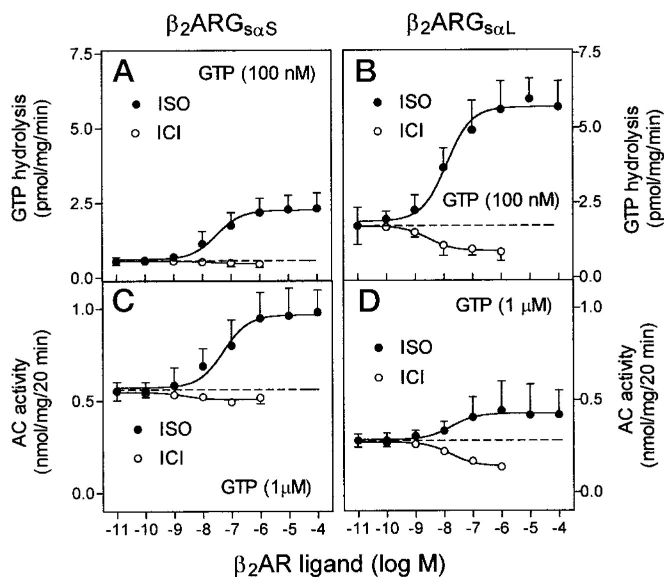

FIG. 3. **Effects of (−)-ISO and ICI on GTPase and AC activity in Sf9 membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$.** GTPase activity (*A* and *B*) and AC activity (*C* and *D*) in $\beta_2ARG_s\alpha_S$ membranes (*A* and *C*) and $\beta_2ARG_s\alpha_L$ membranes (*B* and *D*) were determined as described under "Experimental Procedures" in the presence of (−)-ISO or ICI at various concentrations. For GTPase studies, the expression level of fusion proteins was 4.5–5.0 pmol/mg, and for AC studies, 2.3–2.6 pmol/mg. GTP hydrolysis was determined with 100 nM [γ-^{32}P]GTP as substrate. AC activity was determined in the presence of 1 μM GTP. Data shown are the means ± S.D. of three to five independent experiments performed in duplicate. The *dotted lines* are extrapolations of basal GTPase and AC activities to illustrate the relative contributions of (−)-ISO and ICI at the ligand-regulated enzyme activities.

GTPase stimulation induced by βARs is small relative to the high background GTPase activity of other cellular G-proteins with higher GTP turnover than G_s and the presence of low-affinity nucleotidases (34, 35). In S49 lymphoma cell membranes, a prototypical system for studying β_2AR/G_s interaction (23, 36), the β_2AR and $G_s\alpha$ are expressed at levels of ~0.2 and ~20 pmol/mg, respectively, *i.e.* there is an ~100-fold molar excess of G-protein compared with receptor (37). We co-expressed the β_2AR at a level of 1.4 pmol/mg with $G_s\alpha_L$ at a level of ~100 pmol/mg in Sf9 membranes, achieving a similar receptor/G-protein ratio as in S49 lymphoma cells, and studied the regulation of GTPase activity by (−)-ISO and ICI. However, despite the relatively high expression of β_2AR and $G_s\alpha_L$ at a stoichiometry similar to that in the mammalian cell line, we detected only marginal activation of GTPase by agonist in Sf9 membranes and failed to see inhibition by inverse agonist (Fig. 2*A*). Similar results were obtained when the expression level of β_2AR was increased to 11.8 pmol/mg (data not shown). In marked contrast, (−)-ISO increased GTP hydrolysis in membranes expressing $\beta_2ARG_s\alpha$ (5.0 pmol/mg) by up to 245% above basal, and ICI reduced GTP hydrolysis by up to 50% (Fig. 2*B*). These findings demonstrate that fusion of the β_2AR to $G_s\alpha$ greatly facilitates detection of ligand-regulated GTP hydrolysis in Sf9 membranes.

The regulation of GTPase activity in Sf9 membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ at similar levels (4.5–5.0 pmol/mg) was compared. (−)-ISO increased GTP hydrolysis in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ by up to 315 and 245%, respectively (Fig. 3, *A* and *B*). To the best of our knowledge, these are the highest reported agonist stimulations of GTPase by βARs in a membrane system (28, 34, 35). It should also be noted that Fig. 3, *A* and *B*, show total GTP hydrolysis rates and not only the high-affinity GTPase activity corrected for low-affinity GTPases (34). This fact further underlines the high sensitivity of the GTPase assay with the $\beta_2ARG_s\alpha$ fusion proteins in Sf9 membranes.

The Effects of $G_s\alpha_S$ and $G_s\alpha_L$ on the Efficacy of Agonists and Inverse Agonists at the $\beta_2ARG_s\alpha$ Fusion Proteins—The precise determination of the intrinsic activities of partial agonists constitutes a major problem in the functional characterization of GPCRs, because the intrinsic activity of a given ligand may depend on numerous variables, *i.e.* receptor and G-protein expression level and the availability of effector molecules such as AC (11, 22, 38–40). In most studies, the intrinsic activities of partial β_2AR agonists were characterized by measuring AC activity (7, 10, 11, 41). The AC assay takes advantage of the signal amplification at the G_s level, but it is difficult to control for the impact of G_s and AC availability on intrinsic activities of ligands. We reasoned that with the GTPase activity of $\beta_2ARG_s\alpha$ fusion proteins as parameter, determination of the intrinsic activities of partial agonists should be less ambiguous because of the fixed stoichiometry of the signaling components. Moreover, signal amplification by AC is not required, thereby reducing the number of variables that can influence the determination of intrinsic activity. To validate this assumption, we studied the potencies and intrinsic activities of a series of partial β_2AR agonists at the GTPase of $\beta_2ARG_s\alpha_L$ with expression levels ranging from 0.6 to 7.6 pmol/mg. Within this broad range of expression, we did not observe significant differences in the potency and intrinsic activity of partial β_2AR agonists (data not shown).

Based on the above results, we determined the effects of a series of agonists with different intrinsic activities and of inverse agonists on GTP hydrolysis in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$. The affinity of the β_2AR for (+)-ISO in $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ is substantially lower than the affinity for (−)-ISO (Table I). In agreement with the difference in binding affinity, (+)-ISO activated the GTPase of both fusion proteins more than 10-fold less potently than (−)-ISO (Table II). Notably, the potencies of all agonists studied were higher at $\beta_2ARG_s\alpha_L$ than at $\beta_2ARG_s\alpha_S$. This difference between the two fusion proteins was significant for all ligands studied except for (−)-ISO (Table II). The difference in potency of partial agonists between $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ was most prominent for EPH (24-fold). For most ligands ((+)-ISO, SAL, DOB, and DCI), the difference in potency between $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ was about 3–4-fold.

The intrinsic activities of (−)-ISO and (+)-ISO to activate the GTPase of $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ were similar (Fig. 4*A*).

TABLE II
Potencies of full and partial β_2AR agonists at the GTPase of $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$

For determination of the potency of ligands, GTP hydrolysis was measured as described under "Experimental Procedures" in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ at 4.5–5.0 pmol/mg. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. EC_{50} values were calculated by nonlinear regression. Data shown represent the means ± S.D. of five to seven independent experiments performed in duplicate or triplicate. Potencies are expressed in nanomolar.

Ligand	Potency at $\beta_2ARG_s\alpha_S$	Potency at $\beta_2ARG_s\alpha_L$
(−)-ISO	32 ± 21	13 ± 3
(+)-ISO	530 ± 160	180 ± 57[a]
SAL	200 ± 42	78 ± 10[a]
DOB	200 ± 49	71 ± 39[a]
EPH	20500 ± 5600	870 ± 380[a]
DCI	82 ± 33	21 ± 4[a]
ALP	7.9 ± 1.3	0.6 ± 0.4[a]

[a] $p < 0.05$ for comparison of $\beta_2ARG_s\alpha_S$ *versus* $\beta_2ARG_s\alpha_L$.

Analogous data concerning the intrinsic activities of (−)-ISO and (+)-ISO were obtained for nonfused βARs (28–30). For both $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$, ligands activated GTPase in the rank order of intrinsic activity (−)-ISO ~ (+)-ISO ≥ SAL > DOB > EPH > DCI > ALP > (−)-propranolol (no intrinsic activity). Of interest, the intrinsic activities of SAL, DOB, EPH, DCI, and ALP at $\beta_2ARG_s\alpha_L$ were significantly higher than at $\beta_2ARG_s\alpha_S$. When the intrinsic activities of ligands at the GTPase of $\beta_2ARG_s\alpha_L$ are plotted *versus* the intrinsic activities of ligands at the GTPase of $\beta_2ARG_s\alpha_S$, data are best fitted by a hyperbolic and not a linear function (Fig. 4*B*). A similar hyperbolic relationship in the intrinsic activities of β_2AR ligands was found for a (nonfused) constitutively active mutant of the β_2AR (β_2AR_{CAM}) in comparison with the (nonfused) wild-type β_2AR (7). Thus, the β_2AR in $\beta_2ARG_s\alpha_L$ appears to possess some properties of a constitutively active receptor.

To obtain further evidence for constitutive activation of the β_2AR in $\beta_2ARG_s\alpha_L$, we studied the effects of the inverse agonist ICI on basal GTPase activity. The basal steady-state GTPase activity with 100 nM [γ-^{32}P]GTP as substrate was about 3-fold higher for $\beta_2ARG_s\alpha_L$ than for $\beta_2ARG_s\alpha_S$ (Fig. 3, *A* and *B*). In membranes expressing $\beta_2ARG_s\alpha_S$, ICI had a smaller inhibitory effect (15% reduction) on GTP hydrolysis than in membranes expressing $\beta_2ARG_s\alpha_L$ (50% reduction). Similar results were obtained with timolol, another inverse agonist at the β_2AR (10) (data not shown). These inverse agonist studies show that the higher basal GTPase activity in membranes expressing $\beta_2ARG_s\alpha_L$ compared with membranes expressing $\beta_2ARG_s\alpha_S$ is largely attributable to the activity of the agonist-free β_2AR.

Regulation of High-affinity Agonist Binding at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ by Guanine Nucleotides—High-affinity agonist binding to GPCRs depends on their interaction with G-protein α-subunits, presumably in the nucleotide-free state (1, 42). Occupation of the guanine nucleotide-binding site of α-subunits disrupts high-affinity agonist binding (1, 7). To determine the guanine nucleotide binding affinities of $G_s\alpha_S$ and $G_s\alpha_L$ in $\beta_2ARG_s\alpha$ fusion proteins, we examined binding of a fixed concentration of the antagonist [^{3}H]DHA in the presence of a subsaturating concentration of the strong partial agonist SAL in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha$. Various nucleotides at increasing concentrations were added to the binding assays. Guanine nucleotide binding to $G_s\alpha$ reduces the affinity of the β_2AR for agonist and, thereby, increases [^{3}H]DHA binding (Fig. 5). In this way, the affinity of G-proteins for nucleotides can be measured. It should be noted that our binding experiments were performed in the absence of a nucleotide-regenerating system, excluding the possibility that effects caused by nucleoside 5′-monophosphates and -diphosphates are due to transphosphorylation. GTP was similarly potent at inhibiting high-affinity agonist binding at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ (EC_{50}, 59 ± 15 and 49 ± 20 nM, respectively). In contrast, GDP was far more potent at $\beta_2ARG_s\alpha_S$ (EC_{50}, 83 ± 23 nM) than at $\beta_2ARG_s\alpha_L$ (EC_{50}, 1.8 ± 0.2 μM). Like GDP, its nucleotidase-resistant phosphorothioate analog, guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), inhibited agonist binding at $\beta_2ARG_s\alpha_S$ more potently than at $\beta_2ARG_s\alpha_L$ (EC_{50}, 490 ± 150 nM and 2.2 ± 0.3 μM, respectively). These data show that in the $\beta_2ARG_s\alpha$ fusion proteins, $G_s\alpha_S$ has a higher affinity for guanosine 5′-diphosphates than $G_s\alpha_L$ and are in agreement with data obtained with purified $G_s\alpha_L$ and $G_s\alpha_S$ (18).

Of interest, even GMP inhibited high-affinity agonist binding at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ to some extent, although less potently than GDP (EC_{50}, 55 ± 12 and 15 ± 7 μM, respectively). Substitution of the phosphate group in GMP by a phosphorothioate group, yielding the nucleotidase-resistant GMPS, substantially enhanced the potency and efficacy of the nucleotide to disrupt high-affinity agonist binding at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ (EC_{50}, 4.5 ± 1.2 μM and 6.3 ± 3.3 μM, respectively). The data obtained with GMP and GMPS provide strong support for the suggestion that it is the nucleotide-free form of $G_s\alpha$, which confers high agonist-affinity to the β_2AR (1, 42).

Regulation of AC Activity in Sf9 Membranes Expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ by Agonist and Inverse Agonist—The analysis of AC activity in Sf9 membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ must take into consideration the fact that Sf9 cells express endogenous $G_s\alpha$-like G-proteins (10, 11, 30, 31). This is of particular relevance because for AC studies, we expressed fusion proteins at relatively low levels to avoid AC availability becoming the limiting factor (38). However, the AC activity in membranes from uninfected Sf9 cells in the presence of 10 μM GTPγS was ~5.5-fold lower than in Sf9 membranes expressing $\beta_2ARG_s\alpha_L$ at 2.6 pmol/mg (0.089 ± 0.015 nmol/mg/20 min *versus* 0.491 ± 0.054 nmol/mg/20 min). These data show that even under maximal stimulation of AC, the contribution of endogenous $G_s\alpha$-like G-proteins in Sf9 cells to total AC activity is small.

When the basal AC activity in the presence of 1 μM GTP in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ at a similar level (2.3–2.6 pmol/mg) was compared, substantial differences between the two fusion proteins became apparent. Specifically, the AC activity in membranes expressing $\beta_2ARG_s\alpha_S$ was almost twice as high as in membranes expressing $\beta_2ARG_s\alpha_L$ (Fig. 3, *C* and *D*). (−)-ISO increased AC activity in $\beta_2ARG_s\alpha_S$ membranes by up to 80%, while in membranes expressing $\beta_2ARG_s\alpha_L$, (−)-ISO increased AC activity only by 45%. The EC_{50} values of (−)-ISO were 51 ± 17 nM for $\beta_2ARG_s\alpha_S$ and 17 ± 18 nM for $\beta_2ARG_s\alpha_L$. Despite the fact that the basal AC activity in membranes expressing $\beta_2ARG_s\alpha_L$ was considerably lower than in membranes expressing $\beta_2ARG_s\alpha_S$, the inhibitory effect of ICI in membranes expressing $\beta_2ARG_s\alpha_L$ (50% reduction) was substantially greater than in membranes expressing $\beta_2ARG_s\alpha_S$ (10% reduction). Similar results were obtained with the inverse agonist timolol (data not shown). Thus, the AC data corroborate the GTPase data, pointing to constitutive activity of the β_2AR in $\beta_2ARG_s\alpha_L$.

AC Regulation in Sf9 Membranes Expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ in the Absence of Exogenous Guanine Nucleotides—In the presence of GTP, agonists at G_s-coupled GPCRs cause AC activation (1, 2). However, in the absence of added guanine nucleotides, agonists at G_s-coupled receptors can *reduce* AC activity (43, 44). The most likely explanation for these observations is that agonists induce release of prebound guanine nucleotide from $G_s\alpha$, generating guanine nucleotide-free $G_s\alpha$ and, thereby, reducing AC activity. Indeed, (−)-ISO re-

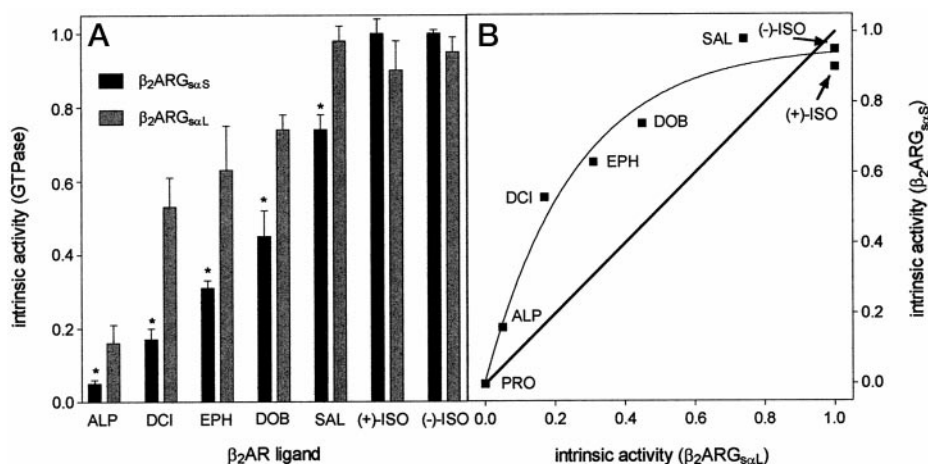

FIG. 4. **Intrinsic activities of full and partial β_2AR agonists at the GTPase of $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$.** For determination of the intrinsic activities of ligands, GTP hydrolysis was measured as described under "Experimental Procedures" in membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ at 4.5–5.0 pmol/mg. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. The intrinsic activities were derived from plateau values of concentration-response curves generated by nonlinear regression analysis. *A*, comparison of the intrinsic activities of various ligands at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$. *, $p < 0.05$. Data shown are the means ± S.D. of five to seven independent experiments performed in duplicate. *B*, replot of the data shown in *A*. The intrinsic activities of ligands at $\beta_2ARG_s\alpha_L$ were plotted against their intrinsic activities at $\beta_2ARG_s\alpha_S$. Data points were best fitted by a hyperbolic function as assessed by nonlinear regression. The *straight line* represents the theoretical curve that would have fitted data best if the intrinsic activities of ligands at $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ had been the same. For comparison, the data for the neutral antagonist (propranolol) (*PRO*) are included in the panel.

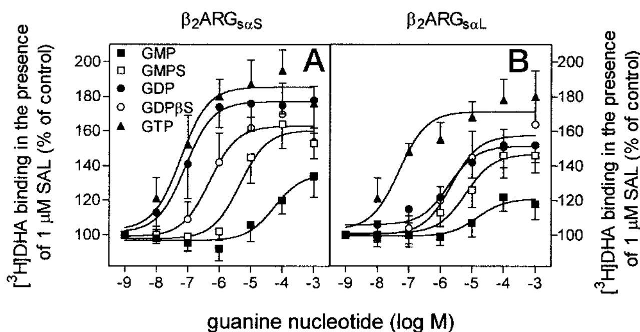

FIG. 5. **Effects of guanine nucleotides on high-affinity agonist binding in Sf9 membranes expressing $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$.** Binding experiments were carried out as described under "Experimental Procedures" with $\beta_2ARG_s\alpha_S$ membranes (*A*) or $\beta_2ARG_s\alpha_L$ membranes (*B*). Reactions mixtures additionally contained 1 nM [^{3}H]DHA, 1 μM SAL, and guanine nucleotides at increasing concentrations. Data shown are the means ± S.D. of two independent experiments performed in triplicate.

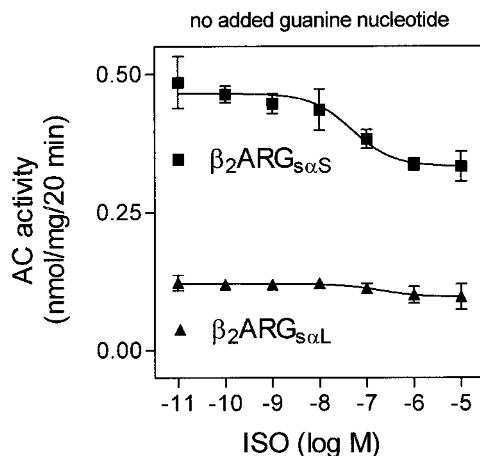

FIG. 6. **Deactivation of AC by (−)-ISO in the absence of exogenous nucleotides.** AC activity in $\beta_2ARG_s\alpha_S$ and $\beta_2ARG_s\alpha_L$ membranes was determined as described under "Experimental Procedures" in the absence of exogenous guanine nucleotides and in the presence of (−)-ISO at different concentrations. The expression level of fusion proteins was 2.3–2.6 pmol/mg. Data shown are the means ± S.D. of three to four independent experiments performed in duplicate.

duced the basal AC activity in membranes expressing $\beta_2ARG_s\alpha_S$ by about 30% and with an IC_{50} of 40 ± 12 nM (Fig. 6). In contrast, (−)-ISO had no significant deactivating effect on AC activity in membranes expressing $\beta_2ARG_s\alpha_L$. These results suggest that the nucleotide-binding pocket of $G_s\alpha_L$ in $\beta_2ARG_s\alpha_L$ is already nucleotide-free.

DISCUSSION

The β_2AR Fused to $G_s\alpha_L$ Has Properties of a Constitutively Active Receptor—Previous studies have shown that there are subtle differences in the GDP affinities of purified $G_s\alpha_S$ and $G_s\alpha_L$ (18) and that $G_s\alpha_S$ may activate AC more efficiently than $G_s\alpha_L$ (19). However, studies aiming to reveal differences between $G_s\alpha_S$ and $G_s\alpha_L$ in their coupling to the β_2AR have remained inconclusive because of the difficulties to ensure exactly defined receptor/G-protein stoichiometry (18, 20, 21). This is important because functional interactions between GPCRs and G-proteins are strongly influenced by their relative expression levels (11, 22, 40). To circumvent this problem, we constructed fusion proteins in which the C terminus of the β_2AR was linked to the N terminus of $G_s\alpha_S$ or $G_s\alpha_L$ (Fig. 1*A*), thereby guaranteeing a defined stoichiometry of receptor to G-protein and increasing the efficiency of receptor/G-protein coupling. Using this approach we observed that the efficacy and potency of partial agonists acting on the β_2AR were significantly higher when the receptor was fused to $G_s\alpha_L$ than when the receptor was fused to $G_s\alpha_S$ (Fig. 4 and Table II). Moreover, the basal GTPase and AC activities in membranes expressing $\beta_2ARG_s\alpha_L$ were more sensitive to inverse agonists than the corresponding activities in membranes expressing $\beta_2ARG_s\alpha_S$ (Fig. 3). These functional properties of the β_2AR fused to $G_s\alpha_L$ are similar to those of the β_2AR_{CAM} (7, 8, 13).

According to the two-state model of receptor activation, GPCRs exist either in an inactive state R or an active state R*. These two states are in equilibrium, and the R* state can be stabilized by agonists, partial agonists, and nucleotide-free $G_s\alpha$, while the R state is stabilized by inverse agonists (7–14). The results of our agonist binding studies are in agreement with the two-state model (Table I) and strongly support the suggestion that guanine nucleotide-free $G_s\alpha$ is necessary to

form a high-affinity complex with the β_2AR (Fig. 5) (1, 42). It has been proposed that in constitutively active receptor mutants, the equilibrium between R and R* is shifted toward R* (7, 8, 13). Experimentally, this results in an increased potency and efficacy of partial agonists, increased efficacy of inverse agonists, and increased basal G-protein activity (7, 8, 11, 13).

The apparent stabilization of the β_2AR in the R* state in β_2ARG$_s\alpha_L$ relative to the β_2AR in β_2ARG$_s\alpha_S$ can be revealed in experiments in which the outcome of multiple G-protein activation/deactivation cycles is monitored, *i.e.* the steady-state GTPase assay and the AC assay. As shown in Fig. 4 and Table II, the potency and intrinsic activity of a series of partial β_2AR agonists to activate GTPase was significantly higher for β_2ARG$_s\alpha_L$ than for β_2ARG$_s\alpha_S$. Moreover, the basal GTPase activity in membranes expressing β_2ARG$_s\alpha_L$ was approximately 3-fold higher than in membranes expressing β_2ARG$_s\alpha_S$ at a comparable level (Fig. 3, *A* and *B*). This elevated basal GTPase activity in membranes expressing β_2ARG$_s\alpha_L$ can be reduced by ICI to a level near the basal level of membranes expressing β_2ARG$_s\alpha_S$. In contrast to membranes expressing β_2ARG$_s\alpha_L$, ICI has little effect on the basal GTPase activity in membranes expressing β_2ARG$_s\alpha_S$. These properties of constitutive activity of the β_2AR in β_2ARG$_s\alpha_L$ may be due to differences in the way G$_s\alpha_L$ and G$_s\alpha_S$ interact with the β_2AR. In particular, G$_s\alpha_L$ has a lower affinity for GDP than G$_s\alpha_S$ (Fig. 5) (18). Therefore, G$_s\alpha_L$ may be more often guanine nucleotide-free and more often available for stabilizing R* than G$_s\alpha_S$.

In contrast to basal GTPase activity, membranes expressing β_2ARG$_s\alpha_S$ had a higher basal and (−)-ISO-stimulated AC activity than membranes expressing β_2ARG$_s\alpha_L$ (Fig. 3, *C* and *D*). However, ICI inhibited the elevated basal AC activity in membranes expressing β_2ARG$_s\alpha_S$ by only 10%, while ICI inhibited the lower basal AC activity in membranes expressing β_2ARG$_s\alpha_L$ by 50%. Therefore, the elevated basal AC activity in membranes expressing β_2ARG$_s\alpha_S$ is likely due to the intrinsic properties of G$_s\alpha_S$ rather than to the β_2AR in the fusion protein. A previous study had already shown that G$_s\alpha_S$ is more effective in activating AC than G$_s\alpha_L$ (19). Since GTP hydrolysis is the major mechanism by which G-proteins are deactivated (1, 2, 28, 34), the higher basal and (−)-ISO-stimulated GTPase activity in membranes expressing β_2ARG$_s\alpha_L$ could indicate that G$_s\alpha_L$ spends less time in the active GTP-bound state than G$_s\alpha_S$ and, therefore, is less effective in stimulating AC.

The data shown in Fig. 6 suggest that G$_s\alpha$ in its GDP-liganded form may be able to stimulate AC and, thereby, to contribute to the higher basal AC activity in membranes expressing β_2ARG$_s\alpha_S$. Specifically, in the absence of added guanine nucleotides, (−)-ISO reduces basal AC activity in membranes expressing β_2ARG$_s\alpha_S$. Under these conditions, (−)-ISO can promote dissociation of previously bound GDP, but binding of GTP cannot occur. In contrast, in the absence of added guanine nucleotides, AC activity in membranes expressing β_2ARG$_s\alpha_L$ is lower, and there is no significant reduction in basal activity following the addition of (−)-ISO. This observation is consistent with the lower affinity of G$_s\alpha_L$ for GDP compared with G$_s\alpha_S$ (Fig. 5) (18) and indicates that most of the G$_s\alpha_L$ in β_2ARG$_s\alpha_L$ has already released its GDP.

Of interest, there is no major differences in the apparent ability of G$_s\alpha_L$ and G$_s\alpha_S$ to stabilize the β_2AR in the R* state in binding experiments (Table I). Similar results were previously obtained by Freissmuth *et al.* (20) in a reconstituted system. These data can be explained by the fact that agonist competition studies were performed at equilibrium and in the absence of exogenous guanine nucleotides. Under these conditions, G$_s\alpha_L$ in β_2ARG$_s\alpha_L$ membranes is already largely GDP-free so that the R* state accumulates rapidly, while in β_2ARG$_s\alpha_S$ membranes, agonists induce GDP release from G$_s\alpha_S$ and, thereby, facilitate accumulation of the R* state (Fig. 6). In contrast, in GTPase studies and AC experiments with added GTP, there is continuous cycling of the β_2AR between R and R* so that differences in the apparent proportions of the two receptor states can be more readily detected.

Our studies regarding differential effects of G$_s\alpha$ splice variants on β_2AR signaling were facilitated by using receptor/G-protein fusion proteins. However, our data indicate that fusing receptor to G-protein does not alter the fundamental properties of either component. In particular, the binding properties of β_2AR agonists and antagonists were not altered by fusion to G$_s\alpha$ (Table I) (7, 8, 12, 28–30, 45). In addition, GTPγS efficiently activated AC in membranes expressing β_2ARG$_s\alpha_L$, indicating that fusion of G$_s\alpha$ to the β_2AR does not impair the interaction of the G-protein with AC. Moreover, the relative potencies of GTPγS and guanylyl imidodiphosphate to activate AC are preserved in fusion proteins (data not shown). Finally, the K_m values of the (−)-ISO-stimulated GTPases of β_2ARG$_s\alpha_L$ and β_2ARG$_s\alpha_S$ (279 ± 10 and 144 ± 23 nM, respectively) are in agreement with values reported for reconstituted systems (32).

Physiological Considerations—Although constitutive activation of GPCRs is easily observed with high receptor expression levels (10–12), this is not a prerequisite. There are several examples in the literature documenting constitutive GPCR activity at physiological or near-physiological expression levels (5, 6, 9, 14). These data raise the possibility that constitutive activity of GPCRs is of relevance *in vivo* and that the R* state can be more readily stabilized or detected by specific G-protein α-subunits. In agreement with such a concept is the finding that increases in expression of specific G-proteins can increase high-affinity agonist binding and can promote constitutive receptor activation (40, 46).

G$_s\alpha_S$ and G$_s\alpha_L$ are differentially expressed in various tissues (47). In addition, the expression of G$_s\alpha_S$ and G$_s\alpha_L$ changes during erythroid differentiation (48), during multiple passages of HIT insulinoma cells (19), and in uterine smooth muscle during pregnancy (49). These findings could point to different roles of G$_s\alpha_S$ and G$_s\alpha_L$ in cell functions. The expression of G$_s\alpha$ splice variants also changes in pathological situations. Specifically, in preterm labor, only G$_s\alpha_S$ is expressed in the uterus, whereas in the normal pregnant uterus, both G$_s\alpha$ isoforms are present (49). It remains to be determined of whether the lack of G$_s\alpha_L$ expression in preterm labor is the basis for the poor therapeutic efficiency of partial β_2AR agonists as tocolytic drugs (49).

Conclusion—The 15-amino acid insert by which G$_s\alpha_L$ differs from G$_s\alpha_S$ (Fig. 1*B*) lowers the GDP affinity of the G-protein. Using fusion proteins of the β_2AR with G$_s\alpha$ splice variants, which ensure precise receptor/G-protein stoichiometry, we could show that the subtle differences in GDP affinity between G$_s\alpha_S$ and G$_s\alpha_L$ have important consequences for the interaction with the β_2AR, *i.e.* G$_s\alpha_L$ confers to the β_2AR some properties of a constitutively active receptor. Future studies will have to examine the effects of partial and inverse agonists of the β_2AR in tissues and cells expressing G$_s\alpha_S$ and G$_s\alpha_L$ at different levels and to further define the physiological and pharmacological implications of the differences that we have discovered for the interaction of the β_2AR with the two splice variants of G$_s\alpha$. Because the overall properties of the β_2AR and G$_s\alpha$ and their interaction were not changed as a result of fusion, this approach may be applied to a broad variety of receptors and G-proteins to uncover subtle differences in the interaction of closely related G-protein α-subunits with GPCRs.

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