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Similarities and differences in the coupling of human β_1- and β_2-adrenoceptors to G_{s_α} splice variants

Katharina Wenzel-Seifert, Hui-Yu Liu[1], Roland Seifert[*]

Department of Pharmacology and Toxicology, The University of Kansas, Malott Hall, Room 5064, 1251 Wescoe Hall Drive, Lawrence, KS 66045, USA

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Abstract

The human β_1-adrenoceptor (β_1AR) and β_2-adrenoceptor (β_2AR) couple to G_s-proteins to activate adenylyl cyclase (AC). There are differences in desensitization between the β_2AR and the originally cloned Gly389-β_1AR, but with respect to ternary complex formation, constitutive activity, and AC activation the picture is unclear. To learn more about the similarities and differences between the β_1AR and β_2AR, we analyzed coupling of the Gly389-β_1AR to the G_{s_α} splice variants $G_{s_\alpha L}$ and $G_{s_\alpha S}$ using β_1AR-G_{s_α} fusion proteins expressed in Sf9 cells and compared the data with previously published data on β_2AR-G_{s_α} fusion proteins (Seifert *et al.*, J Biol Chem 1998;273:5109–16). Fusion ensures defined receptor/G-protein stoichiometry and efficient coupling. The agonist (−)-isoproterenol stabilized the ternary complex at β_1AR-$G_{s_\alpha S}$, β_1AR-$G_{s_\alpha L}$, β_2AR-$G_{s_\alpha S}$, and β_2AR-$G_{s_\alpha L}$ with similar efficiency. β_1AR-$G_{s_\alpha L}$ but not β_1AR-$G_{s_\alpha S}$ showed the hallmarks of constitutive activity as assessed by increased potencies and efficacies of partial agonists and AC activation by the agonist-free receptor. Similar differences were observed previously for β_2AR-$G_{s_\alpha S}$ and β_2AR-$G_{s_\alpha L}$. β_1AR-$G_{s_\alpha S}$ and β_2AR-$G_{s_\alpha S}$ were similarly efficient at activating AC, but β_1AR-$G_{s_\alpha L}$ was ~4-fold more efficient at activating AC than β_2AR-$G_{s_\alpha L}$. Our data show that (i) the β_1AR and β_2AR are similarly efficient at stabilizing the ternary complex with G_{s_α} splice variants, (ii) $G_{s_\alpha L}$ confers constitutive activity to the β_1AR and β_2AR, and (iii) the β_1AR coupled to $G_{s_\alpha L}$ is more efficient at activating AC than the β_2AR coupled to $G_{s_\alpha L}$. These data help us understand some of the discrepancies regarding similarities and differences between the β_1AR and β_2AR.

Keywords: β_1-Adrenoceptor; β_2-Adrenoceptor; G_{s_α} splice variants; Constitutive activity; Adenylyl cyclase; Fusion protein

1. Introduction

Many hormones and neurotransmitters exert their effects via GPCRs [1–4]. Upon binding of an agonist, GPCRs isomerize from an inactive to an active state, enabling GPCRs to promote GDP dissociation from G-proteins. Not only agonist-occupied but also agonist-free GPCRs can activate G-proteins. Inverse agonists reduce this agonist-independent (constitutive) activity. Agonist-occupied GPCRs form a ternary complex with nucleotide-free G-protein. The ternary complex is characterized by high agonist affinity. GPCRs then promote binding of GTP to G_α, resulting in ternary complex disruption and dissociation of G-protein into $G_{\alpha\text{-GTP}}$ and the $\beta\gamma$-complex. Both $G_{\alpha\text{-GTP}}$ and $\beta\gamma$ regulate the activity of effectors. The GTPase (EC 3.6.1.) of G_α accomplishes G-protein deactivation by hydrolyzing GTP. $G_{\alpha\text{-GDP}}$ and $\beta\gamma$ re-associate, completing the G-protein cycle.

The β_1AR and β_2AR are prototypical GPCRs that couple to G_s-proteins to activate AC (EC 4.6.1.1) [5]. The human

[*] Corresponding author. Tel.: +1-785-864-3525; fax: +1-785-864-5219.
E-mail address: rseifert@ko.edu (R. Seifert).
[1] Present address: Neurodegeneration Group, The John P. Robarts Research Institute, London, Ont., Canada.

Abbreviations: AC, adenylyl cyclase; ALP, (−)-alprenolol; βAR, non-specified human β-adrenoceptor subtype; β_1AR, human β_1-adrenoceptor (unless explicitly stated otherwise, the Gly389 polymorphism of the β_1AR, i.e. the originally cloned "wild-type' β_1AR with an allele frequency of 0.26 is referred to as the β_1AR); β_1AR-$G_{s_\alpha L}$, fusion protein consisting of the human β_1-adrenoceptor and the long splice variant of G_{s_α}; β_1AR-$G_{s_\alpha S}$, fusion protein consisting of the human β_1-adrenoceptor and the short splice variant of G_{s_α}; β_2AR, human β_2-adrenoceptor; β_2AR-$G_{s_\alpha L}$, fusion protein consisting of the human β_2-adrenoceptor and the long splice variant of G_{s_α}; β_2AR-$G_{s_\alpha S}$, fusion protein consisting of the human β_2-adrenoceptor and the short splice variant of G_{s_α}; BET, betaxolol; DCI, dichloroisoproterenol; [^{3}H]DHA, [^{3}H]dihydroalprenolol; DOB, dobutamine; EPH, (−)ephedrine; G_α, non-specified G-protein α-subunit; GPCR, G-protein-coupled receptor; G_{s_α}, non-specified G_s-protein, i.e. either $G_{s_\alpha L}$, $G_{s_\alpha S}$, or $G_{\alpha olf}$; $G_{s_\alpha L}$, long splice variant of G_{s_α}; $G_{s_\alpha S}$, short splice variant of G_{s_α}; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ICI, ICI 118,551 [erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol]; ISO, (−)-isoproterenol; LAB, (±)-labetalol; PRA, practolol; PRO, (−)-propranolol; SAL, salbutamol; XAM, xamoterol.

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β_1AR exists as two polymorphisms. The originally cloned Gly389-β_1AR has an allele frequency of 26% and is less efficient than the Arg389-β_1AR (allele frequency 74%) at stabilizing the ternary complex and activating AC [6]. Since the Arg389-β_1AR has been identified only recently, most, if not all, published studies aimed at comparing the β_1AR and β_2AR have been conducted with Gly389-β_1AR. For this reason, we analyzed Gly389-β_1AR in our present study, although future studies will need to directly compare Arg389-β_1AR with β_2AR, too.

There is agreement in the literature that the β_1AR is less sensitive to desensitization and internalization than the β_2AR [7–10]. However, with respect to other parameters, the picture is unclear. Freissmuth and coworkers [11] observed a higher percentage of high-affinity binding sites with the β_2AR reconstituted with G_s than with the β_1AR reconstituted with G_s using an *Escherichia coli* expression system. In contrast, Green and coworkers [12] found a higher percentage of high-affinity binding sites with the β_1AR than with the β_2AR using CHW cells as the expression system. In the former study, the K_h values for ISO were similar at the β_1AR and β_2AR, whereas in the latter study the K_h value for the β_1AR was ~5-fold higher than the K_h value for the β_2AR. In other studies, it was difficult to discern distinct high-affinity binding sites with the β_1AR [6,13]. When the constitutive activity of the β_1AR and β_2AR is considered, the situation is unclear, too. The β_1AR and β_2AR expressed in HEK293 cells exhibit similarly low extents of constitutive activity [14]. In contrast, the β_2AR expressed in cardiac myocytes from β_1AR/β_2AR double knockout mice exhibits significant constitutive activity, whereas the β_1AR is devoid of any constitutive activity in this expression system [15]. However, when expressed in COS-7 cells, the β_1AR clearly exhibits constitutive activity, although to a lesser extent than the β_2AR [16]. Finally, the data concerning AC activation are controversial. In some studies, the agonist-occupied β_2AR was found to be more efficient at activating AC than the β_1AR [9,17], whereas in other studies the agonist-occupied β_1AR and β_2AR were reported to be similarly efficient at activating AC [7,8,13,14].

The G-protein G_s exists as two splice variants, $G_{s_\alpha L}$ and $G_{s_\alpha S}$, respectively. $G_{s_\alpha L}$ possesses a lower GDP affinity than $G_{s_\alpha S}$ [18,19]. As a result of these biochemical differences between G_{s_α} splice variants, the agonist-free β_2AR and the β_2AR bound to partial agonists are more efficient at promoting GDP/GTP exchange at $G_{s_\alpha L}$ than at $G_{s_\alpha S}$, i.e. $G_{s_\alpha L}$ confers the hallmarks of constitutive activity to the β_2AR [19,20]. In addition, the maximum AC activity induced by the $G_{s_\alpha L}$-coupled β_2AR is lower than the AC activity induced by the $G_{s_\alpha S}$-coupled β_2AR.

Based on these data and the fact that the expression of $G_{s_\alpha S}$ and $G_{s_\alpha L}$ varies substantially in different tissues [21], we asked the question whether the analysis of β_1AR-coupling to G_{s_α} splice variants would help us to understand some of the discrepancies in the literature regarding differences/similarities between the β_1AR and β_2AR. To address this question, we studied β_1AR-G_{s_α} fusion proteins expressed in Sf9 insect cells and compared their properties with the properties of the previously published β_2AR-G_{s_α} fusion proteins [19,20,22]. In GPCR-G_α fusion proteins, the GPCR C-terminus is linked to the G_α N-terminus [23–25]. The fusion guarantees a defined 1:1 stoichiometry of the signaling partners, ensures close proximity of the partners, promotes efficient coupling, and allows for the analysis of the coupling of a given GPCR to various G-proteins under defined experimental conditions [19,22]. The expression level of fusion proteins and, thereby, the expression of a specific G_α, can be precisely determined by GPCR antagonist saturation binding. This information can be used to determine the steady-state GTP turnover of the fusion protein [24,25], the functional integrity of fusion proteins by assessing ligand-regulated GTPγS saturation binding [22,26,27], and the specific efficacies of a given GPCR-G_{s_α} fusion protein at activating AC [27]. Additionally, ternary complex formation, GTP turnover, as well as the efficacies and potencies of partial agonists and inverse agonists in the GTPase and GTPγS binding assays are independent of the expression level of GPCR-G_α fusion proteins [19,22,27,28]. These properties together with the low background signaling of exogenously expressed GPCRs to endogenous insect G_s-like G-proteins and the very stable AC activity in Sf9 cells render these cells a very sensitive system for the analysis of βARs [19,28].

2. Materials and methods

2.1. Materials

The cDNA for the human β_1AR (Gly389 polymorphism) in pUC18 was provided by Dr. M. Bouvier (Department of Biochemistry, University of Montreal). [^{35}S]GTPγS (1100 Ci/mmol), [γ-^{32}P]GTP (6000 Ci/mmol), and [α-^{32}P]ATP (3000 Ci/mmol) were from Perkin Elmer. [^{3}H]DHA (85–90 Ci/mmol) was from Amersham Pharmacia Biotech. Unlabeled GTP, GTPγS, GDP, and ATP [high quality, catalogue No. 519 979; <0.01% (w/w) GTP contamination as assessed by HPLC analysis] were obtained from Roche Diagnostics. ICI 118,551 (ICI) was from RBI. The M1 monoclonal antibody (detecting the FLAG epitope), ISO, SAL, EPH, DOB, and ALP were from the Sigma Chemical Co. DCI was from the Aldrich Chemical Co. BET, PRA, and XAM were purchased from Tocris Cookson. All restriction enzymes, DNA polymerase I, and T4 DNA ligase were from New England Biolabs. Glass fiber filters (GF/C) were from Schleicher & Schuell.

2.2. Construction of the β_1AR-G_{s_α} fusion proteins

In the fusion proteins generated in our laboratory, the N-terminus of GPCRs is routinely tagged with the FLAG

epitope, which is recognized by the anti-FLAG Ig (M1 antibody) [28]. The G_α N-terminus is linked to the C-terminus of the GPCRs via a hexahistidine tag. Fusion of the open reading frames of the β_1AR with $G_{s_\alpha S}$ and $G_{s_\alpha L}$, respectively, was achieved by sequential overlap-extension PCRs using *Pfu* polymerase (Stratagene). pUC-β_1AR was digested with *Nco*I and *Xba*I, and the *Nco*I/*Xba*I fragment encoding the β_1AR was cloned into pGEM-3Z-FLAG-formyl peptide receptor digested with *Nco*I and *Xba*I to generate pGEM-3Z-FLAG-β_1AR. In PCR 1A, the DNA sequence of the C-terminus of β_1AR was amplified with pGEM-3Z-FLAG-β_1AR as template by using a sense primer 5′ of the *Xho*I site in the C-terminus (sense *Xho*I primer) and an antisense primer encoding the hexahistidine tag. In PCR 1B, the cDNA of $G_{s_\alpha L}$ was amplified with pGEM-3Z-FLAG-β_2AR-$G_{s_\alpha L}$ as template by using a sense primer encoding the hexahistidine tag and an antisense primer encoding the last 5 amino acids of the C-terminus of the G_{s_α} followed by the stop codon and an extra *Xba*I site for cloning purposes in the 3′-end extension (antisense *Xba*I primer). In PCR 1C, the cDNA of $G_{s_\alpha S}$ was amplified with pGEM-3Z-FLAG-β_2AR-$G_{s_\alpha S}$ as template by using a sense primer encoding the hexahistidine tag and the antisense *Xba*I primer. In PCR 2A, the cDNA fragments from PCRs 1A and 1B were annealed and amplified using the sense *Xho*I primer and the antisense *Xba*I primer. In this way, a fragment encoding the C-terminus of the β_1AR, a hexahistidine tag, and $G_{s_\alpha L}$ followed by an *Xba*I site was obtained. In PCR 2B, the cDNA fragments from PCRs 1A and 1C were annealed and amplified using the sense *Xho*I primer and the antisense *Xba*I primer. In this way, a fragment encoding the C-terminus of the β_1AR, a hexahistidine tag, and $G_{s_\alpha S}$ followed by an *Xba*I site was obtained. The fragments of PCRs 2A and 2B were digested with *Xho*I and *Xba*I and cloned into pGEM-3Z-FLAG-β_1AR digested with *Xho*I plus *Xba*I. PCR-generated DNA sequences were confirmed by enzymatic sequencing. For generation of the baculovirus expression vector pVL1392, pGEM-3Z-β_1AR-$G_{s_\alpha L}$ and pGEM-3Z-β_1AR-$G_{s_\alpha S}$ were digested with *Sac*I and *Xba*I and subsequently with *Pvu*II to destroy the pGEM-3Z vector, and fusion protein cDNAs were cloned into pVL1392-FLAG-formyl peptide receptor digested with *Sac*I and *Xba*I.

2.3. Generation of recombinant baculoviruses, cell culture, and membrane preparation

Sf9 cells were cultured in 250-mL disposable Erlenmeyer flasks at 28° under rotation at 125 rpm in SF 900 II medium (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (BioWhittaker) and 0.1 mg/mL of gentamicin (BioWhittaker). Cells were maintained at a density of 1.0 to 6.0×10^6 cells/mL. Recombinant baculoviruses encoding β_1AR-G_{s_α} fusion proteins were generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen) according to the instructions of the manufacturer. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. In the first amplification, cells were seeded at 2.0×10^6 cells/mL and infected with a 1:100 dilution of the supernatant fluid from the initial transfection. Cells were cultured for 7 days, resulting in the death of virtually the entire cell population. The supernatant fluid of this infection was harvested and stored under light protection at 4°. In a second amplification, cells were seeded at 3.0×10^6 cells/mL and infected with a 1:20 dilution of the supernatant from the initial amplification. Cells were cultured for 48 hr, and the supernatant fluid was harvested. After the 48-hr culture, the majority of cells showed signs of infections (e.g. altered morphology, viral inclusion bodies), but most of the cells were still intact. The supernatant fluid from the second amplification was also stored under light protection at 4° and was the routine virus stock for membrane preparations. For expression of fusion proteins, Sf9 cells were sedimented by centrifugation (5 min at 500 *g* at 25°) and suspended in fresh medium. Cells were seeded at 3.0×10^6 cells/mL and infected with 1:100 or 1:1000 dilutions of high-titer baculovirus stocks encoding β_1AR-$G_{s_\alpha L}$ or β_1AR-$G_{s_\alpha S}$ fusion proteins. Cells were cultured for 48 hr before membrane preparation. Sf9 membranes were prepared as described previously [28], using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL of benzamidine, and 10 μg/mL of leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris/HCl, pH 7.4), and were stored at −80° for periods of up to 1 year (longer periods of time were not analyzed in this study) without loss of functional activity in the various assays employed.

2.4. [^{3}H]DHA binding assay

Membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous guanine nucleotides as far as possible, and resuspended in binding buffer. Expression levels of fusion proteins were determined by incubating Sf9 membranes (25–30 μg protein/tube) in the presence of [^{3}H]DHA at concentrations from 0.1 to 10 nM. The total volume of the binding reaction was 500 μL. Incubations were performed for 90 min at 25° on a shaker set at 250 rpm. Non-specific [^{3}H]DHA binding was determined in the presence of [^{3}H]DHA at various concentrations plus 10 μM (±)-alprenolol. Non-specific [^{3}H]DHA binding amounted to less than 10–15% of total [^{3}H]DHA binding even with the highest radioligand concentration (10 nM [^{3}H]DHA). In agonist-competition studies, tubes contained Sf9 membranes expressing fusion proteins at 3.6–7.5 pmol/mg (25–30 μg protein/tube), 1 nM [^{3}H]DHA, and agonists at increasing concentrations. Reaction mixtures additionally contained solvent (control) or GTPγS (10 μM). Bound [^{3}H]DHA was separated from free [^{3}H]DHA by filtration through GF/C filters using a 48-well harvester (model

M-48R, Brandel), followed by three washes with 2 mL of binding buffer (4°). Filter-bound radioactivity was determined by liquid scintillation counting using Cytoscint fluid from ICN. The experimental conditions chosen ensured that not more than 10% of the total amount of [^{3}H]DHA added to binding tubes was bound to filters.

2.5. [^{35}S]GTPγS binding assay

Membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous guanine nucleotides as far as possible, and resuspended in binding buffer. For GTPγS saturation binding studies, reaction mixtures (500 μL total volume) contained Sf9 membranes expressing fusion proteins at 3.6–7.5 pmol/mg (15–30 μg protein/tube) in binding buffer supplemented with 0.05% (w/v) BSA, 1 μM GDP, and 0.2–1.0 nM [^{35}S]GTPγS plus unlabeled GTPγS at increasing concentrations to give the final ligand concentrations of up to 10 nM. Reaction mixtures additionally contained distilled water (basal) or ISO (10 μM). Incubations were performed at 25° for 60 min on a shaker set at 250 rpm. For the analysis of ligand potencies and efficacies, reaction mixtures (500 μL total volume) contained Sf9 membranes expressing fusion proteins at 3.6–7.5 pmol/mg (15 μg protein/tube) in binding buffer supplemented with 0.05% (w/v) BSA, 1 μM GDP, and 0.4 nM [^{35}S]GTPγS. Reaction mixtures additionally contained ligands at various concentrations. Incubations were performed at 25° for 60 min on a shaker set at 250 rpm. Non-specific [^{35}S]GTPγS binding was determined in the presence of 10 μM GTPγS and amounted to less than 0.1% of total [^{35}S]GTPγS binding. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration through GF/C filters, followed by three washes with 2 mL of binding buffer (4°). Filter-bound radioactivity was determined by liquid scintillation counting using Cytoscint fluid. The experimental conditions chosen ensured that no more than 10% of the total amount of [^{35}S]GTPγS added was bound to filters.

2.6. AC activity assay

Membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous guanine nucleotides as far as possible, and resuspended in binding buffer. Tubes contained Sf9 membranes expressing fusion proteins at 0.8–3.8 pmol/mg (15–30 μg protein/tube), 5 mM $MgCl_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, and GTP at various concentrations without or with ISO (10 μM). Assay tubes containing membranes and additions in a total volume of 30 μL were incubated for 3 min at 37° before starting reactions by the addition of 20 μL of reaction mixture containing (final) [α-^{32}P]ATP (1.0–1.5 μCi/tube) plus 40 μM unlabeled ATP, 0.1 mM cAMP, and a regenerating system consisting of 2.7 mM mono(-cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU pyruvate kinase, and 1 IU myokinase. We used only high quality ATP from Roche (catalogue No. 519 979; <0.01% GTP content as assessed by HPLC analysis) to minimize exogenous GTP contamination as far as possible. Reactions were conducted for 20 min at 37° and were terminated by the addition of 20 μL of 2.2N HCl. Denatured protein was sedimented by a 3-min centrifugation at 25° and 15,000 *g*, and reaction supernatants (65 μL) were applied onto disposable columns filled with 1.3 g of neutral alumina (Sigma A-1522, super I, WN-6). [^{32}P]cAMP was separated from [α-^{32}P]ATP by elution of [^{32}P]cAMP with 4 mL of 0.1 M ammonium acetate, pH 7.0. Recovery of [^{32}P]cAMP was ~80%. Blank values were routinely ~0.01% of the total amount of [α-^{32}P]ATP added. [^{32}P]cAMP was determined by liquid scintillation counting using ScintiSafe Econo2 scintillation fluid from Fisher. The experimental conditions chosen ensured that not more than 1–3% of the total amount of [α-^{32}P]ATP added was converted to [^{32}P]cAMP.

2.7. Steady-state GPase activity assay

Membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous guanine nucleotides as far as possible, and resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained Sf9 membranes expressing fusion proteins at 3.6–7.5 pmol/mg (10 μg protein/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, and 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4. Tubes additionally contained unlabeled GTP at concentrations between 30 nM and 1 μM without or with ISO (10 μM). Reaction mixtures (80 μL) were incubated for 3 min at 25° before the addition of 20 μL of [γ-^{32}P]GTP (0.2–0.5 μCi/tube). Reactions were conducted for 20 min at 25°, and were terminated by the addition of 900 μL of a suspension consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not P_i. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000 *g*. Seven hundred microliters of the supernatant fluid of reaction mixtures was removed carefully to avoid any aspiration of charcoal, and $^{32}P_i$ was determined by liquid scintillation counting using ScintiSafe Econo2 scintillation fluid. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$.

2.8. SDS–PAGE and immunoblot analysis

Membrane proteins were separated on SDS gels containing 10% (w/v) acrylamide. Proteins were then transferred onto Immobilon-P transfer membranes (Millipore) according to the instructions of the manufacturer. Membranes were reacted with anti-FLAG Ig or anti-G_{s_α} Ig (1:1000

each). Immunoreactive bands were visualized by sheep anti-mouse IgG (anti-FLAG Ig, 1:1000) and donkey anti-rabbit IgG (anti-G_{s_α} Ig, 1:1000), respectively, coupled to peroxidase, using *o*-dianisidine and H_2O_2 as substrates.

2.9. Miscellaneous

Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad). Data shown in Figs. 2, 3B, and 4 were analyzed by non-linear regression using the Prism III program (GraphPad, Prism). Statistical comparisons were performed using the *t*-test.

3. Results and discussion

3.1. Analysis of the expression of β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ in Sf9 membranes by antagonist saturation binding, immunoblotting, and GTPγS saturation binding

Membranes from Sf9 cells infected with high-titer baculovirus stocks encoding β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ were prepared, and the expression levels of constructs were determined by [^{3}H]DHA saturation binding. With a 1:100 dilution of virus stocks, the B_{max} value (expression levels) of β_1AR-G_{s_α} fusion proteins was 4.5 ± 1.7 pmol/mg (mean ± SD, N = 6). With a 1:1000 dilution of virus stocks, the expression level of β_1AR-G_{s_α} fusion proteins was 1.1 ± 0.4 pmol/mg (mean ± SD, N = 4). β_1AR-$G_{s_\alpha L}$ bound [^{3}H]DHA with a K_d value of 1.6 ± 0.4 nM (mean ± SD, N = 5), and β_1AR-$G_{s_\alpha S}$ bound [^{3}H]DHA with a K_d value of 1.9 ± 0.3 nM (mean ± SD, N = 5). These data show that the expression levels achieved for β_1AR-G_{s_α} are comparable with the expression levels of β_2AR-G_{s_α} fusion proteins [19,27,28] and that the antagonist binding properties of β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ are similar. Our data also show that by lowering the virus titer by 10-fold, the expression of β_1AR-G_{s_α} fusion proteins was reduced by ~4-fold, which was important for the conduction of AC studies (see below). The maximum expression levels achieved for β_2AR-$G_{s_\alpha L}$ were substantially higher (~20 pmol/mg) than the expression levels of β_2AR-$G_{s_\alpha S}$ (~6 pmol/mg), but we did not observe such a difference in the maximum expression level for β_1AR-G_{s_α} fusion proteins.

Sf9 membranes were prepared and analyzed by SDS–PAGE and immunoblotting with the anti-FLAG Ig and the anti-G_{s_α} Ig (C-terminal). $G_{s_\alpha L}$ and $G_{s_\alpha S}$ possess molecular masses of 52 and 45 kDa, respectively [18]. The β_1AR possesses a predicted molecular mass of ~51 kDa [29]. β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ exhibited apparent molecular masses of ~109–111 and 102–104 kDa, respectively, when probed with the anti-FLAG Ig (Fig. 1A) and anti-G_{s_α} Ig (Fig. 1B). The fact that the observed molecular masses of β_1AR-G_{s_α} fusion proteins were slightly higher than the sum of the predicted masses of the β_1AR and G_{s_α} is presumably due to covalent modifications of the β_1AR. Specifically, the β_1AR possesses one consensus site for *N*-glycosylation [29], and *N*-glycosylation tends to increase the apparent molecular masses of GPCR-G_α fusion proteins in SDS–PAGE [27]. We did not observe immunoreactive bands below the β_1AR-G_{s_α} fusion proteins with either of the two antibodies. These findings indicate that the conditions

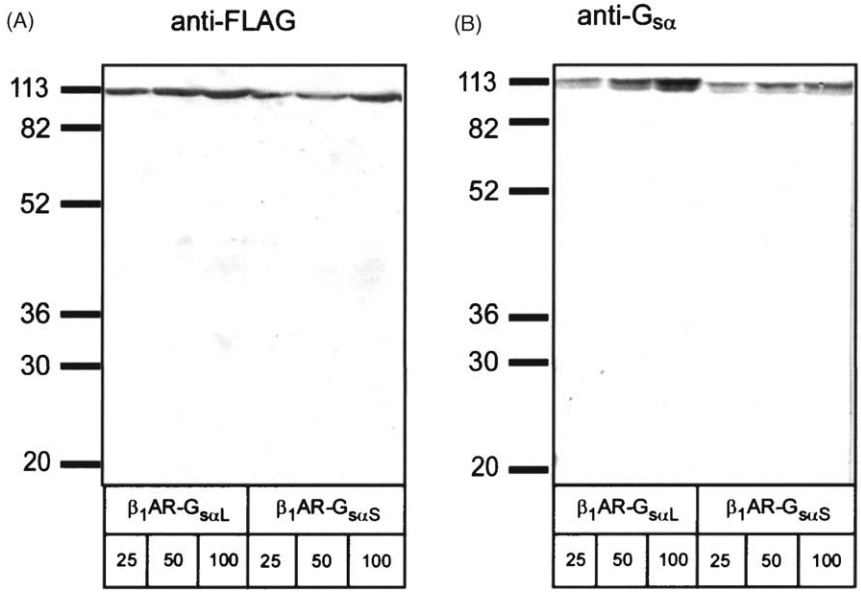

Fig. 1. Analysis of the expression of β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ in Sf9 membranes. Sf9 cell membranes expressing β_1AR-$G_{s_\alpha L}$ (4.2 pmol/mg) and β_1AR-$G_{s_\alpha S}$ (3.0 pmol/mg) were prepared and separated on SDS gels containing 10% (w/v) acrylamide as described in Section 2. Proteins were transferred onto Immobilon-P transfer membranes and probed with anti-FLAG Ig (A) or anti-G_{s_α} Ig (B). Shown are the horseradish peroxidase-reacted Immobilon-P transfer membranes. Numbers below fusion proteins indicate the amount of protein (in μg) applied to each lane. Numbers on the left indicate molecular masses of marker proteins.

chosen for cell culture and membrane preparation (see Section 2) efficiently prevented the occurrence of degradation products.

Fusion proteins exhibit a 1:1 GPCR/G_α stoichiometry [23–25]. Thus, 1 mol of GPCR-G_α can maximally bind 1 mol of GTPγS provided that both the GPCR and G_α moieties are functionally intact and that interaction of the GPCRs with the endogenous G-proteins of Sf9 cells is minimal. Inefficient coupling of the β_2AR to the endogenous G-proteins of the Sf9 cells was documented earlier [19,22,28]. The ratio of the B_{max} of ligand-regulated GTPγS binding and the B_{max} of receptor antagonist binding is referred to as a coupling factor [22,27]. To calculate the coupling factor in membranes expressing β_1AR-$G_{s_\alpha S}$ and β_1AR-$G_{s_\alpha L}$, we divided the B_{max} of ISO-stimulated [^{35}S]GTPγS binding by the B_{max} of [^{3}H]DHA binding of the respective membrane preparation. The coupling factors were 0.88 ± 0.16 (mean ± SD, N = 3) for β_1AR-$G_{s_\alpha L}$ and 0.91 ± 0.19 (mean ± SD, N = 3) for β_1AR-$G_{s_\alpha S}$. The K_d values of ISO-stimulated [^{35}S]GTPγS binding were 0.45 ± 0.08 nM (mean ± SD, N = 3) for β_1AR-$G_{s_\alpha L}$ and 0.81 ± 0.43 nM (mean ± SD, N = 3) for β_1AR-$G_{s_\alpha S}$. These data indicate that the majority, if not all of the expressed β_1AR-G_{s_α} fusion protein molecules were functionally intact. Similar coupling factors as for β_1AR-G_{s_α} fusion proteins were obtained for β_2AR-G_{s_α} fusion proteins [22,27]. Additionally, the K_d values of agonist-stimulated [^{35}S]GTPγS binding at β_1AR-G_{s_α} and β_2AR-G_{s_α} fusion proteins are very similar [22,27], indicating that fusion of G_{s_α} to different βARs does not change GTPγS affinity of G_{s_α}.

3.2. Analysis of the agonist binding properties of β_1AR-G_{s_α} and β_2AR-G_{s_α} expressed in Sf9 membranes

Ternary complex formation in β_2AR-G_{s_α} fusion proteins is independent of the fusion protein expression level [19,27,28]. Nonetheless, for the most accurate comparison of data for the β_1AR and β_2AR, we ensured that the expression levels of β_1AR-G_{s_α} fusion proteins were in a similar range (3.6–7.5 pmol/mg) as the expression levels of β_2AR-G_{s_α} fusion proteins (3.3–7.5 pmol/mg) [19]. As for β_2AR-G_{s_α} [19,28], ternary complex formation at β_1AR-G_{s_α} was determined indirectly by agonist-competition of [^{3}H]DHA binding. We analyzed the agonists ISO, SAL, and DOB. Fig. 2 shows the agonist-competition isotherms, and Table 1 summarizes the non-linear regression analysis of the binding data. For all three agonists, we obtained biphasic competition curves at β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ in the absence of GTPγS. These data show that ISO, SAL, and DOB stabilized the ternary complex with a fraction of the available β_1AR-G_{s_α} molecules. The question as to why agonists do not stabilize the ternary complex in all fusion protein molecules present has been discussed earlier [30,31]. In accordance with previous results obtained for the β_2AR [19] and turkey βAR [32], GTPγS shifted the agonist-competition curves to the right and rendered them monophasic, indicative of ternary complex disruption. The efficiency of a given agonist at stabilizing the ternary complex was similar at β_1AR-$G_{s_\alpha S}$ and β_1AR-$G_{s_\alpha L}$ as expressed by the percentage of high-affinity binding sites. Similar data were obtained for β_2AR-$G_{s_\alpha S}$ and β_2AR-$G_{s_\alpha L}$ [19]. The K_h values for ISO at β_1AR-G_{s_α} fusion proteins were similar to the corresponding K_h values for ISO at β_2AR-G_{s_α} fusion proteins (Table 1) [19]. Moreover, the percentages of high-affinity binding sites at β_1AR-G_{s_α} and β_2AR-G_{s_α} fusion proteins with ISO were very similar (~40–50%). Taken together, the ability of a given βAR at stabilizing the ternary complex with a specific ligand is independent of the particular G_{s_α} splice variant to which the GPCR is fused. In addition, ISO is similarly efficient at stabilizing the ternary complex at the two β_1AR-G_{s_α} and the two β_2AR-G_{s_α} fusion proteins. Previous studies yielded inconsistent results regarding the ability of ISO at stabilizing the ternary complex at the β_1AR and β_2AR [6,11–13]. An explanation for these differences could be different βAR/G_s stoichiometries and/or different βAR/G_s compartmentalizations in the various assay systems. Our data show that annihilation of differences in stoichiometry and/or compartments by the use of the GPCR-G_α fusion protein

Table 1
Analysis of the agonist binding properties of β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ expressed in Sf9 membranes

Agonist	K_h (nM)	K_l (nM)	$\%R_h$	$K_{l\text{GTP}\gamma\text{S}}$ (nM)
β_1AR-$G_{s_\alpha L}$				
ISO	2.0 (0.6–6.8)	90 (40–200)	42.2 (23.4–61.0)	46 (30–70)
SAL	19 (8.0–35)	3900 (2500–6200)	13.6 (4.0–23.5)	4900 (3900–6100)
DOB	9.5 (3.5–25)	1700 (1200–2400)	25.1 (18.5–31.6)	1400 (1200–1800)
β_1AR-$G_{s_\alpha S}$				
ISO	0.9 (0.7–1.1)	90 (78–100)	39.6 (66.9–42.2)	54 (42–68)
SAL	2.9 (0.7–13)	4700 (3700–6100)	16.5 (11.9–21.1)	5500 (4700–6400)
DOB	8.6 (2.4–28)	1800 (1200–2700)	23.2 (15.6–30.6)	3600 (2400–5300)

Agonist-competition binding in Sf9 membranes expressing β_1AR-G_{s_α} was performed as described in Section 2. The data shown in Fig. 2 were analyzed by non-linear regression for the best fit to monophasic or biphasic competition curves. Data shown are the means of 3–4 experiments performed in triplicate. Number in parentheses represent the 95% confidence intervals. K_h and K_l designate the dissociation constants for the high- and low-affinity state of the β_1AR, respectively. $\%R_h$ indicates the percentage of high-affinity binding sites. The dissociation constants obtained in the presence of GTPγS (10 μM) are listed under $K_{l\text{GTP}\gamma\text{S}}$.

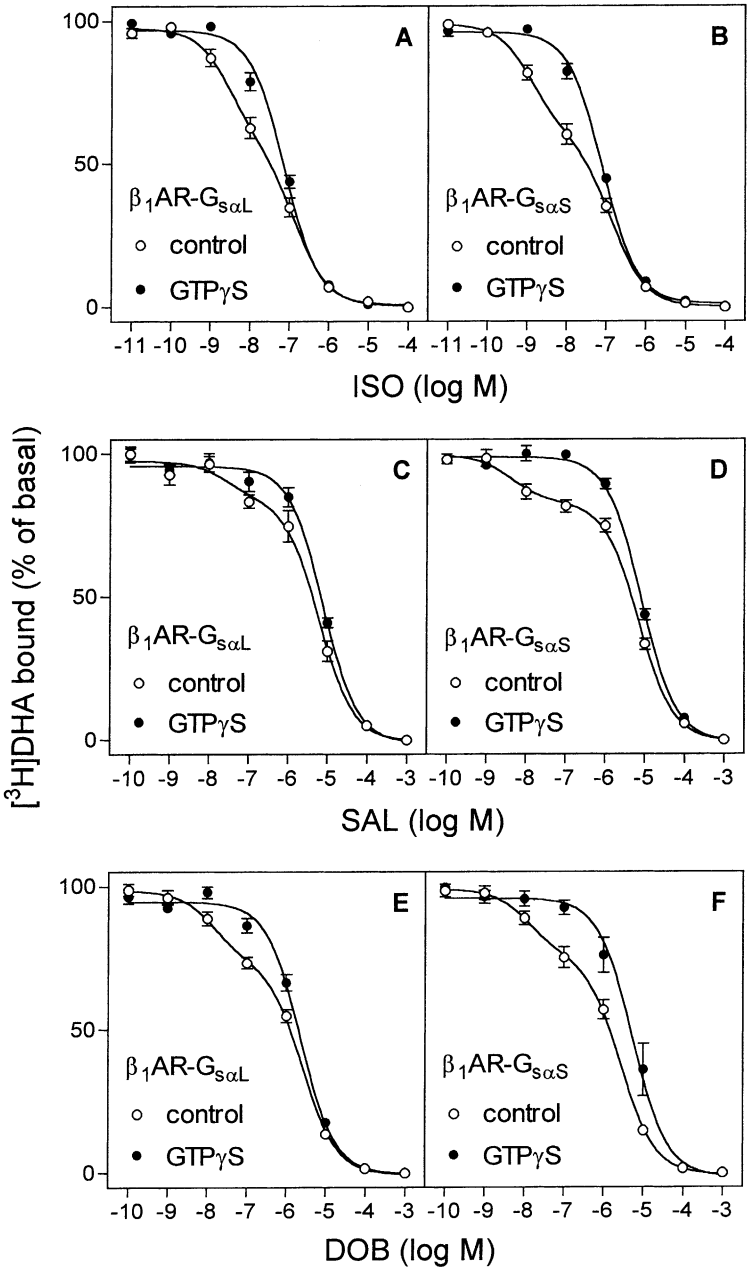

Fig. 2. Competition by ISO, SAL, and DOB of [^{3}H]DHA binding in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$: effect of GTPγS. [^{3}H]DHA binding was performed as described in Section 2. Reaction mixtures contained Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ (A, C, and E) or β_1AR-$G_{s_\alpha S}$ (B, D, and F), 1 nM [^{3}H]DHA, and agonists at increasing concentrations. Reaction mixtures additionally contained distilled water (control, ○) or 10 μM GTPγS (●). Data points shown are the means ± SD of 3–4 experiments. Data were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. The results of this analysis are summarized in Table 1. Typical absolute [^{3}H]DHA binding values in the absence of competitor were ~2000–6000 cpm, depending upon the amount of protein added and the expression level of fusion proteins.

technique abolishes potential differences in the ability of the β_1AR and β_2AR at stabilizing the ternary complex with ISO.

3.3. Ligand potencies and efficacies at β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ expressed in Sf9 membranes

The steady-state GTPase assay and GTPγS binding assay are equally feasible for determining ligand potencies and efficacies at GPCR-G_α fusion proteins [19,22,26,33]. In the present study, we determined ligand efficacies in the GTPγS binding assay. At β_1AR-$G_{s_\alpha L}$, agonists-stimulated GTPγS binding in the order of efficacy ISO ~ SAL ~ DOB > XAM > EPH > DCI > PRA > LAB ~ ALP > PRO (Fig. 3A). ICI and BET acted as weak inverse agonists at β_1AR-$G_{s_\alpha L}$. With the exception of ISO, being a full agonist at both fusion proteins, and ICI, the efficacies of all other ligands were significantly lower at β_1AR-$G_{s_\alpha S}$ than at β_1AR-$G_{s_\alpha L}$. The differences in agonist/inverse agonist efficacies at β_1AR-$G_{s_\alpha L}$ versus β_1AR-$G_{s_\alpha S}$ resulted in a

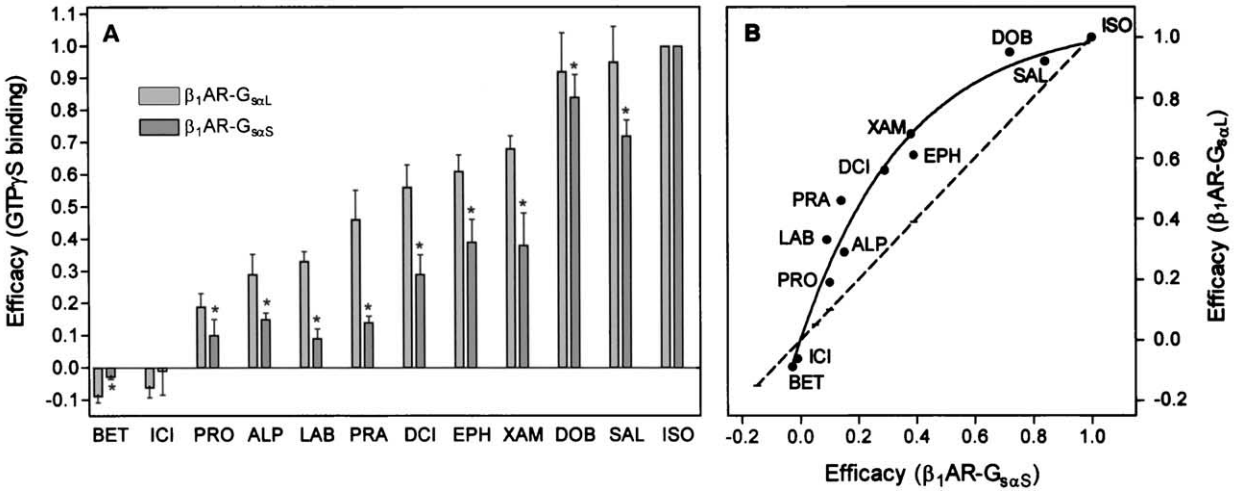

Fig. 3. Efficacies of partial agonists at β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ in the GTPγS binding assay. GTPγS binding in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_2AR-$G_{s_\alpha S}$ was determined as described in Section 2. Reaction mixtures contained Sf9 membranes expressing fusion proteins, 1 μM GDP, 0.4 nM [^{35}S]GTPγS, and ISO, SAL, DOB, EPH, and DCI at concentrations between 0.1 nM and 100 μM as appropriate to obtain saturated concentration/response curves. Efficacies were determined by using the baseline and plateau values of the non-linear regression analysis of concentration/response curves. For determination of the efficacies of XAM, PRA, LAB, ALP, PRO, ICI, and BET, we used ligands at a single fixed concentration (10 μM). The effect of ISO at each fusion protein was set at 1.00, and the effects of other ligands were referred to this value. Data shown in panel A represent the means ± SD of 4–5 experiments performed in triplicate. The effects of a given ligand at β_1AR-$G_{s_\alpha L}$ versus β_1AR-$G_{s_\alpha S}$ were compared using the *t*-test. Key: (∗) $P < 0.05$. In panel B, the efficacies of ligands at β_1AR-$G_{s_\alpha S}$ were plotted against the efficacies of ligands at β_1AR-$G_{s_\alpha L}$. Data were best fitted to a monophasic saturation hyperbola (r^2, 0.97). The straight dashed line represents the theoretical curve that would have resulted if the efficacies of ligands at β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ had been identical.

hyperbolic relation when the ligand efficacies at β_1AR-$G_{s_\alpha S}$ were plotted against the efficacies at β_1AR-$G_{s_\alpha L}$ (Fig. 3B). Thus, the increase in efficacy of agonists at β_1AR-$G_{s_\alpha L}$ was most pronounced for agonists with intermediate efficacies (LAB → XAM). The differences in agonist efficacies were accompanied by similar changes in agonist potencies, i.e. at β_1AR-$G_{s_\alpha L}$ the potencies of partial agonists and the full agonist ISO were all higher than at β_1AR-$G_{s_\alpha S}$ (Table 2). These data together with the strong stimulatory effect of GTP on basal AC activity in membranes expressing β_1AR-$G_{s_\alpha L}$ (Fig. 4A), reflecting GDP/GTP exchange catalyzed by the agonist-free β_1AR, show that the β_1AR coupled to $G_{s_\alpha L}$ exhibits the hallmarks of constitutive activity [19,34]. For the β_2AR-$G_{s_\alpha L}$/β_2AR-$G_{s_\alpha S}$ couple, we observed similar differences in efficacies and potencies for SAL, DOB, EPH, and DCI as for the β_1AR-$G_{s_\alpha L}$/β_1AR-$G_{s_\alpha S}$ couple (Fig. 3) [19]. Additionally, the relative stimulatory effects of GTP on AC activity are similar in membranes expressing β_1AR-$G_{s_\alpha L}$ and β_2AR-$G_{s_\alpha L}$ (Fig. 4A) [20,35]. Collectively, our data indicate that $G_{s_\alpha L}$ confers similar extents of constitutive activity to the β_1AR and β_2AR.

Table 2
Potencies of full and partial agonists at stimulating GTPγS binding in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$

Ligand	β_1AR-$G_{s_\alpha L}$ (EC_{50}, nM)	β_1AR-$G_{s_\alpha S}$ (EC_{50}, nM)
ISO	2.8 ± 1.5	10.5 ± 2.1*
SAL	530 ± 231	1370 ± 303*
DOB	133 ± 62	400 ± 117*
EPH	1670 ± 503	4720 ± 2860*
DCI	13.1 ± 2.5	39 ± 15*

Measurement of GTPγS binding in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ was performed as described under Section 2. Reaction mixture contained ligands at concentrations between 0.1 nM and 100 μM as appropriate to obtain saturated concentration/response curves. Data were best fitted to sigmoidal concentration/response curves. Data shown are the mean ± SD of 4–5 experiments performed in triplicate.

* The EC_{50} values of ligands at β_1AR-$G_{s_\alpha L}$ were compared versus the corresponding EC_{50} values of ligands at β_1AR-$G_{s_\alpha S}$ with *t*-test; $P < 0.05$.

Our data may help in reconciling some of the divergent results in the literature regarding the constitutive activity of the β_1AR compared with the constitutive activity of the β_2AR [14–16]. Specifically, the β_1AR expressed in cardiac myocytes from β_1AR/β_2AR double knockout mice could be exclusively coupled to $G_{s_\alpha S}$, resulting in the absence of constitutive activity [15]. In contrast, in this system, the β_2AR could efficiently couple to $G_{s_\alpha L}$, resulting in significant constitutive activity. In transfected HEK293 cells, both the β_1AR and β_2AR may couple more efficiently to $G_{s_\alpha S}$ than to $G_{s_\alpha L}$, resulting in low but measurable constitutive activity of both βARs [14]. Finally, the β_1AR expressed in COS-7 cells may couple quite efficiently to $G_{s_\alpha L}$, resulting in significant constitutive activity of the β_1AR [16]. However, in this system, the β_2AR could couple more efficiently to $G_{s_\alpha L}$ than the β_1AR, since the β_2AR exhibits higher constitutive activity than the β_1AR. Taken together, our results and the results from other investigators suggest that in different expression systems, the β_1AR and β_2AR have differential access to G_{s_α} splice variants, resulting in differential activation of $G_{s_\alpha S}$ and $G_{s_\alpha L}$ by the two βARs. In fact, there is precedence for differential activation of G_{s_α} splice variants by various GPCRs in native systems [36]. Future studies will have

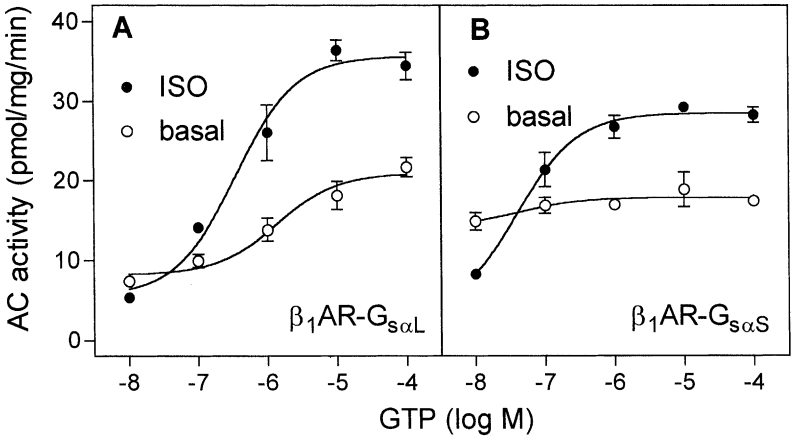

Fig. 4. Regulation of AC activity in Sf9 membranes by β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$. AC activity in Sf9 membranes was determined as described in Section 2. Reaction mixtures contained Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ (0.8 pmol/mg, panel A) or β_1AR-$G_{s_\alpha S}$ (1.1 pmol/mg, panel B), GTP at the concentrations indicated on the abscissa (10^{-8} designates the absence of added GTP) plus solvent (basal, ○) or ISO (10 μM, ●). The commercial ATP preparation used (Roche No. 519 979) contains <0.01% (w/w) GTP. Given an ATP concentration of 40 μM, the maximum GTP concentration in the absence of added GTP was <4 nM. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 3 experiments performed in duplicate.

to determine the expression levels of G_{s_α} splice variants in various expression systems and the localization of G_{s_α} splice variants relative to the β_1AR and β_2AR. Moreover, differential activation of G_{s_α} splice variants by the β_1AR and β_2AR has to be verified in those systems.

3.4. Regulation of AC activity in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$

For comparing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ in terms of AC regulation it was important to express fusion proteins at similar levels, ensuring comparable fusion protein/AC ratios and to avoid excessively high expression levels because this leads to a depletion of the available AC molecules [27,28,37]. We performed AC studies at low (0.8–1.3 pmol/mg) and intermediate (3.6–3.8 pmol/mg) β_1AR-G_{s_α} expression levels. Fig. 4 shows the GTP-dependency of AC activity in membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ at a low level, and Table 3 shows results obtained for membranes expressing β_1AR-G_{s_α} at different levels. In membranes expressing β_1AR-$G_{s_\alpha L}$, GTP increased basal AC activity with an EC_{50} of 1.4 μM (95% confidence interval, 0.64–3.1 μM) and by ∼250%. The stimulatory effect of GTP on basal AC activity reflects the constitutive activity of the β_1AR (see above). ISO shifted the concentration/response curve for GTP in membranes expressing β_1AR-$G_{s_\alpha L}$ ∼4-fold to the left (EC_{50}, 0.35 μM; 95% confidence interval, 0.19–0.66 μM) and increased AC activity by ∼100 and 60% above basal with GTP concentrations of 10 and 100 μM, respectively.

The basal AC activity in membranes expressing β_1AR-$G_{s_\alpha S}$ was ∼2-fold higher than in membranes expressing β_1AR-$G_{s_\alpha L}$. GTP had a much smaller stimulatory effect on basal AC activity in membranes expressing β_1AR-$G_{s_\alpha S}$ than in membranes expressing β_1AR-$G_{s_\alpha L}$, reflecting the low constitutive activity of the $G_{s_\alpha S}$-coupled β_1AR (see above). In the presence of GTP, ISO exhibited a significant stimulatory effect on AC activity in membranes expressing β_1AR-$G_{s_\alpha S}$ (∼45%), but the maximum ISO-stimulated AC activities in membranes expressing β_1AR-$G_{s_\alpha S}$ at a low

Table 3
Regulation of AC activity in Sf9 membranes expressing β_1AR-$G_{s_\alpha L}$ and β_1AR-$G_{s_\alpha S}$ at low and intermediate levels

Expression level (pmol/mg)	AC activity (pmol/mg/min)				Stimulatory effect of GTP (%)	Stimulatory effect of ISO with GTP (%)
	−GTP, −ISO	−GTP, +ISO	+GTP, −ISO	+GTP, +ISO		
β_1AR-$G_{s_\alpha L}$						
0.8	6.6 ± 1.1	5.3 ± 0.1	23.4 ± 2.3	37.5 ± 4.2	254.5	60.3
3.6	9.8 ± 1.7	9.3 ± 1.0	37.4 ± 2.4	45.9 ± 3.8	281.6	22.7
β_1AR-$G_{s_\alpha S}$						
1.1	14.0 ± 1.3	8.6 ± 2.7	17.6 ± 0.3	26.8 ± 2.1	25.7	46.0
3.8	25.3 ± 1.1	18.9 ± 0.8	36.0 ± 2.2	45.5 ± 4.6	42.2	26.3

Membranes from Sf9 cells expressing β_1AR-G_{s_α} at various levels as determined by [^{3}H]DHA saturation binding were prepared. AC activity in membranes expressing β_1AR-G_{s_α} was determined as described in Section 2. Reaction mixtures contained no addition (−GTP, −ISO), 10 μM ISO (−GTP, +ISO), 100 μM GTP (+GTP, −ISO), or 100 μM GTP plus 10 μM ISO (+GTP, +ISO). The relative stimulatory effects of GTP (+ GTP, −ISO versus −GTP, −ISO) and ISO (+GTP, +ISO versus +GTP, −ISO) were calculated as well. Data shown are the means ± SD of three experiments performed in duplicate.

level were lower than the AC activities observed with β_1AR-$G_{s_\alpha L}$ expressed at a similar level.

In membranes expressing β_1AR-$G_{s_\alpha S}$, ISO exhibited a profound *inhibitory* effect on AC activity in the absence of GTP. This inhibition is explained by the fact that in the absence of GTP, the agonist-occupied β_1AR can only promote GDP dissociation from, but not GTP binding to, G_{s_α}. Since G_{s_α}-GDP is more efficient at activating AC than nucleotide-free G_{s_α} [19,27], agonist stimulation actually decreases AC activity. The lower GDP affinity of $G_{s_\alpha L}$ relative to $G_{s_\alpha S}$ explains the lower basal AC activity in membranes expressing β_1AR-$G_{s_\alpha L}$ and the lack of inhibitory effect of ISO on basal AC activity in the absence of GTP [18,19]. A very similar pattern of AC regulation in the absence of GTP as for β_1AR-G_{s_α} was observed for β_2AR-G_{s_α} fusion proteins [19,27].

With β_1AR-$G_{s_\alpha L}$ expressed at ~1 pmol/mg, we obtained ISO-stimulated AC activity of ~35–40 pmol/mg/min. If there had been a linear relation between fusion protein expression level and AC activation, we would have expected to observe ISO-stimulated AC activities of ~125–140 pmol/mg/min in membranes expressing β_1AR-$G_{s_\alpha L}$ at ~3.5 pmol/mg. However, the maximum ISO-stimulated AC activities in membranes expressing β_1AR-$G_{s_\alpha L}$ did not exceed ~45 pmol/mg/min. These data indicate depletion of AC molecules with β_1AR-$G_{s_\alpha L}$ expressed at high levels. With respect to basal AC activities, ISO inhibition of AC in the absence of GTP, and the stimulatory effects of GTP on basal AC activity, similar patterns of AC regulation were observed with β_1AR-G_{s_α} expressed at low and intermediate levels. However, because of AC depletion, the relative stimulatory effects of ISO were smaller in membranes expressing fusion proteins at intermediate levels than in membranes expressing β_1AR-G_{s_α} at low levels. The depletion of AC molecules with β_1AR-$G_{s_\alpha L}$ at a level of 3.6 pmol/mg was, nonetheless, surprising, given the fact that with β_2AR-$G_{s_\alpha L}$, AC depletion is only observed with fusion protein expression levels of >7.0 pmol/mg [28]. We also noted that with β_1AR-$G_{s_\alpha L}$ expressed at ~1 pmol/mg, the maximum ISO-stimulated AC activities were almost 2-fold higher than the corresponding AC activities with β_2AR-$G_{s_\alpha L}$ expressed at ~2.5 pmol/mg [35].

To compare the efficacies of β_1AR-G_{s_α} and β_2AR-G_{s_α} at activating AC in an expression level-independent manner, we divided AC activities by the expression levels of fusion proteins (Table 4). The numbers obtained represent the specific efficacies of a given fusion protein at activating AC [27]. For these calculations, we considered only AC activities obtained with fusion proteins expressed at levels that did not deplete AC molecules. β_2AR-$G_{s_\alpha L}$ exhibited a 2- to 5-fold lower specific efficacy than β_2AR-$G_{s_\alpha S}$ at activating AC, depending on the particular experimental condition considered. The specific efficacy of β_1AR-$G_{s_\alpha L}$ at activating AC in the absence of GTP was 30% lower than that of β_1AR-$G_{s_\alpha S}$, whereas the specific efficacy of β_1AR-$G_{s_\alpha L}$ in the presence of GTP and GTP plus ISO was ~70–80% higher than the specific efficacy of β_1AR-$G_{s_\alpha S}$. The specific efficacies of β_1AR-$G_{s_\alpha S}$ and β_2AR-$G_{s_\alpha S}$ at activating AC were very similar, whereas the specific efficacy of β_1AR-$G_{s_\alpha L}$ was ~4-fold higher than the efficacy of β_2AR-$G_{s_\alpha L}$ under all experimental conditions analyzed.

Table 4
Specific efficacies of β_1AR-G_{s_α} and β_2AR-G_{s_α} at activating AC in Sf9 membranes

Fusion protein	Specific efficacy at activating AC (min^{-1})		
	−GTP, −ISO	+GTP, −ISO	+GTP, +ISO
β_1AR-$G_{s_\alpha L}$	7.3 ± 2.8	24.9 ± 3.1	39.9 ± 4.5
β_1AR-$G_{s_\alpha S}$	11.7 ± 0.3	14.8 ± 2.1	22.4 ± 0.8
β_2AR-$G_{s_\alpha L}$	2.0 ± 0.3	6.8 ± 0.9	9.4 ± 1.7
β_2AR-$G_{s_\alpha S}$	9.6 ± 1.4	14.3 ± 2.1	22.5 ± 2.3

AC activity in Sf9 membranes expressing β_1AR-G_{s_α} (0.81–1.3 pmol/mg) or β_2AR-G_{s_α} (2.0–3.0 pmol/mg) was determined as described in Section 2. Reaction mixtures contained no addition (−GTP, −ISO), 100 μM GTP (+GTP, −ISO), or 100 μM GTP plus 10 μM ISO (+GTP, +ISO). AC activities (in pmol/mg/min) were divided by the respective expression levels of fusion protein (in pmol/mg) to obtain the specific efficacies of fusion proteins at activating AC. The raw data for β_2AR-$G_{s_\alpha L}$ and β_2AR-$G_{s_\alpha S}$ were taken from [19,20,27]. Data shown are the means ± SD of 3–5 experiments with different membrane preparations performed in duplicate.

Several possibilities have to be discussed to explain the differences in specific efficacies of fusion proteins at activating AC. First, differences in G-protein deactivation have to be considered. Specifically, $G_{s_\alpha L}$ coupled to the β_1AR could be ~4 times more frequent in the active GTP-bound state than $G_{s_\alpha L}$ coupled to the β_2AR. If this model were correct, we would expect considerably higher ISO-stimulated GTPase activities with β_2AR-$G_{s_\alpha L}$ than with β_1AR-$G_{s_\alpha L}$. In fact, an inverse relation between maximum agonist-stimulated steady-state GTP hydrolysis and effector system activation was observed for various systems [27,38]. The V_{max} of the ISO-stimulated GTP hydrolysis of β_1AR-$G_{s_\alpha L}$ was 2.90 ± 0.16 min^{-1} (mean ± SD, N = 3) and was actually ~2-fold higher than the V_{max} of ISO-stimulated GTP hydrolysis of β_2AR-$G_{s_\alpha L}$ (1.37 ± 0.11 min^{-1}) [35]. ISO-stimulated GTP hydrolysis in β_1AR-$G_{s_\alpha S}$ with a V_{max} of 2.66 ± 0.10 min^{-1} (mean ± SD, N = 3). These data argue against the hypothesis that differences in G-protein deactivation account for the differences in specific efficacies of β_1AR-G_{s_α} and β_2AR-G_{s_α} at activating AC. Second, one has to consider that βARs and G_{s_α} are in contact with each other during the entire G-protein cycle and that GPCRs confer specific conformations to a given G-protein. Specifically, the β_1AR may confer to $G_{s_\alpha L}$ a more active conformation with respect to AC activation than the β_2AR. Additionally, the $G_{s_\alpha L}$ conformation stabilized by the β_1AR may be more active than the corresponding $G_{s_\alpha S}$ conformation, whereas the opposite may be true for the β_2AR. There is already evidence for continuous interaction of GPCRs with G-proteins and effector during the entire G-protein cycle [22,35,39–41]. Third, one must consider that the individual

βAR-G_{s_α} fusion proteins and the endogenous AC molecules of the insect cells are localized in different membrane microcompartments. At this time, we cannot decide whether the second or third explanation is correct or whether both explanations are correct.

Our data on the differential activation of AC by β_1AR-G_{s_α} and β_2AR-G_{s_α} fusion proteins may help us to understand some of the controversial results regarding AC activation by those GPCRs in non-fused systems. Specifically, several studies have shown that the agonist-occupied β_1AR and β_2AR are similarly efficient at activating AC [7,8,13,14]. These data would be compatible with the assumption that the β_1AR and β_2AR preferentially couple to $G_{s_\alpha S}$ in those systems (Table 4). However, our data cannot readily explain results showing that the β_2AR is more efficient at activating AC than the β_1AR [9,17] since neither of the two β_2AR-G_{s_α} fusion proteins was more efficient at activating AC than the corresponding β_1AR-G_{s_α} fusion proteins. It is possible that in a mammalian expression system, the compartmentalization of AC molecules relative to GPCRs and/or G_{s_α} differs from the compartmentalization in the insect cell expression system used in this study. Given the fact that the availability of AC molecules limits signal output in βAR/G_{s_α} signaling [37,42], it is likely that the localization and/or number of available AC molecules play a major role in determining the efficiencies of βARs at activating AC in various systems.

In conclusion, coupling of the β_1AR and β_2AR to G_{s_α} splice variants is similar in terms of ternary complex formation. $G_{s_\alpha L}$ confers the properties of constitutive activity to the β_1AR and β_2AR, whereas the β_1AR and β_2AR coupled to $G_{s_\alpha S}$ are not constitutively active. β_1AR-$G_{s_\alpha L}$ and β_2AR-$G_{s_\alpha L}$ differ from each other in their specific efficacies at activating AC. Our data could explain some of the discrepancies in the literature regarding similarities/dissimilarities in ternary complex formation, constitutive activity, and AC activation between the β_1AR and the β_2AR.

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