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Co-expression of the β_2-adrenoceptor and dopamine D_1-receptor with $G_{s\alpha}$ proteins in Sf9 insect cells: limitations in comparison with fusion proteins

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Abstract

The G-protein $G_{s\alpha}$ exists in three isoforms, the $G_{s\alpha}$ splice variants $G_{s\alpha short}$ ($G_{s\alpha S}$) and $G_{s\alpha long}$ ($G_{s\alpha L}$), and the G-protein $G_{\alpha olf}$ that is not only involved in olfactory signaling but also in extrapyramidal motor regulation. Studies with β_2-adrenoceptor (β_2AR)-$G_{s\alpha}$ fusion proteins showed that $G_{s\alpha}$ proteins activate adenylyl cyclase (AC) in the order of efficacy $G_{s\alpha S} > G_{s\alpha L} \sim G_{\alpha olf}$ and that $G_{s\alpha}$ proteins confer the hallmarks of constitutive activity to the β_2AR in the order of efficacy $G_{s\alpha L} > G_{\alpha olf} > G_{s\alpha S}$. However, it is unclear whether such differences between $G_{s\alpha}$ proteins also exist in the nonfused state. In the present study, we co-expressed the β_2AR and dopamine D_1-receptor (D_1R) with $G_{s\alpha}$ proteins at different ratios in Sf9 insect cells. In agreement with the fusion protein studies, nonfused $G_{\alpha olf}$ was less efficient than nonfused $G_{s\alpha S}$ and $G_{s\alpha L}$ at activating AC, but otherwise, we did not observe differences between the three $G_{s\alpha}$ isoforms. Thus, it is much easier to dissect differences between $G_{s\alpha}$ isoforms using β_2AR-$G_{s\alpha}$ fusion proteins than nonfused $G_{s\alpha}$ isoforms.

Keywords: Adenylyl cyclase; β_2-adrenoceptor; Dopamine D_1-receptor; $G_{s\alpha}$ splice variant; $G_{\alpha olf}$; Sf9 insect cell

1. Introduction

G-proteins are heterotrimeric membrane-bound proteins that mediate signal transfer from receptors to cellular effectors [1–3]. Upon binding of agonist to receptors, receptors undergo a conformational change, allowing them to promote the dissociation of GDP from G_α. GDP dissociation from G_α is the rate-limiting step of the G-protein cycle. Agonist-occupied receptors then form a ternary complex with the nucleotide-free G-protein that exhibits high agonist-affinity. Subsequent GTP binding to G_α disrupts the ternary complex and the G-protein dissociates into $G_{\alpha\text{-}GTP}$ and the $\beta\gamma$-complex. Both $G_{\alpha\text{-}GTP}$ and $\beta\gamma$ can regulate the activity of effector systems. G-protein deactivation is accomplished by the GTPase of G_α, cleaving GTP into GDP and P_i. Subsequently, $G_{\alpha\text{-}GDP}$ and the $\beta\gamma$-complex reassociate, closing the G-protein cycle. Commonly employed methods for the analysis of the G-protein cycle are the measurements of (i) ternary complex formation, (ii) binding of the GTPase-resistant GTP analog GTPγS, (iii) effector activity and (iv) steady-state GTP hydrolysis.

The G-protein $G_{s\alpha}$ mediates signal transfer from numerous receptors including the β_2-adrenoceptor (β_2AR) and dopamine D_1-receptor (D_1R) to adenylyl cyclase (AC) [1–3]. There are three $G_{s\alpha}$ isoforms: $G_{\alpha olf}$ and the $G_{s\alpha}$ splice variants $G_{s\alpha S}$ and $G_{s\alpha L}$ [4–8]. $G_{s\alpha}$ proteins are differentially expressed in various organs including heart, liver and brain, and $G_{s\alpha}$ isoform expression changes during development and aging [9–19]. Additionally, $G_{s\alpha}$ expression changes in various pathological conditions [20–25]. Moreover, the phenotype of the $G_{\alpha olf}$ knockout mouse points to a specific role of $G_{\alpha olf}$ in olfactory signal transduction and extrapyramidal motor regulation [26]. Collectively, all these data

Abbreviations: AC, adenylyl cyclase; β_2AR, β_2-adrenoceptor; β_2AR-$G_{\alpha olf}$, fusion protein consisting of the β_2-adrenoceptor and the G-protein $G_{\alpha olf}$; β_2AR-$G_{s\alpha S}$, fusion protein consisting of the β_2-adrenoceptor and the short splice variant of $G_{s\alpha}$; β_2AR-$G_{s\alpha L}$, fusion protein consisting of the β_2-adrenoceptor and the long splice variant of $G_{s\alpha}$; D_1R, dopamine D_1-receptor; $G_{\alpha olf}$, a $G_{s\alpha}$ protein that is not only involved in olfactory signal transduction but also in extrapyramidal motor regulation; $G_{s\alpha S}$, short splice variant of $G_{s\alpha}$; $G_{s\alpha L}$, long splice variant of $G_{s\alpha}$; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ICI 118,551, [*erythro*-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol]; SCH 23390, 7-chloro-8-hydroxy-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1 *H*-3-benzazepine; SP followed by a three-digit number, a specific Sf9 cell membrane preparation of our consecutively labeled membrane inventory

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point to different physiological and pathophysiological roles of the individual $G_{s\alpha}$ proteins.

However, it has been difficult to answer the question whether biochemical differences between the three $G_{s\alpha}$ proteins exist. Subtle differences in the interaction of the β_2AR with $G_{s\alpha}$ splice variants were observed in some but not all studies [4,27–29]. Similar inconsistencies were reported with respect to the efficacy of $G_{s\alpha}$ splice variants at activating AC [27–30]. A problem in some of the previous studies using mammalian expression systems was to achieve comparable expression levels of the $G_{s\alpha}$ proteins and receptor/$G_{s\alpha}$ stoichiometries [28,29].

In recent studies, we took advantage of the receptor-G_α fusion protein technique to analyze the biochemical properties of $G_{s\alpha S}$, $G_{s\alpha L}$ and $G_{\alpha olf}$. In fusion proteins, the receptor C-terminus is linked to the G_α N-terminus [31,32]. Compared to conventional co-expression systems, signaling efficiency is enhanced in fusion proteins [31,32]. Presumably, the enhanced signaling efficiency in fusion proteins is due to tight tethering of $G_{s\alpha}$ to the plasma membrane [33]. The enhanced signaling efficiency facilitated comparison of receptor-coupling to various G_α proteins [34–36]. When expressed at comparable levels in Sf9 insect cells, β_2AR-$G_{s\alpha}$ fusion proteins activate AC in the order of efficacy β_2AR-$G_{s\alpha S}$>β_2AR-$G_{s\alpha L}$ ~ β_2AR-$G_{\alpha olf}$ [34,37–39]. Moreover, $G_{s\alpha}$ proteins confer to the β_2AR the hallmarks of constitutive (agonist-independent) activity in the order of efficacy $G_{s\alpha L}$>$G_{\alpha olf}$>$G_{s\alpha S}$ [34,37]. However, fusion proteins do not occur naturally, and one may question the physiological relevance of the results obtained with fusion proteins [31,32,40].

The critique regarding the fusion protein technique prompted us to reexamine the question whether biochemical differences between the three $G_{s\alpha}$ proteins in the nonfused state can be unmasked. To address the question we analyzed ternary complex formation, GTPγS binding, GTP hydrolysis and AC activity in Sf9 insect cell membranes co-expressing the β_2AR with $G_{s\alpha S}$, $G_{s\alpha L}$ and $G_{\alpha olf}$ at different G-protein/receptor ratios and compared the data with those obtained for β_2AR-$G_{s\alpha}$ fusion proteins. We also included the D_1R into our present study since this receptor may specifically couple to $G_{\alpha olf}$ in the basal ganglia of the brain [25,41].

2. Materials and methods

2.1. Materials

Rat $G_{\alpha olf}$ cDNA in pBluescript KS [8] was kindly provided by Dr. R.R. Reed (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD, USA). Baculovirus encoding the human D_1R was kindly provided by Dr. M. Bouvier (Department of Biochemistry, University of Montreal, Quebec, Canada). Baculovirus encoding $G_{s\alpha S}$ was provided by Dr. A.G. Gilman (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, USA). [α-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]GTP (6000 Ci/mmol), [^{35}S]GTPγS (1100 Ci/mmol), [^{3}H]dihydroalprenolol (110 Ci/mmol) and [^{3}H]SCH 23390 (75 Ci/mmol) were from Perkin Elmer (Boston, MA, USA). Adenylyl imididiphosphate, GDP, GTP, GTPγS and ATP (special quality, catalogue #519,979; <0.01% (wt/wt) GTP contamination as assessed by HPLC analysis) were obtained from Roche Diagnostics (Indianapolis, IN, USA). ICI 118,551 was from RBI (Natick, MA, USA). Chlorpromazine, dopamine, (−)-isoproterenol and (±)-alprenolol were from Sigma (St. Louis, MO, USA). All restriction enzymes, DNA polymerase Klenov fragment A and T4 DNA ligase were from New England Biolabs (Beverly, MA, USA). Glass fiber filters (GF/C) were from Schleicher and Schuell (Dassel, Germany). The anti-$G_{s\alpha}$ Ig (C-terminal) was from Calbiochem (La Jolla, CA, USA). The anti-$G_{s\alpha}$ Ig against an epitope in the α-helical domain of $G_{s\alpha S/L}$ (K-20) and the anti-$G_{\alpha olf}$ Ig against an epitope in the α-helical domain of $G_{\alpha olf}$ (K-19) [8] were from Santa Cruz Biochemicals (Santa Cruz, CA, USA).

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

The generation of baculoviruses encoding β_2AR, $G_{s\alpha L}$, β_2AR-$G_{s\alpha L}$, β_2AR-$G_{s\alpha S}$ and β_2AR-$G_{\alpha olf}$ was described previously [34,37,42]. For generation of a baculovirus encoding $G_{\alpha olf}$, pBluescript KS-$G_{\alpha olf}$ was digested with *Eco*RI and *Bsr*GI, and the overlapping fragments were filled with DNA polymerase Klenov fragment A. This DNA fragment was then digested with *Eco*NI and cloned into the baculovirus expression vector pVL1392-β_2AR-$G_{i\alpha 2}$ that had been opened with *Sac*I, filled with DNA polymerase Klenov fragment A and further digested with *Eco*NI. Recombinant baculoviruses were generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen) according to the manufacturer's instructions. Sf9 cells were cultured in 250-ml disposable Erlenmeyer flasks at 28 °C under rotation at 125 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% (vol/vol) fetal calf serum (Bio Whittaker, Walkersville, MD, USA) and 0.1 mg/ml gentamicin (Bio Whittaker). Cells were maintained at a density of $0.5–6.0 \times 10^6$ cells/ml. After initial transfection, virus work 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 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 °C. 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 h, and the supernatant fluid was harvested. After the 48-h 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 °C and was the routine virus stock for membrane preparations. For infection, cells were sedimented by centrifugation and resuspended in fresh medium. Cells were seeded at 3.0×10^6 cells/ml and infected with 1:100, 1:1000 or 1:10,000 dilutions of baculovirus work stocks encoding β_2AR, D_1R, β_2AR-$G_{s\alpha}$ fusion proteins or $G_{s\alpha}$ proteins. Sf9 cells were cultured for 48 h before membrane preparation. Sf9 membranes were prepared as described [43]. Cells were disrupted with 25 strokes in a Dounce homogenizer in a buffer consisting of 10 mM Tris/HCl, pH 7.4 and 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine and 10 μg/ml leupeptin as protease inhibitors. Nuclei and unbroken cells were removed by centrifugation for 5 min at 500 × *g* at 4 °C. The supernatant fluid was centrifuged for 30 min at 40,000 × *g* 4 °C. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Membranes were stored at − 80 °C.

2.3. [^{3}H]dihydroalprenolol and [^{3}H]SCH 23390 binding assays

Membranes were thawed and sedimented by a 12-min centrifugation at 4 °C and 15,000 × *g* to remove residual endogenous guanine nucleotides as far as possible and resuspended in binding buffer. Expression levels of β_2AR and β_2AR-$G_{s\alpha}$ fusion proteins were determined by incubating Sf9 membranes (10–30 μg protein/tube) in the presence of 10 nM [^{3}H]dihydroalprenolol and 0.05% (wt/vol) bovine serum albumin. Nonspecific binding was determined in the presence of 10 nM [^{3}H]dihydroalprenolol plus 10 μM (±)-alprenolol. The total volume of binding reactions was 500 μl. Incubations were conducted for 90 min at 25 °C and shaking at 250 rpm. For determination of the agonist-affinities of the β_2AR, reaction mixtures contained Sf9 membranes (15–30 μg protein/tube), 1 nM [^{3}H]dihydroalprenolol and (−)-isoproterenol at various concentrations in the absence or presence of GTPγS (10 μM). For determination of the K_d and B_{max} values of [^{3}H]SCH 23390 binding, reaction mixtures contained Sf9 membranes (2.5–20 μg protein/tube), 0.1–10 nM [^{3}H]SCH 23390 and 0.05% (wt/vol) bovine serum albumin. Nonspecific binding was determined in the presence of 0.1–10 nM [^{3}H]SCH 23390 plus 1 mM dopamine. For determination of the agonist-affinities of the D_1R, reaction mixtures contained Sf9 membranes (15–30 μg protein/tube), 1 nM [^{3}H]SCH 23390 and dopamine at various concentrations in the absence or presence of GTPγS (10 μM). Bound radioligand was separated from free radioligand by filtration through GF/C filters using a 48-well harvester (model M-48R, Brandel, Gaithersburg, MD, USA), followed by three washes with 2 ml of binding buffer (4 °C). Filter-bound radioactivity was determined by liquid scintillation counting using Cytoscint cocktail from ICN (Irvine, CA, USA).

2.4. [^{35}S]GTPγs binding assay

[^{35}S]GTPγS binding was determined as described [36,43]. Briefly, membranes were thawed and sedimented by a 12-min centrifugation at 4 °C and 15,000 × *g* to remove residual endogenous guanine nucleotides as far as possible and resuspended in binding buffer. Reaction mixtures (total volume 500 μl) contained Sf9 membranes (30–50 μg protein/tube) in binding buffer supplemented with 0.05% (wt/vol) BSA, 1.0 nM [^{35}S]GTPγS, 9 nM unlabeled GTPγS (to give a final GTPγS concentration of 10 nM) and 1 μM GDP. Previous studies showed that these conditions are appropriate to determine [^{35}S]GTPγS saturation binding to $G_{s\alpha}$ proteins in Sf9 membranes [36,37]. Reaction mixtures additionally contained solvent (basal), inverse agonist or agonist. Incubations were conducted for 90 min at 25 °C and shaking at 250 rpm. 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 °C). Filter-bound radioactivity was determined by liquid scintillation counting. Nonspecific [^{35}S]GTPγS binding was determined in the presence of 10 μM unlabeled GTPγS.

2.5. Steady-state GTPase activity assay

GTPase activity was determined as described [37]. Briefly, membranes were thawed, sedimented by a 12-min centrifugation at 4 °C 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 (10 μg protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase and 0.2% (wt/vol) BSA in 50 mM Tris/HCl, pH 7.4. Reaction mixtures additionally contained solvent (basal) or the appropriate agonists at a maximally effective concentration. Reaction mixtures (80 μl) were incubated for 3 min at 25 °C before the addition of 20 μl of [γ-^{32}P]GTP (0.2–0.5 μCi/tube). All stock and work dilutions of [γ-^{32}P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min at 25 °C. Reactions were terminated by the addition of 900 μl of slurry consisting of 5% (wt/vol) 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, and $^{32}P_i$ was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation was determined in tubes containing all of the above described components plus a very high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{32}P]GTP, prevents [γ-^{32}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP degradation was < 1% of the total amount

of radioactivity added using 20 mM Tris/HCl, pH 7.4, as solvent for [γ-^{32}P]GTP. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$.

2.6. AC activity assay

Membranes were thawed and sedimented by a 12-min centrifugation at 4 °C and 15,000 × *g* to remove residual endogenous guanine nucleotides as far as possible and resuspended in binding buffer. Tubes contained Sf9 membranes (15–50 μg protein/tube) expressing various proteins, 5 mM $MgCl_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4 and GTPγS or GTP at various concentrations in the absence or presence of receptor ligands. Assay tubes containing membranes and various additions in a total volume of 30 μl were incubated for 3 min at 37 °C before initiating 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, 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU pyruvate kinase, 1 IU myokinase and 0.1 mM cAMP. Reactions were conducted for 20 min at 37 °C. Reactions were terminated by the addition of 20 μl of 2.2 N HCl. Denatured protein was sedimented by a 3-min centrifugation at 25 °C and 15,000 × *g*. Sixty-five microliters of the supernatant fluid was applied onto 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 [44]. Recovery of [^{32}P]cAMP was ~ 80%. Blank values were ~ 0.01% of the total amount of [α-^{32}P]ATP added. [^{32}P]cAMP was determined by liquid scintillation counting.

2.7. SDS polyacrylamide gel electrophoresis and immunoblot analysis

Membrane proteins were separated on SDS polyacrylamide gels containing 10% (wt/vol) acrylamide [37]. Proteins were then transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA) according to the manufacturer's instructions. Membranes were reacted with anti-$G_{s\alpha}$ Ig (C-terminal) (1:1000) or with K-19 Ig or K-20 Ig (1:100 each). Immunoreactive bands were visualized with anti-rabbit IgG coupled to peroxidase, using *o*-dianisidine and H_2O_2 as substrates. For quantification of $G_{s\alpha}$ proteins, we used Sf9 membranes (25, 50, 75 and 100 μg protein/lane) expressing the appropriate β_2AR-$G_{s\alpha}$ fusion protein at a defined level ({^{3}H}dihydroalprenolol saturation binding) as standards [35,43]. For $G_{s\alpha}$-expressing membranes, 25–75 μg of protein was loaded per gel. $G_{\alpha olf}$ was quantified using the K-19 Ig. $G_{s\alpha S}$ and $G_{s\alpha L}$ were quantified using the K-20 Ig. Immunoblots were scanned using a Molecular Imager FX and evaluated with the Quantity One image analysis software (version 4.3) (Bio-Rad, Hercules, CA, USA).

2.8. Miscellaneous

Protein was determined using the Bio-Rad DC protein assay kit. Data shown in Figs. 2–5 were analyzed by nonlinear regression using the Prism III program (GraphPad, Prism, San Diego, CA, USA).

3. Results

3.1. Expression of $G_{s\alpha}$ proteins, D_1R and β_2AR in Sf9 membranes

Sf9 cells were infected with baculoviruses encoding various β_2AR-$G_{s\alpha}$ fusion proteins and nonfused $G_{s\alpha}$ proteins, and the expression of proteins was analyzed by immunoblotting. When expressed in mammalian cells, $G_{s\alpha S}$ and $G_{s\alpha L}$ exhibit apparent molecular masses of 45 and 52 kDa, respectively [28–30]. In agreement with those data, the anti-$G_{s\alpha}$ Ig (C-terminal) (Fig. 1A) and K-20 Ig (Fig. 1C) recognized 45- and 52-kDa proteins in Sf9 membranes expressing $G_{s\alpha S}$ and $G_{s\alpha L}$, respectively. In membranes expressing $G_{s\alpha L}$, the anti-$G_{s\alpha}$ Ig and K-20 Ig recognized a proteolytic fragment of ~ 40 kDa that was also observed in another study [45]. The C terminus of $G_{\alpha olf}$ differs from the C terminus of $G_{s\alpha S/L}$ only in one amino acid [8]. Therefore, it was not surprising that the anti-$G_{s\alpha}$ Ig against the C terminus of $G_{s\alpha S/L}$ also recognized $G_{\alpha olf}$ (Fig. 1A). In agreement with a previous expression study using S49 *cyc*$^-$ lymphoma cells [28], $G_{\alpha olf}$ expressed in Sf9 membranes exhibited a slightly higher molecular mass than $G_{s\alpha S}$. $G_{s\alpha S/L}$ and $G_{\alpha olf}$ differ significantly from each other in the α-helical domain [8]. Accordingly, this domain is feasible for the generation of $G_{s\alpha}$ subtype-specific antibodies [8]. Indeed, the K-19 Ig against a specific epitope of the α-helical domain of $G_{\alpha olf}$ strongly reacted with $G_{\alpha olf}$ and the β_2AR-$G_{\alpha olf}$ fusion protein expressed in Sf9 membranes but not with $G_{s\alpha}$ splice variants or the β_2AR-$G_{s\alpha L}$ fusion protein (Fig. 1B). In contrast, the $G_{s\alpha S/L}$-specific K-20 Ig did not detect $G_{\alpha olf}$ and the β_2AR-$G_{\alpha olf}$ fusion protein (Fig. 1C).

For quantification of nonfused $G_{s\alpha}$ proteins in immunoblotting, we used β_2AR-$G_{s\alpha}$ fusion proteins expressed at a defined level ([^{3}H]dihydroalprenolol saturation binding) as standard. Fig. 1A and C illustrates a typical problem of protein expression studies with Sf9 cells. Specifically, in the initial phase of the project, we used identical dilutions (1:100) of virus work stocks encoding $G_{s\alpha}$ proteins. It emerged that $G_{s\alpha S}$ was expressed at lower levels than $G_{s\alpha L}$ and $G_{\alpha olf}$. Attempts to achieve identical $G_{s\alpha}$ expression levels by titration of virus stocks were only partially successful (Tables 1–4).

[^{3}H]Dihydroalprenolol binding to the human β_2AR expressed in Sf9 membranes was characterized in a previous study [43]. The human D_1R expressed in Sf9 membranes bound the antagonist radioligand [^{3}H]SCH 23390 with a K_d

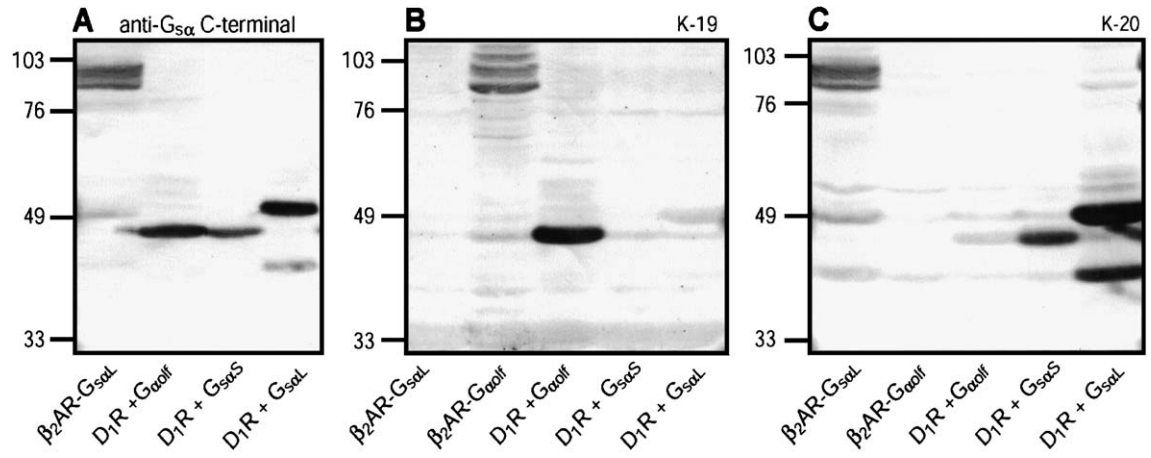

Fig. 1. Analysis of the expression of $G_{s\alpha}$-proteins in Sf9 membranes. Sf9 cells were infected with the baculoviruses indicated below panels A–C, and incubated for 48 h before membrane preparation. Sf9 cell membranes (50 μg of protein/lane) were separated on SDS gels containing 10% (wt/vol) acrylamide as described in Materials and methods. Proteins were transferred onto Immobilon-P transfer membranes and probed with anti-$G_{s\alpha}$ Ig (C-terminal (A), K-19 Ig (anti-$G_{\alpha olf}$) (B) or K-20 Ig (anti-$G_{s\alpha}$) (C). The expression levels of fusion proteins were assessed by [^{3}H]dihydroalprenolol saturation binding (β_2AR-$G_{s\alpha L}$, 7.0 pmol/mg; β_2AR-$G_{\alpha olf}$, 19.5 pmol/mg). Shown are the horseradish peroxidase-reacted Immobilon-P transfer membranes. Numbers on the left indicate molecular masses of marker proteins.

value of 0.92 ± 0.15 nM (mean ± S.D. of three experiments performed in triplicates). This K_d value fits well to the K_d value for the D_1R expressed in rat brain (0.7 nM) [46]. Depending on the baculovirus dilution used, the D_1R was expressed with B_{max} values of 1.5–24.8 pmol/mg (Tables 1–4 and Fig. 5). These expression levels are comparable with the expression levels obtained for the β_2AR (0.9–24.0 pmol/mg, Tables 1–4 and Fig. 5).

3.2. Agonist binding properties of the D_1R and β_2AR

We studied ternary complex formation with the D_1R and β_2AR indirectly by competing radioligand antagonist binding with unlabeled agonist in the absence and presence of GTPγS. Figs. 2 and 3 show representative agonist competition curves for the D_1R and β_2AR co-expressed with the three $G_{s\alpha}$ proteins at various G-protein/receptor ratios, and Table 1 provides a summary of the nonlinear regression analysis of the agonist competition curves. Table 1 also contains the nonlinear regression analysis for additional membrane preparations not shown in Figs. 2 and 3.

An increase in the G-protein/receptor ratio should facilitate detection of high-affinity agonist binding [47,48]. We varied the $G_{\alpha olf}$/receptor ratio between 0.3 and 5.9, the $G_{s\alpha S}$/receptor between 0.4 and 2.1 and the $G_{s\alpha L}$/receptor ratio between 0.7 and 11. In a previous study we reported that a $G_{s\alpha L}/\beta_2$AR ratio of ~100 allowed for the detection of high-affinity agonist binding [43]. The data of our present study show that a $G_{s\alpha L}/\beta_2$AR ratio of ~10 is sufficient to detect ternary complex formation. However, the data for the β_2AR/$G_{s\alpha L}$ couple cannot be extrapolated to other receptor/$G_{s\alpha}$ couples. Specifically, ternary complex formation was detected in the membrane with the lowest $G_{s\alpha L}/D_1$R ratio (SP366, Fig. 2F), but not in the membrane with the highest $G_{s\alpha L}/D_1$R ratio (SP386, Fig. 2E). With respect to $G_{s\alpha S}$, in one membrane preparation with a G-protein/D_1R ratio of 0.7 (SP474), no ternary complex formation was detected, whereas in another preparation with the same G-protein/receptor ratio (SP339), high-affinity binding was clearly detected. Additionally, with several receptor/G-protein pairs, an increase in the G-protein/receptor ratio by a factor of ~2 did not result in an equivalent increase in high-affinity agonist binding (D_1R plus $G_{\alpha olf}$, compare SP378 with SP384; β_2AR plus $G_{s\alpha S}$, compare SP161 with SP410). Moreover, even at $G_{s\alpha S}/\beta_2$AR ratio as low as 0.4 (SP161), ternary complex formation was detected. Taken together, our data show that all $G_{s\alpha}$ proteins are capable of stabilizing the ternary complex with the D_1R and β_2AR, indicating that at least a fraction of the expressed $G_{s\alpha}$ proteins is functional. However, there is no correlation between G-protein/receptor ratio and ternary complex formation.

3.3. GTPγs binding studies

The measurement of ligand-regulated GTPγS saturation binding provides a method to determine the number of G-proteins activated by a given receptor [49,50]. As predicted by the receptor/G_α stoichiometry, 1 mol of β_2AR-$G_{s\alpha}$ fusion protein binds ~1 mol of GTPγS [36,37]. A previous study suggested that in nonfused systems, signal transfer between receptors and G-proteins is catalytic, i.e. one receptor molecule activates multiple G-proteins [49], but subsequent studies rather suggested that signal transfer is linear or even sublinear [35,36].

When using Sf9 cells as expression proteins for mammalian $G_{s\alpha}$ proteins, one has to keep in mind that the D_1R and β_2AR can also couple to the $G_{s\alpha}$-like proteins of the insect cells, providing the background signaling activity [42,51,52]. In membranes expressing the β_2AR alone (SP46), the ligand-regulated increase in GTPγS binding

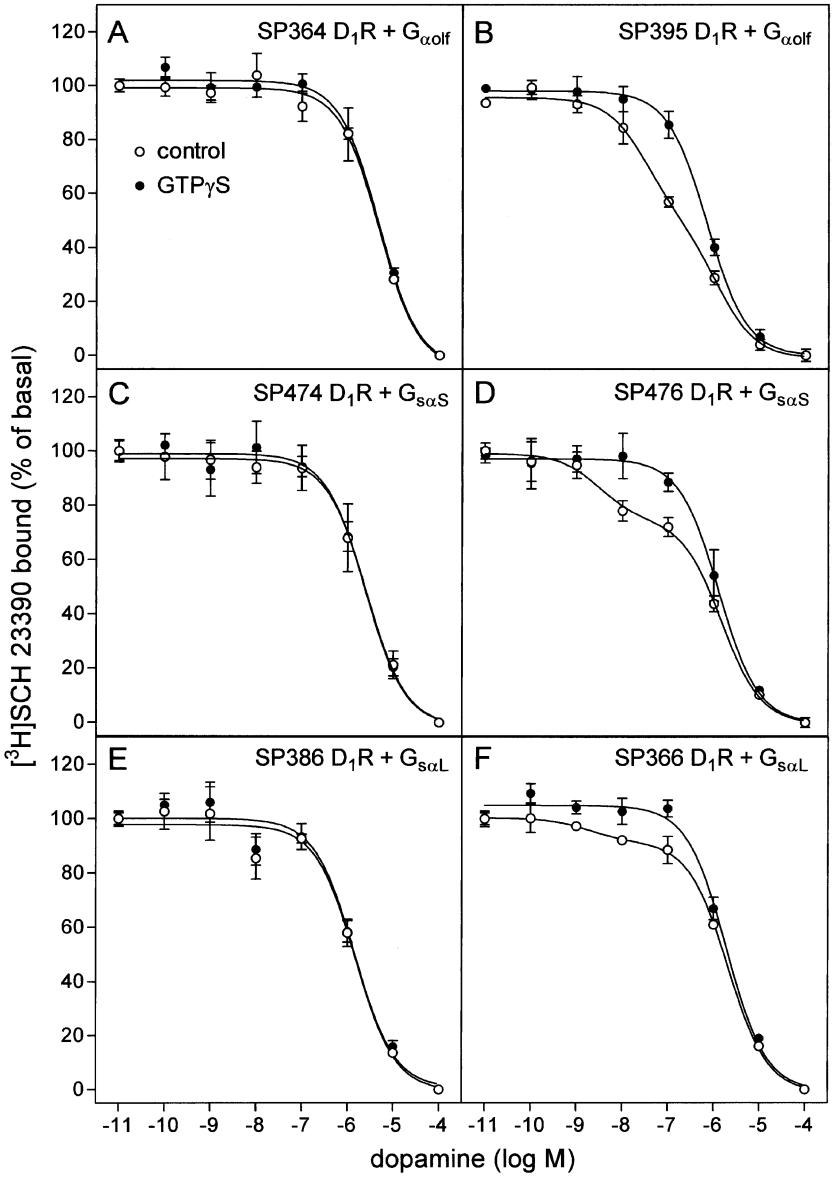

Fig. 2. Competition of [^{3}H]SCH 23390 binding by dopamine in Sf9 membranes expressing the D_1R and various $G_{s\alpha}$ proteins. [^{3}H]SCH 23390 binding in Sf9 membranes was determined as described in Materials and methods. Reaction mixtures contained membranes expressing the proteins indicated in panels A–F, 1 nM [^{3}H]SCH 23390 and dopamine at increasing concentrations. Tubes additionally contained solvent (control) (○) or GTPγS (10 μM) (●). Data points are the means ± S.D. of three to four experiments performed in triplicates. Data were analyzed for best fit to monophasic or biphasic competition isotherms. The results of the nonlinear regression analysis and the receptor- and $G_{s\alpha}$ expression levels of the various membrane preparations are summarized in Table 1. " − 11" designates the absence of dopamine.

amounted to 0.17 pmol/mg of membrane protein, corresponding to 0.015 activated G-protein molecules per β_2AR molecule (Table 2). Thus, only a very small fraction of the expressed β_2AR molecules actually couples to the $G_{s\alpha}$-like proteins of the insect cells. The latter number is also referred to as coupling factor [35,36]. In membranes expressing the D_1R and β_2AR with $G_{s\alpha}$ proteins, ligand-regulated GTPγS binding did not generally exceed the values observed for membranes expressing receptor alone (Table 2). In no case, ligand-regulated GTPγS binding in the co-expression membranes approached the values observed for β_2AR-$G_{s\alpha}$ fusion proteins, i.e. the coupling factors reached ~ 0.24 at best. When expressed as the percentage of $G_{s\alpha}$ proteins activated, the highest values were generally observed for $G_{s\alpha S}$ (up to 11%). Thus, only a small fraction of the expressed $G_{s\alpha}$ proteins participated in receptor-regulated GDP/GTPγS exchange.

3.4. GTPase studies

The steady-state GTPase assay measures the outcome of multiple G-protein cycles and can be readily applied to Sf9 membranes since this system possesses a very low basal GTPase activity, providing an excellent signal-to noise ratio

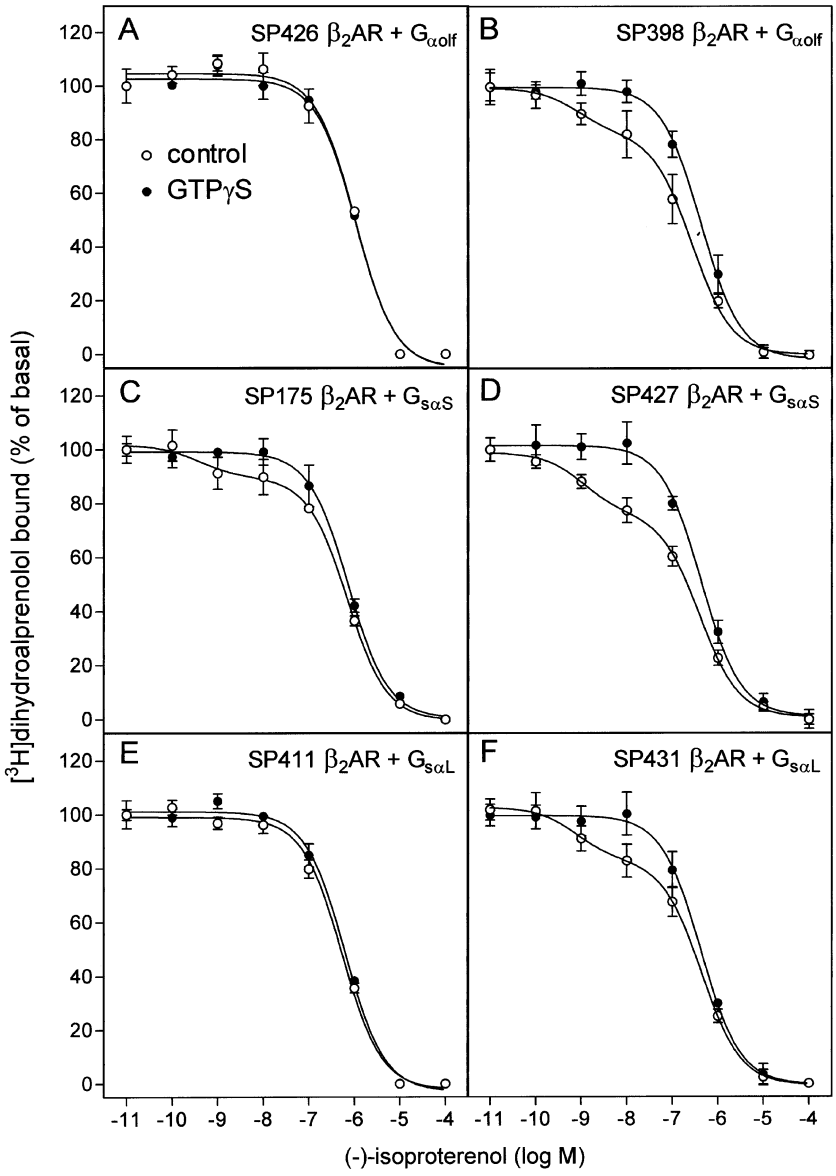

Fig. 3. Competition of [^{3}H]dihydroalprenolol binding by (−)-isoproterenol in Sf9 membranes expressing the β_2AR and various $G_{s\alpha}$ proteins. [^{3}H]Dihydroalprenolol binding in Sf9 membranes was determined as described in Materials and methods. Reaction mixtures contained membranes expressing the proteins indicated in panels A–F, 1 nM [^{3}H]dihydroalprenolol and (−)-isoproterenol at increasing concentrations. Tubes additionally contained solvent (control) (○) or GTPγS (10 μM) (●). Data points are the means ± S.D. of three to four experiments performed in triplicates. Data were analyzed for best fit to monophasic or biphasic competition isotherms. The results of the nonlinear regression analysis and the receptor- and $G_{s\alpha}$ expression levels of the various membrane preparations are summarized in Table 1. "−11" designates the absence of (−)-isoproterenol.

[43,53]. In membranes expressing the β_2AR alone (SP48), (−)-isoproterenol increased GTP hydrolysis by 0.30 pmol/mg/min (Table 3). In Sf9 membranes expressing the D_1R or β_2AR plus $G_{s\alpha}$ proteins, the absolute increases in agonist-stimulated GTP hydrolysis were not consistently larger than in membranes expressing receptor alone.

3.5. Analysis of AC activity in the absence of receptors

In membranes from uninfected Sf9 cells, GTPγS stimulated AC with an EC_{50} of ~40 nM (95% confidence interval, 17–112 nM) and a maximum activity of 5.9 pmol/mg/min (95% confidence interval, 5.2–6.7 pmol/mg/min) (Fig. 4A). This AC activation represents the background mediated by the endogenous $G_{s\alpha}$-like proteins of Sf9 cells. In membranes expressing $G_{\alpha olf}$, GTPγS activated AC with similar potency (EC_{50}, 36 nM; 95% confidence interval, 20–62 nM) as in membranes from uninfected Sf9 cells, but the maximum AC activity achieved with $G_{\alpha olf}$ was only moderately higher (10.4 pmol/mg/min; 95% confidence interval, 9.6–11.1 pmol/mg/min) than in membranes from uninfected cells (Fig. 4B). In membranes expressing $G_{s\alpha S}$ or

Table 1
Nonlinear regression analysis of the agonist binding properties of the D_1R and β_2AR in Sf9 membranes co-expressed with various $G_{s\alpha}$ proteins at different G-protein/receptor ratios

Constructs (receptor level in pmol/mg)	$G_{s\alpha}$ (pmol/mg)	G/R ratio	Membrane preparation	K_h (nM)	K_l (μM)	R_h (%)	$K_{GTP\gamma S}$ (μM)
$D_1R + G_{\alpha olf}$							
19.9	7.5	0.3	SP364	–	2.1 (1.4–3.2)	–	2.0 (1.4–3.0)
7.8	8.1	1.0	SP378	27 (5.0–150)	1.5 (1.1–2.0)	12 (2–25)	1.1 (0.9–1.4)
4.9	10.5	2.1	SP384	0.2 (0.01–5.6)	0.6 (0.5–0.7)	6 (2–11)	0.6 (0.5–0.8)
1.5	8.9	5.9	SP395	14 (7.0–27)	0.5 (0.3–1.0)	47 (33–60)	0.3 (0.3–0.4)
$D_1R + G_{s\alpha S}$							
13.5	8.8	0.7	SP474	–	1.1 (0.8–1.6)	–	1.1 (0.7–1.7)
11.8	10.5	0.9	SP475	3.0 (0.3–19)	0.7 (0.5–1.1)	16 (9–24)	0.5 (0.3–0.8)
6.3	11.9	1.9	SP476	1.3 (0.2–8.8)	0.7 (0.4–1.2)	25 (20–29)	0.6 (0.5–0.7)
5.7	4.0	0.7	SP339	0.4 (0.1–4.5)	0.6 (0.4–0.8)	15 (10–20)	0.6 (0.5–0.8)
$D_1R + G_{s\alpha L}$							
10.9	10.7	1.0	SP366	0.8 (0.1–4.8)	0.9 (0.8–1.1)	9 (5–12)	0.9 (0.7–1.1)
3.8	11.5	3.0	SP380	–	0.5 (0.3–0.7)	–	0.7 (0.5–0.9)
2.2	11.5	5.2	SP386	–	0.7 (0.4–1.0)	–	0.6 (0.4–0.9)
$\beta_2AR + G_{\alpha olf}$							
24.0	6.1	0.3	SP426	–	0.3 (0.2–0.4)	–	0.3 (0.2–0.3)
17.5	6.5	0.4	SP367	–	0.3 (0.2–0.4)	–	0.4 (0.3–0.5)
17.1	6.8	0.4	SP409	–	0.3 (0.2–0.4)	–	0.2 (0.2–0.3)
1.7	8.2	4.8	SP398	0.4 (0.1–2.3)	0.1 (0.1–0.2)	17 (10–24)	0.2 (0.2–0.2)
$\beta_2AR + G_{s\alpha S}$							
12.1	5.0	0.4	SP161	3.8 (1.2–12)	0.2 (0.2–0.3)	13 (8–19)	0.2 (0.2–0.2)
9.5	6.4	0.7	SP175	0.1 (0.01–1.0)	0.2 (0.1–0.2)	12 (7–17)	0.2 (0.2–0.2)
8.7	6.5	0.8	SP410	0.1 (0.04–0.4)	0.2 (0.2–0.3)	14 (11–17)	0.3 (0.2–0.3)
1.1	2.3	2.1	SP427	0.5 (0.2–1.0)	0.2 (0.1–0.2)	23 (19–27)	0.2 (0.2–0.2)
$\beta_2AR + G_{s\alpha L}$							
14.6	9.7	0.7	SP411	–	0.2 (0.1–0.2)	–	0.2 (0.1–0.2)
13.8	10.7	0.8	SP369	–	0.3 (0.3–0.4)	–	0.4 (0.3–0.4)
12.8	10.8	0.8	SP428	–	0.1 (0.1–0.1)	–	0.2 (0.1–0.2)
0.9	9.7	11	SP431	0.3 (0.1–1.0)	0.2 (0.1–0.3)	19 (15–24)	0.2 (0.2–0.2)

Agonist binding was determined as described in Materials and methods. The data shown in Figs. 2 and 3 were analyzed by nonlinear regression for best fit to monophasic or biphasic competition isotherms (one-site or two-site competition). The table also contains the nonlinear regression analysis of additional membrane preparations not shown in Figs. 2 and 3. G-protein/receptor ratios (G/R ratios) were calculated by dividing the $G_{s\alpha}$ expression level by the receptor expression level. Data shown are the means from three to four independent experiments performed in triplicates. Numbers in parentheses represent the 95% confidence intervals. K_h and K_l designate the dissociation constants for the high- and low-affinity states of the receptors, R_h (%) the percentage of high-affinity binding sites and $K_{GTP\gamma S}$ the dissociation constant for the low-affinity state of the D_1R and β_2AR in the presence of 10 μM GTPγS.

$G_{s\alpha L}$, the basal AC activity was already as high as the GTPγS-stimulated AC activity in membranes expressing $G_{\alpha olf}$ (Fig. 4B–C). These data support the view that even $G_{s\alpha S/L\text{-}GDP}$ is quite efficient at activating AC [54]. In membranes expressing $G_{s\alpha S}$ and $G_{s\alpha L}$, GTPγS activated AC with similar potency (EC_{50} $G_{s\alpha S}$, 16 nM; 95% confidence interval, 8–34 nM) (EC_{50} $G_{s\alpha L}$, 31 nM; 95% confidence interval, 27–34 nM) as in the other membranes. However, the maximum GTPγS-stimulated AC activities in membranes expressing $G_{s\alpha S}$ and $G_{s\alpha L}$ were ~3–3.5-fold higher than in membranes expressing $G_{\alpha olf}$. Taken together, these data indicate that $G_{\alpha olf}$ is considerably less efficient at activating AC than $G_{s\alpha S}$ and $G_{s\alpha L}$, i.e. $G_{\alpha olf}$ just exceeds the efficacy of the endogenous $G_{s\alpha}$-like proteins of the insect cells.

Numerous receptors exhibit constitutive activity [55,56]. Constitutive receptor activity increases GDP/GTP exchange at G-proteins in an agonist-independent manner. Accordingly, constitutive activity of G_s-coupled receptors results in strong stimulatory effects of GTP on basal AC activity [38,57–59]. However, GTP was essentially devoid of stimulatory effects on AC in membranes from uninfected cells and membranes from cells expressing mammalian $G_{s\alpha}$ proteins (Fig. 4). These data indicate that Sf9 cells do not endogenously express sufficiently high levels of constitutively active receptors that couple to $G_{s\alpha}$-like proteins or mammalian G_s-proteins. Thus, Sf9 membranes provide an excellent background for the analysis of constitutively active mammalian receptors.

3.6. Analysis of AC activity in the presence of receptors

Fig. 5 shows representative concentration/response curves for the stimulatory effects of GTP on AC activity in the absence and presence of agonist and inverse agonist in Sf9 membranes expressing the D_1R or β_2AR alone and together with mammalian $G_{s\alpha}$ proteins. Table 4 summarizes the AC activities in Sf9 membranes expressing the β_2AR and D_1R with $G_{s\alpha}$ splice variants at different $G_{s\alpha}$/receptor ratios (~0.4–2.0 for $G_{s\alpha S}$ and ~0.7–12 for $G_{s\alpha L}$). In

Table 2
GTPγS binding in Sf9 membranes expressing the D_1R and β_2AR, respectively, and various $G_{s\alpha}$ proteins at different G-protein/receptor ratios

Constructs (receptor level in pmol/mg)	$G_{s\alpha}$ (pmol/mg)	G/R ratio	Membrane preparation	GTPγS bound (basal) (pmol/mg)	GTPγS bound (+inv. ago.) (pmol/mg)	GTPγS bound (+ago.) (pmol/mg)	GTPγS bound (Δinv. ago.) (pmol/mg)	GTPγS bound (Δago.) (pmol/mg)	GTPγS bound (Δtotal) (pmol/mg)	Coupling factor	Receptor-activated $G_{s\alpha}$ (percent of total)
$D_1R + G_{\alpha olf}$											
4.9	10.5	2.1	SP384	0.40 ± 0.04	0.35 ± 0.03	0.47 ± 0.03	− 0.05	0.07	0.12	0.024	1.1
1.5	8.9	5.9	SP395	1.65 ± 0.08	1.78 ± 0.18	2.00 ± 0.19	0.13	0.35	0.35	0.233	3.9
$D_1R + G_{s\alpha S}$											
13.5	8.8	0.7	SP474	1.91 ± 0.14	1.56 ± 0.11	2.38 ± 0.13	− 0.35	0.47	0.82	0.061	9.3
11.8	10.5	0.9	SP475	1.74 ± 0.27	1.30 ± 0.18	1.97 ± 0.25	− 0.45	0.23	0.67	0.057	6.4
6.3	11.9	1.9	SP476	1.63 ± 0.21	1.50 ± 0.22	2.30 ± 0.28	− 0.13	0.68	0.81	0.129	6.8
$D_1R + G_{s\alpha L}$											
10.9	10.7	1.0	SP366	0.57 ± 0.22	0.61 ± 0.01	0.84 ± 0.06	− 0.04	0.27	0.27	0.025	2.5
$\beta_2AR - G_{\alpha olf}$											
19.8	3.0	0.2	SP429	1.41 ± 0.19	1.38 ± 0.10	1.54 ± 0.07	− 0.03	0.13	0.16	0.008	5.3
$\beta_2AR + G_{s\alpha S}$											
4.4	10.4	2.4	SP368	1.25 ± 0.09	1.25 ± 0.07	1.57 ± 0.10	0.00	0.32	0.32	0.073	3.1
12.1	5.0	0.4	SP161	1.51 ± 0.19	1.45 ± 0.08	1.93 ± 0.12	− 0.06	0.42	0.48	0.040	9.6
1.1	2.3	2.1	SP427	0.95 ± 0.19	0.91 ± 0.25	1.17 ± 0.28	− 0.04	0.22	0.26	0.236	11.3
$\beta_2AR + G_{s\alpha L}$											
13.8	10.7	0.8	SP369	1.22 ± 0.18	1.19 ± 0.04	1.45 ± 0.21	− 0.03	0.23	0.26	0.019	2.4
12.8	10.8	0.8	SP428	0.91 ± 0.06	0.82 ± 0.08	1.05 ± 0.12	− 0.09	0.14	0.23	0.018	2.1
0.9	9.7	11	SP431	0.50 ± 0.23	0.46 ± 0.06	0.64 ± 0.16	− 0.04	0.15	0.19	0.211	2.0
β_2AR											
12.2	n.a.	n.a.	SP46	0.78 ± 0.19	0.77 ± 0.10	0.93 ± 0.08	− 0.01	0.17	0.18	n.a.	n.a.

GTPγS binding in Sf9 membranes expressing various proteins was determined as described in Materials and methods. G-protein/receptor ratios (G/R ratios) were calculated by dividing the $G_{s\alpha}$ expression level by the receptor expression level. Reaction mixtures contained 1 nM [^{35}S]GTPγS, 9 nM unlabeled GTPγS and 1 μM GDP in the presence of solvent (basal), 1 μM chlorpromazine/1 μM ICI 118,551 (+inv. ago.) or 10 μM dopamine/10 μM (−)-isoproterenol (+ago.). Data shown are the means of three independent experiments performed in triplicates. The differences between basal GTPγS binding and GTPγS binding in the presence of inverse agonist (Δinv. ago.), the differences between basal GTPγS binding and GTPγS binding in the presence of agonist (Δago.) and the maximum receptor-regulated GTPγS binding (Δtotal) were calculated. The coupling factor was calculated by dividing the maximum receptor-regulated GTPγS binding by the receptor expression level. The percentage of receptor-activated G-proteins was calculated by dividing the maximum receptor-regulated GTPγS binding by the $G_{s\alpha}$ expression level. n.a., not applicable.

membranes expressing the D_1R or β_2AR alone, GTP had only a small stimulatory effect on basal AC activity (Fig. 5A and E). Accordingly, the effects of the inverse agonists were small. However, agonist substantially increased AC activity in membranes expressing D_1R and β_2AR, reflecting the coupling of those receptors to $G_{s\alpha}$-like proteins. In membranes co-expressing the D_1R or β_2AR with $G_{\alpha olf}$, the maximum agonist-stimulated AC activities did not exceed the AC activities in the presence of receptors alone (compare Fig. 5A with B and Fig. 5E with F). Similarly low AC activities were obtained when additional membranes co-expressing receptors and $G_{\alpha olf}$ (SP367, SP378, SP384, SP395, SP398 and SP426) were analyzed (data not shown). In contrast, the AC activities in membranes co-expressing receptors and $G_{s\alpha}$ splice variants exceeded the AC activities determined with receptors alone (Fig. 5 and Table 4). These

Table 3
GTPase activity in Sf9 membranes expressing the D_1R and β_2AR, respectively, and various $G_{s\alpha}$ proteins

Constructs	Receptor (pmol/mg)	$G_{s\alpha}$ (pmol/mg)	G/R ratio	Membrane preparation	GTPase activity (basal) (pmol/mg/min)	GTPase activity (+ago.) (pmol/mg/min)	Agonist-stimulation (percent of basal)	GTPase activity (Δago.) (pmol/mg/min)
$D_1R + G_{\alpha olf}$	19.9	7.5	0.3	SP364	0.21 ± 0.05	0.38 ± 04	182	0.17
$D_1R + G_{s\alpha S}$	4.2	10.2	2.4	SP365	0.52 ± 0.11	1.37 ± 0.21	164	0.85
$D_1R + G_{s\alpha L}$	10.9	10.7	1.0	SP366	0.65 ± 0.05	1.12 ± 0.09	173	0.47
$\beta_2AR + G_{\alpha olf}$	17.5	6.5	0.4	SP367	0.80 ± 0.10	1.10 ± 0.15	138	0.30
$\beta_2AR + G_{s\alpha S}$	4.4	10.4	2.4	SP368	0.79 ± 0.09	1.54 ± 0.10	194	0.75
$\beta_2AR + G_{s\alpha L}$	13.8	10.7	0.8	SP369	1.47 ± 0.09	2.01 ± 0.12	112	0.54
β_2AR	7.5	n.a.	n.a.	SP48	0.80 ± 0.08	1.10 ± 0.08	138	0.30

GTPase activity in Sf9 membranes expressing various proteins was determined as described in Materials and methods. G-protein receptor ratios (G/R ratios) were calculated by dividing the $G_{s\alpha}$ expression level by the receptor expression level. Reaction mixtures contained 0.2–0.5 μCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) or 10 μM dopamine/10 μM (−)-isoproterenol (+ago.). Data shown are the means of three to four independent experiments for each membrane preparation performed in quadruplicates. The relative agonist-stimulation of GTP hydrolysis (percent of basal) and the absolute agonist-stimulation of GTP hydrolysis (Δago.) were calculated.

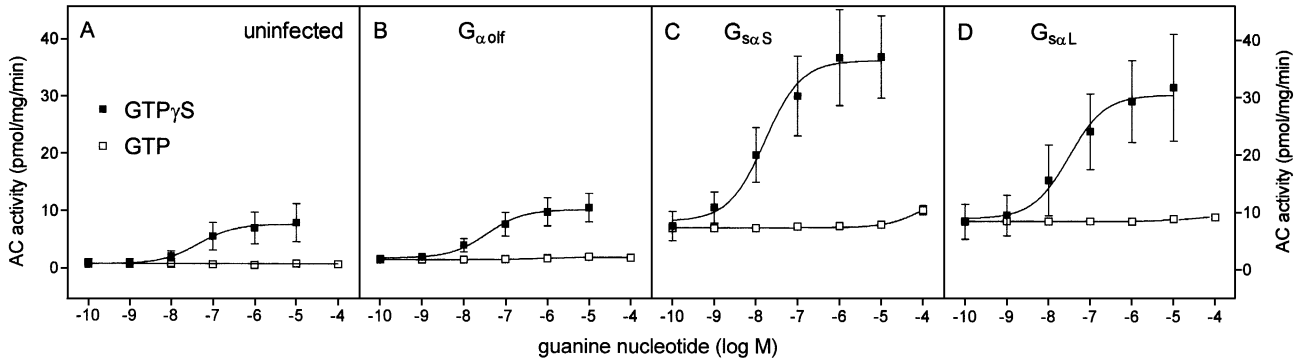

Fig. 4. Effects of GTP and GTPγS on AC activity in Sf9 membranes expressing various G-proteins. AC activity in membranes from uninfected Sf9 cells (A), Sf9 membranes expressing $G_{\alpha olf}$ (10.5 ± 2.0 pmol/mg) (B), Sf9 membranes expressing $G_{s\alpha S}$ (5.9 ± 0.3 pmol/mg) (C) and Sf9 membranes expressing $G_{s\alpha L}$ (10.1 ± 1.6 pmol/mg) (D) was determined as described in Materials and methods. Reaction mixtures contained GTPγS (■) or GTP (□) at the concentrations indicated on the abscissa. Data shown are the means ± S.D. of three to four experiments performed in duplicates with three to five membrane preparations. " − 10" designates the absence of guanine nucleotide.

data fit to the AC data obtained with GTPγS in the absence of receptors (Fig. 4).

In Sf9 membranes co-expressing the D_1R or β_2AR with $G_{s\alpha S}$ or $G_{s\alpha L}$, GTP exhibited profound stimulatory effects on basal AC activity (Fig. 5C, D, G and H) (Table 4). Moreover, the inverse D_1R agonist, chlorpromazine [60], and the inverse β_2AR agonist, ICI 118,551 [38,59,61], reduced the stimulatory effects of GTP on basal AC activity. Furthermore, GTP did not substantially increase AC activity in Sf9 membranes expressing $G_{s\alpha}$ splice variants alone (Fig. 4C and D). These data indicate that the stimulatory effects of GTP on AC activity in the co-expression membranes are attributable to constitutive activity of the D_1R and β_2AR. However, chlorpromazine and ICI 118,551 inhibited the

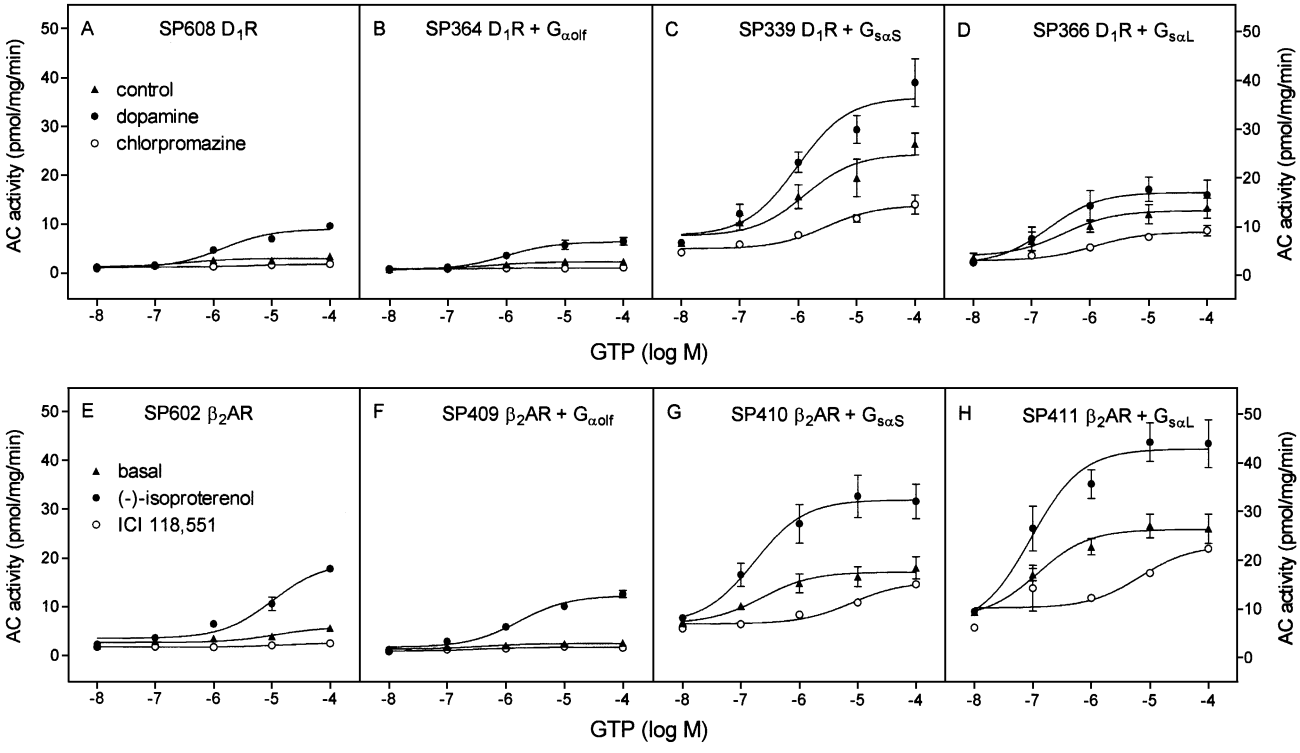

Fig. 5. Effects of GTP, agonist and inverse agonist on AC activity in Sf9 membranes expressing the D_1R or β_2AR with various $G_{s\alpha}$ proteins. AC activity in Sf9 membranes expressing various proteins was determined as described in Materials and methods. Reaction mixtures contained GTP at the concentrations indicated on the abscissa. Experiments shown in panels A–D were conducted in the presence of solvent (control) (▲), 10 μM dopamine (●) or 1 μM chlorpromazine (○). Experiments shown in panels E–H were conducted in the presence of solvent (control) (▲), 10 μM (−)-isoproterenol (●) or 1 μM ICI 118,551 (○). Data shown are the means ± S.D. of five to ten experiments performed in duplicates. The expression level of D_1R (SP608) (A) was 24.8 pmol/mg. The expression level of β_2AR (SP602) (E) was 23.9 pmol/mg. Receptor- and $G_{s\alpha}$ expression levels of the other membrane preparations are summarized in Table 4. " − 8" designates the absence of GTP.

Table 4
AC activation in Sf9 membranes expressing the D_1R and β_2AR, respectively, and various $G_{s\alpha}$ proteins at different G-protein/receptor ratios

Constructs (receptor level in pmol/mg)	$G_{s\alpha}$ (pmol/mg)	G/R ratio	Membrane preparation	− GTP, − agonist (pmol/mg/min)	+ GTP, − agonist (pmol/mg/min)	Const. act. (percent of total)	+ GTP, + agonist (pmol/mg/min)	Ago. - stim. (percent of control)	+ GTPγS (pmol/mg/min)
$D_1R + G_{s\alpha S}$									
13.5	8.8	0.65	SP474	10.9 ± 0.7 (8)	28.2 ± 3.4 (6)	83.2	31.7 ± 3.2 (6)	113	34.6 ± 1.1 (6)
11.8	10.5	0.89	SP475	10.3 ± 1.9 (12)	26.4 ± 6.9 (12)	77.8	31.0 ± 6.5 (14)	117	34.2 ± 2.5 (6)
6.3	11.9	1.89	SP476	10.1 ± 1.7 (8)	23.5 ± 4.6 (6)	56.8	33.7 ± 4.4 (6)	144	39.1 ± 2.9 (6)
5.7	4.0	0.70	SP339	6.4 ± 0.7 (13)	26.9 ± 2.2 (9)	62.1	39.4 ± 4.9 (9)	147	49.4 ± 4.8 (4)
$D_1R + G_{s\alpha L}$									
10.9	10.7	0.98	SP366	3.5 ± 0.8 (12)	13.8 ± 2.0 (8)	79.2	16.5 ± 3.0 (8)	120	26.8 ± 4.5 (8)
3.8	11.5	3.03	SP380	2.4 ± 0.7 (10)	7.4 ± 2.1 (6)	76.9	8.9 ± 2.6 (6)	119	16.1 ± 3.1 (8)
2.2	11.5	5.23	SP386	2.5 ± 0.8 (14)	9.4 ± 3.4 (12)	73.4	11.9 ± 3.8 (13)	126	15.6 ± 3.8 (8)
$\beta_2AR + G_{s\alpha S}$									
12.1	5.0	0.41	SP161	22.5 ± 9 (4)	54.4 ± 20.3 (4)	60.4	75.3 ± 18.1 (4)	138	77.1 ± 7.7 (4)
9.5	6.4	0.67	SP175	8.0 ± 2.3 (7)	22.1 ± 6.4 (7)	47.3	37.8 ± 9.7 (7)	171	32.3 ± 1.3 (4)
8.7	6.5	0.75	SP410	6.6 ± 1.7 (10)	18.4 ± 5.6 (6)	46.5	32.0 ± 8.7 (6)	174	32.5 ± 2.1 (4)
1.1	2.3	2.09	SP427	2.5 ± 1.6 (6)	7.7 ± 1.6 (5)	36.1	14.4 ± 1.8 (6)	187	18.7 ± 0.1 (2)
$\beta_2AR + G_{s\alpha L}$									
14.6	9.7	0.66	SP411	8.4 ± 1.9 (16)	26.5 ± 9.5 (10)	50.8	44.0 ± 15.5 (10)	166	37.6 ± 7.6 (8)
13.8	10.7	0.78	SP369	6.5 ± 1.9 (12)	21.1 ± 7.1 (8)	57.7	31.8 ± 12.3 (7)	151	31.4 ± 8.2 (8)
12.8	10.8	0.85	SP428	4.1 ± 1.7 (10)	9.8 ± 2.2 (7)	37.0	19.5 ± 5.6 (8)	198	22.2 ± 9.6 (4)
0.9	9.7	10.8	SP431	3.1 ± 0.7 (8)	9.5 ± 3.4 (6)	46.7	16.8 ± 6.0 (6)	176	11.9 ± 0.3 (2)

AC activity in Sf9 membranes expressing various proteins was determined as described in Materials and methods. Reaction mixtures contained no addition (− GTP, − agonist), 100 μM GTP (+ GTP, − agonist), 100 μM GTP + 10 μM dopamine or 10 μM (−)-isoproterenol (+ GTP, + agonist) and 10 μM GTPγS (+ GTPγS), respectively. The number of independent experiments (each performed in duplicates) is shown in parentheses after each data set. Data shown are the means ± S.D. of the individual experiments. G-protein/receptor ratios (G/R ratios) were calculated by dividing the $G_{s\alpha}$ expression level by the receptor expression level. The constitutive activities of the D_1R and β_2AR (const. act. (percent of total)) were calculated by dividing the difference of the absolute AC activities in the presence of GTP and absence of agonist (+ GTP, − agonist) and the AC activities in the absence of GTP and agonist (− GTP, − agonist) by the difference of the absolute AC activities in the presence of GTP and agonist (+ GTP, + agonist) and the AC activities in the absence of GTP and agonist (− GTP, − agonist). The relative stimulatory effects of agonists on AC activity (ago. - stim. (%)) were calculated by dividing the AC activity in the presence of GTP and agonist (+ GTP, + agonist) by the AC activity in the presence of GTP and absence of agonist (+ GTP, − agonist).

stimulatory effects of GTP only incompletely, indicating that these ligands are partial inverse agonists at the D_1R and β_2AR, respectively. In view of these data, the most accurate measure of constitutive receptor activity in our system is the stimulatory effect of GTP on basal AC activity relative to the combined stimulatory effects of GTP plus agonist. The constitutive activity of the D_1R coupled to $G_{s\alpha}$ splice variants amounted to 57–83% of total receptor activity in various membrane preparations, whereas the corresponding values for the β_2AR were generally lower (36–60%) (Table 4). In agreement with these data, the relative stimulatory effects of agonist were larger in membranes expressing the β_2AR (38–98%) than in membranes expressing the D_1R (13–47%). In the co-expression system, we did not observe a difference in apparent constitutive activity of the β_2AR coupled to $G_{s\alpha}$ splice variants (Fig. 5G and H), whereas the β_2AR fused to $G_{s\alpha L}$ exhibits higher constitutive activity than the β_2AR fused to $G_{s\alpha S}$ [34,38].

In studies on the coupling of the β_2AR to $G_{s\alpha}$-like proteins, a linear relation between receptor expression level and AC activity was observed [58]. In contrast, the highest agonist- and GTPγS-stimulated AC activities regarding $D_1R/G_{s\alpha S}$ couples were observed with SP339 that exhibited the lowest absolute receptor and $G_{s\alpha}$ level. In addition, reduction of the β_2AR expression level by 15-fold (compare SP411 with SP431) did not result in a proportional decrease in AC activities. Similarly, a fivefold decrease in D_1R expression level (compare SP366 with SP386) was only accompanied by moderate reductions of agonist- and GTPγS-stimulated AC activities.

4. Discussion

4.1. Inefficiency of $G_{\alpha olf}$ at activating AC

Previous studies from our laboratory using β_2AR-$G_{s\alpha}$ fusion proteins uncovered differences between $G_{s\alpha L}$, $G_{s\alpha S}$ and $G_{\alpha olf}$ in terms of their efficacy at activating AC [34,37–39]. In view of concerns regarding the physiological relevance of fusion proteins [31,32,40], the aim of our present study was to dissect differences between $G_{s\alpha}$ isoforms using nonfused $G_{s\alpha}$ proteins. Immunoblotting studies showed that $G_{s\alpha S}$, $G_{s\alpha L}$ and $G_{\alpha olf}$ were all expressed in Sf9 membranes (Fig. 1). As predicted from the β_2AR-$G_{s\alpha}$ fusion protein studies, nonfused $G_{\alpha olf}$ was much less efficient at activating AC than $G_{s\alpha}$ splice variants both in the absence and presence of receptors (Figs. 3 and 4). When expressed in $G_{s\alpha}$-deficient S49 *cyc*$^-$ lymphoma cells, $G_{\alpha olf}$ was also less efficient than $G_{s\alpha L}$ and $G_{s\alpha S}$ at activating AC [28]. Thus, with regard to the inefficiency of $G_{\alpha olf}$ at activating AC, the data obtained in various expression systems are consistent.

Future studies will have to address the question whether the structural differences between the α-helical domains of $G_{s\alpha S}$ and $G_{\alpha olf}$ contribute to the differences in AC activation between these $G_{s\alpha}$ isoforms [8,62].

4.2. Analysis of constitutive receptor activity

The β_2AR is the most extensively studied receptor exhibiting constitutive activity [56]. Our present study corroborates the notion that the β_2AR possesses substantial constitutive activity (Fig. 5 and Table 4). The co-expression system allows for sensitive analysis of constitutive receptor activity (Figs. 4 and 5 and Table 4). Using this system and comparing multiple membrane preparations, we found that the D_1R possesses an even higher constitutive activity than the β_2AR. These data point to the importance of agonist-independent dopaminergic signal transduction for the proper function of neurons. The incomplete inhibition of GTP-dependent AC activation by ICI 118,551 and chlorpromazine demonstrates that these ligands are only partial inverse agonists at the β_2AR and D_1R, respectively. Taken together, the system devised in this study is useful to compare constitutive activity of various G_s-coupled receptors and to assess the inverse agonistic efficacy of compounds.

4.3. Limitations of the co-expression approach

We conducted a large body of work to dissect differences between the three $G_{s\alpha}$ proteins, analyzing multiple parameters repeatedly in numerous membrane preparations. However, overall, the results of our study were very disappointing. Regardless of whether ternary complex formation (Figs. 2 and 3 and Table 1), GTPγS binding (Table 2), GTP hydrolysis (Table 3) and AC activity (Figs. 4 and 5 and Table 4) were analyzed, no differences between $G_{\alpha olf}$ and $G_{s\alpha}$ splice variants, between $G_{s\alpha S}$ and $G_{s\alpha L}$ and $G_{s\alpha}$-coupling of the β_2AR and D_1R except for the few differences discussed above were detected. We also noted a lack of relation between expression level and functional activity of $G_{s\alpha}$-proteins in the various assays, rendering data interpretation very difficult if not impossible. Our results are reminiscent to the results of previous expression studies using S49 *cyc*$^-$ lymphoma cells [28,29]. Moreover, even reconstitution studies with purified receptors and G-proteins did not uncover differences between $G_{s\alpha S}$ and $G_{s\alpha L}$ [63,64].

Several factors that are not mutually exclusive may have contributed to the largely negative outcome of our study. First, we quantitated $G_{s\alpha}$ protein expression in immunoblotting studies using β_2AR-$G_{s\alpha}$ fusion proteins as standard (Fig. 1). However, such studies do not provide information about the functional integrity of the expressed G-proteins. The GTPγS saturation binding studies showed that only a minority of the expressed $G_{s\alpha}$ proteins participated in receptor coupling, but we cannot distinguish whether this is because of inefficient receptor-activation of $G_{s\alpha}$ proteins or functional inactivity of $G_{s\alpha}$ proteins (Table 2). Second, one could explain our data by differential compartmentation of receptors, $G_{s\alpha}$ proteins and AC in specific membrane microcompartments [65–67]. In fact, it has already been shown that the glucagon receptor, β_2AR and $G_{s\alpha}$ splice variants are differentially compartmentalized in the regenerating rat liver [68]. Future studies will have to assess the specific localization of receptors and $G_{s\alpha}$ proteins in Sf9 cell membranes, for example by using fluorescent dye-tagged proteins [69]. It is possible that even in reconstitution systems with purified proteins, receptors and G-proteins do not interact freely with each other. Third, it is well known that $G_{s\alpha}$ splice variants differentially distribute between membrane and cytosol in various systems [19,70,71]. Regardless of which of the three explanations is correct, the co-expression approach using non-modified $G_{s\alpha}$ proteins, although apparently closer to the physiological situation than fusion proteins or tethered $G_{s\alpha}$ proteins, does not provide a sensitive approach to dissect biochemical differences between $G_{s\alpha}$ proteins.

4.4. Comparison of the co-expression and fusion protein approach

In a previous study we showed that signaling in the β_2AR-$G_{s\alpha L}$ fusion protein in terms of ternary complex formation, GTPγS binding, GTP hydrolysis and AC activation was much more efficient than in the β_2AR/$G_{s\alpha L}$ co-expression system [43]. This conclusion can now be extended to the other two $G_{s\alpha}$ proteins, $G_{s\alpha S}$ and $G_{\alpha olf}$ (Tables 1–4) [37]. Fusion proteins are particularly more sensitive than co-expression systems with respect to GTPγS binding and GTP hydrolysis. Most likely, the high signaling efficiency in β_2AR-$G_{s\alpha}$ fusion proteins is the result of tight membrane-tethering of $G_{s\alpha}$ [33]. The similarities between $G_{s\alpha}$ isoforms in terms of signaling in the fused versus nonfused state indicate that the fundamental mechanisms governing receptor/G-protein coupling are similar for the three $G_{s\alpha}$ isoforms.

The fusion protein approach allowed us to dissect multiple biochemical differences between $G_{s\alpha}$ isoforms, including kinetics of GTPγS binding and GTP hydrolysis, GDP-affinity, efficacy at activating AC and their ability to confer constitutive activity to the β_2AR [34,37,38]. The only biochemical difference between nonfused $G_{s\alpha}$ splice variants that we could unmask in the present study concerns the lower efficacy of $G_{\alpha olf}$ at activating AC relative to $G_{s\alpha}$ splice variants. This difference was observed in the fusion proteins as well. In conclusion, it is much easier to dissect differences between $G_{s\alpha}$ isoforms using β_2AR-$G_{s\alpha}$ fusion proteins than nonfused $G_{s\alpha}$ isoforms.

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