0026-895X/00/050954-13$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:954–966, 2000

Vol. 58, No. 5
13106/857840
Printed in U.S.A.

Molecular Analysis of β_2-Adrenoceptor Coupling to G_s-, G_i-, and G_q-Proteins

KATHARINA WENZEL-SEIFERT and ROLAND SEIFERT

Howard Hughes Medical Institute, Stanford University Medical School, Stanford, California; and Department of Pharmacology and Toxicology, The University of Kansas, Lawrence, Kansas

Received November 18, 1999; accepted July 31, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The β_2-adrenoceptor (β_2AR) couples to the G-protein G_s to activate adenylyl cyclase. Intriguingly, several studies have demonstrated that the β_2AR can also interact with G-proteins of the G_i- and G_q-family. To assess the efficiency of β_2AR interaction with various G-protein α-subunits ($G_{x\alpha}$), we expressed fusion proteins of the β_2AR with the long ($G_{s\alpha L}$) and short ($G_{s\alpha S}$) splice variants of $G_{s\alpha}$, the G_i-proteins $G_{i\alpha 2}$ and $G_{i\alpha 3}$, and the G_q-proteins $G_{q\alpha}$ and $G_{16\alpha}$ in Sf9 cells. Fusion proteins provide a rigorous approach for comparing the coupling of a given receptor to $G_{x\alpha}$ because of the defined 1:1 stoichiometry of receptor and G-protein and the efficient coupling. Here, we show that the β_2AR couples to G_s-, G_i-, and G_q-proteins as assessed by ternary complex formation and ligand-regulated guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding. The combined analysis of ternary complex formation, GTPγS binding, agonist efficacies, and agonist potencies revealed substantial differences in the interaction of the β_2AR with the various classes of G-proteins. Comparison of the coupling of the β_2AR and formyl peptide receptor to $G_{i\alpha 2}$ revealed receptor-specific differences in the kinetics of GTPγS binding. We also detected highly efficient stimulation of GTPγS dissociation from $G_{s\alpha L}$, but not from $G_{q\alpha}$ and $G_{16\alpha}$, by a β_2AR agonist. Moreover, we show that the 1:1 stoichiometry of receptor to G-protein in fusion proteins reflects the in vivo stoichiometry of receptor/G-protein coupling more closely than was previously assumed. Collectively, our data show 1) that the β_2AR couples differentially to G_s-, G_i-, and G_q-proteins, 2) that there is ligand-specific coupling of the β_2AR to G-proteins, 3) that receptor-specific G-protein conformational states may exist, and 4) that nucleotide dissociation is an important mechanism for G-protein deactivation.

The β_2-adrenoceptor (β_2AR) is a prototypical G-protein-coupled receptor that interacts with the stimulatory G-protein of adenylyl cyclase, G_s (Gilman, 1987; Kobilka, 1992). Intriguingly, studies of intact cells, cell membranes, and purified proteins have shown that the β_2AR can also interact with G_i-proteins (Katada et al., 1982; Asano et al., 1984; Xiao et al., 1995, 1999; Daaka et al., 1997; Pavoine et al., 1999). In addition, the β_2AR can activate phospholipase C-β via G-proteins of the G_q-family, e.g., $G_{16\alpha}$ and $G_{q\alpha}$ (Zhu et al., 1994; Offermanns and Simon, 1995; Wu et al., 1995).

In recent studies (Seifert et al., 1998a,b; Wenzel-Seifert et al., 1998b), we analyzed the coupling of the β_2AR to $G_{s\alpha}$ using fusion proteins. In fusion proteins, the receptor C terminus is covalently linked to the N terminus of $G_{x\alpha}$. Fusion ensures a defined 1:1 stoichiometry of receptor to G-protein and promotes efficient coupling without altering the fundamental properties of the signaling partners. The fusion protein approach has been successfully applied to various receptors and G-proteins (Seifert et al., 1999c; Milligan, 2000). With the fusion protein approach we could dissect subtle differences in the coupling of the β_2AR to $G_{s\alpha S}$ and $G_{s\alpha L}$ (Seifert et al., 1998b). In the latter study, we analyzed receptor-G-protein coupling by measuring ternary complex formation, i.e., the complex of agonist, receptor, and nucleotide-free G-protein displaying high agonist affinity, steady-state GTP hydrolysis, and adenylyl cyclase activation.

The goal of our present study was to quantitatively compare the coupling of the β_2AR to G_s-, G_i-, and G_q-proteins. To achieve this aim, we needed a system that ensures defined receptor-G-protein stoichiometry and efficient coupling.

This work was supported by The New Faculty Award of the University of Kansas and the J.R. and Inez Jay BioMedical Research Award of The Higuchi Biosciences Center of the University of Kansas to R.S. While working in Stanford, R.S. and K.W.S. were supported by a research fellowship of the Deutsche Forschungsgemeinschaft.

ABBREVIATIONS: β_2AR, β_2-adrenoceptor; β_2AR-$G_{i\alpha 2}$ (-$G_{i\alpha 3}$, -$G_{q\alpha}$, -$G_{s\alpha L}$, -$G_{s\alpha S}$, -$G_{16\alpha}$), fusion proteins consisting of the β_2-adrenoceptor and $G_{i\alpha 2}$, $G_{i\alpha 3}$, $G_{q\alpha}$, the short splice variant of $G_{s\alpha}$, the long splice variant of $G_{s\alpha}$, and $G_{16\alpha}$, respectively; DHA, [^{3}H]dihydroalprenolol; DCI, dichloroisoproterenol; DOB, dobutamine; EPH, (−)-ephedrine; FPR, formyl peptide receptor; FPR-$G_{i\alpha 2}$, fusion protein consisting of the FPR and $G_{i\alpha 2}$; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); $G_{x\alpha}$, nonspecified G-protein α-subunit; ISO, (−)-isoproterenol; ICI, ICI 118,55 ([erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol]); SAL, salbutamol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription.

Therefore, we constructed various β_2AR-$G_{x\alpha}$ fusion proteins and analyzed those proteins in Sf9 insect cells. To validate the results obtained with the fusion protein consisting of the β_2AR and $G_{i\alpha2}$ (β_2AR-$G_{i\alpha2}$), we also coexpressed the β_2AR with $G_{i\alpha2}$. Moreover, we compared β_2AR-$G_{i\alpha2}$ coupling with formyl peptide receptor (FPR)-$G_{i\alpha2}$ coupling in the fused and nonfused state because the FPR is a prototypical G_i-protein-coupled receptor (Gierschik et al., 1991; Wenzel-Seifert et al., 1998a, 1999). Here, we report differential coupling of the β_2AR to G_s-, G_i-, and G_q-proteins and differences in the coupling of the β_2AR and FPR to G_i-proteins.

Experimental Procedures

Materials. The cDNAs of $G_{i\alpha2}$ and $G_{i\alpha3}$ in pGEM-2 were kindly provided by Dr. R. Reed (Howard Hughes Medical Institute, Johns-Hopkins-University, Baltimore, MD) (Jones and Reed, 1987). The cDNA of $G_{16\alpha}$ in pCMV was a gift from Dr. D. Wu (Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY) (Amatruda et al., 1991). The cDNA of $G_{q\alpha}$ in pVL1392 was kindly provided by Dr. E. M. Ross (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX) (Biddlecome et al., 1996). Recombinant baculovirus encoding the unmodified versions of the G-protein subunits $\beta_1\gamma_2$ was a kind gift of Dr. P. Gierschik (Abteilung für Pharmakologie und Toxikologie, Universität Ulm, Ulm, Germany). The $G_{i\alpha2}$ baculovirus was kindly provided by Dr. A. G. Gilman (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX). Antibodies recognizing $G_{i\alpha3}$ (anti-$G_{i\alpha3}$, AS 86, C terminal) (Leopoldt et al., 1997), all $G_{i\alpha}$ subunits (anti-$G_{i\alpha common}$, AS 266) (Leopoldt et al., 1997), $G_{16\alpha}$ (anti-$G_{16\alpha}$, AS 339) (Spicher et al., 1994), and $G_{q\alpha}$ (anti-$G_{q\alpha}$, AS 369) (Spicher et al., 1994) were generously provided by Drs. B. Nürnberg and G. Schultz (Institut für Pharmakologie, Freie Universität Berlin, Germany). The antibody recognizing $G_{i\alpha1/2}$ was from Calbiochem (La Jolla, CA). [^{35}S]Guanosine 5′-*O*-(3-thiotriphosphate (GTPγS; 1000–1500 Ci/mmol) was from NEN Life Science Products (Boston, MA). [^{3}H]Dihydroalprenolol (DHA; 85–90 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). Unlabeled GTPγS and GDP were obtained from Roche Diagnostics (Indianapolis, IN). ICI 118,55 ([erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol]) (ICI) was from Research Biochemicals International (Natick, MA). The M1 monoclonal antibody (detecting the FLAG epitope), (−)-isoproterenol (ISO), salbutamol (SAL), (−)-ephedrine (EPH), and (±)-alprenolol were from Sigma (St. Louis, MO). Dichloroisoproterenol (DCI) was from Aldrich (Milwaukee, WI). All restriction enzymes, DNA polymerase I, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Glass fiber filters (GF/C) were from Schleicher & Schuell (Dassel, Germany). All other reagents were of the highest purity available and from standard suppliers.

Construction of the cDNAs Encoding β_2AR-$G_{x\alpha}$ Fusion Proteins. The cDNA of β_2AR-$G_{\alpha sL}$ in pGEM-3Z (Seifert et al., 1998a,b) was used as a template to amplify the C-terminal portions of the β_2AR. In this construct the β_2AR is tagged at the 5′ end with a DNA sequence encoding the cleavable signal peptide (S) from influenza hemagglutinin, which facilitates correct insertion of the receptor into the plasma membrane, followed by the FLAG epitope (F), which can be recognized by the M1 antibody. The C terminus of the receptor is tagged with a hexahistidine tag. Fusion of the β_2AR with different $G_{x\alpha}$-subunits was achieved by sequential overlap-extension polymerase chain reactions (PCRs) using *Pfu* polymerase (Stratagene, La Jolla, CA). In PCR 1A, the DNA sequence of the C terminus of β_2AR was amplified with pGEM3Z-SF-β_2AR-$G_{\alpha sL}$ as a template by using a sense primer 5′ of the *Sac*I site in the C terminus (sense *Sac*I primer) and an antisense primer encoding the hexahistidine tag. The cDNAs of the four different $G_{x\alpha}$-subunits were amplified in four different PCR reactions (PCR $1B_{1-4}$) using pGEM-2-$G_{i\alpha2}$, pGEM-2-$G_{i\alpha3}$, pCMV-$G_{16\alpha}$, and pVL 1392-$G_{q\alpha}$, respectively, as templates. The sense primers annealed with the first 18 bp of the 5′ end of $G_{x\alpha}$ and included the 18 bp of the hexahistidine tag in their 5′ extensions. The antisense primers encoded the last five amino acids of the C terminus of the $G_{x\alpha}$, followed by the stop codon and an extra *Xba*I site for cloning purposes in the 3′-end extension. In the case of $G_{q\alpha}$, a *Bam*HI site instead of an *Xba*I was included in the 3′-end extension of the antisense primer. In PCR 2 the cDNA fragments from PCR 1A and PCR $1B_{1-4}$ were annealed and amplified using the sense *Sac*I primer and the antisense primers of PCR $1B_{1-4}$. In this way, fragments encoding the C terminus of β_2AR, a hexahistidine tag, and the $G_{x\alpha}$ followed by an *Xba*I or *Bam*HI site were obtained. The fragment for β_2AR-$G_{i\alpha2}$ was digested with *Eco*RV and *Xba*I and cloned into pGEM3Z-β_2AR digested with *Sac*I and *Sal*I together with an oligonucleotide linker encoding (5′→3′) an *Xba*I site, a *Bam*HI site, and a *Sal*I site. The fragments for β_2AR-$G_{i\alpha3}$, β_2AR-$G_{16\alpha}$, and β_2AR-$G_{q\alpha}$ were digested with *Eco*RV plus *Xba*I or *Eco*R V plus *Bam*HI, respectively, and cloned into pGEM-3Z-β_2AR-$G_{i\alpha2}$ digested with *Eco*RV plus *Xba*I or *Eco*RV plus *Bam*HI, respectively. PCR-generated DNA sequences were confirmed by enzymatic sequencing using Sequenase version 2.0 Sequencing kit (USB, Cleveland, OH). For cloning into the baculovirus expression vector pVL 1392, the cDNAs encoding β_2AR-$G_{x\alpha}$-fusion proteins in pGEM-3Z were digested with *Hin*dIII at the 5′-end of the SF region, blunted with DNA polymerase I (Klenow fragment), and then digested with *Xba*I or *Bam*HI at the 3′-end of $G_{x\alpha}$. Digested fusion protein DNAs were then ligated into pVL 1392 that had been digested with *Bgl*II, blunted with Klenow fragment, and subsequently digested with *Xba*I or *Bam*HI.

Generation of Recombinant Baculoviruses and Cell Culture and Membrane Preparation. Recombinant baculoviruses encoding the β_2AR-$G_{x\alpha}$ fusion proteins were generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions. After initial transfection, working virus stocks were generated by three sequential virus amplifications. Sf9 cells were cultured in 250-ml disposable Erlenmeyer flasks at 28°C under rotation at 125 rpm in SF 900 II medium (Life Technologies, Grand Island, NY) supplemented with 5% (v/v) fetal calf serum (Gemini, Calabasa, CA) and 0.1 mg/ml gentamicin (Roche Diagnostics). Cells were maintained at a density of 0.5 to 6.0 $\times$ 10^6 cells/ml. For infection, cells were sedimented by centrifugation and suspended in fresh medium. Cells were seeded at 3.0 $\times$ 10^6 cells and infected with a 1:100 dilutions of high-titer baculovirus stocks encoding β_2AR-$G_{x\alpha}$ fusion proteins or nonfused β_2AR plus $G_{i\alpha2}$. Except for the experiments shown in Fig. 5, all cultures were also coinfected with a baculovirus encoding $\beta_1\gamma_2$-subunits at a 1:100 dilution. Cells were cultured for 48 h before membrane preparation.

Sf9 membranes were prepared as described (Seifert et al., 1998a), using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, and 10 μg/ml leupeptin as protease inhibitors.

DHA Binding. Before experiments, membranes were pelleted by a 15-min centrifugation at 4°C and 15,000*g* and resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris-HCl, pH 7.4). B_{max} values were determined by incubating Sf9 membranes (10–40 μg of protein/tube, depending on the specific expression level) with a single saturating concentration of DHA (10 nM). Nonspecific binding was determined in the presence of DHA (10 nM) plus 10 μM (±)-alprenolol. Incubations were performed for 90 min at 25°C and shaking at 200 rpm. Competition binding experiments were carried out with 1 nM DHA in the presence of ISO at various concentrations with or without GTPγS (10 μM). Bound DHA was separated from free DHA by filtration through GF/C filters and washed three times with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

GTPγS Binding and GTPγS Dissociation. Membranes were thawed, pelleted by a 15-min centrifugation at 4°C and 15,000*g*, and resuspended in binding buffer. For GTPγS saturation binding studies, reaction mixtures (500 μl) contained membranes (10–81 μg of

protein/tube) in binding buffer supplemented with 0.05% (w/v) bovine serum albumen, 1 μM GDP, and 0.2 to 1 nM [^{35}S]GTPγS plus unlabeled GTPγS at increasing concentrations to give the desired final ligand concentrations. Reaction mixtures additionally contained distilled water (control) and β_2AR ligands at a saturating concentration (ISO, 10 μM; ICI, 1 μM). Incubations were performed at 25°C and shaking at 200 rpm for various periods, depending on the specific properties of the fusion protein. For time-course studies, Sf9 membranes were suspended in 1500 μl of binding buffer supplemented with 1 to 2 nM [^{35}S]GTPγS plus 9 to 48 nM unlabeled GTPγS, 1 μM GDP, and distilled water (control) or β_2AR ligands at a saturating concentration (ISO, 10 μM; ICI, 1 μM). Aliquots of 200 μl (containing 15–70 μg of protein) were taken at seven different time points. In the experiments shown in Fig. 5B, the [^{35}S]GTPγS concentration was 0.4 nM. Assays were conducted in the absence of GDP or in the presence of GDP at 1 nM to 10 μM. In the experiments shown in Tables 2 and 3, reaction mixtures contained 0.4 nM [^{35}S]GTPγS, 1 μM GDP, and different β_2AR ligands at increasing concentrations. Nonspecific [^{35}S]GTPγS binding was determined in the presence of 10 μM GTPγS and was less than 0.1% of total 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°C). Filter-bound radioactivity was determined by liquid scintillation counting.

For assessing GTPγS dissociation, GTPγS (0.5 nM [^{35}S]GTPγS plus 9.5 nM unlabeled GTPγS) was allowed to associate to membranes for 60 min in the absence of ligand. [^{35}S]GTPγS dissociation was initiated by the addition of 20 μM unlabeled GTPγS in the absence or presence of ISO (10 μM). Aliquots of 150 μl (containing 20–36 μg of protein) were taken from reaction mixtures at different time points, and bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS as described above.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis. SDS-PAGE and immunoblotting were performed as described (Wenzel-Seifert et al., 1998a). Solubilized membrane proteins were separated on gels containing 10% (w/v) acrylamide. Proteins were then transferred onto nitrocellulose filters (Fig. 1, A and C) or Immobilon-P transfer membranes (Millipore, Bedford, MA) (Fig. 1B). Membranes were reacted with M1 antibody (1:1000) or the antibodies directed against different G-protein subunits (1:500 each). Immunoreactive bands were visualized by sheep anti-mouse IgG (M1 antibody) and donkey anti-rabbit IgG (G-protein antibodies), respectively, coupled to peroxidase, using *o*-dianisidine and H_2O_2 as substrates.

Analysis of β_2AR-G_{xa} Fusion Proteins by Reverse Transcription (RT)-PCR and Digestion with Restriction Enzymes. mRNA from Sf9 cells infected with recombinant baculoviruses was isolated with the RNeasy kit from Qiagen (Valencia, CA) and treated with RNase-free DNase. mRNA was reverse-transcribed using the First Strand cDNA synthesis kit from Amersham Pharmacia Biotech. The cDNAs of G-protein α-subunits in β_2AR-$G_{x\alpha}$ fusion proteins were amplified with *Taq* polymerase (Sigma) using appropriate primer pairs. PCR products were digested with various restriction enzymes, separated on gels containing 2% (w/v) agarose, and visualized by ethidium bromide staining.

Miscellaneous. Protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Data were analyzed by nonlinear regression, using the Prism program (GraphPad, San Diego, CA).

Results and Discussion

Analysis of the Expression of β_2AR-$G_{x\alpha}$ Fusion Proteins by Receptor Antagonist Saturation Binding. The expression levels of β_2AR-$G_{x\alpha}$ fusion proteins as determined by DHA saturation binding (lowest and highest value and mean ± S.D.) were as follows: β_2AR-$G_{s\alpha L}$ (6.1–10.6 pmol/mg, 7.3 ± 1.8 pmol/mg); β_2AR-$G_{s\alpha S}$ (2.1–4.6 pmol/mg, 2.9 ± 1.4 pmol/mg); β_2AR-$G_{i\alpha 2}$ (3.5–18.6 pmol/mg, 7.6 ± 5.9 pmol/mg); β_2AR-$G_{i\alpha 3}$ (4.5–20.2 pmol/mg; 9.6 ± 7.3 pmol/mg); β_2AR-$G_{q\alpha}$ (2.7–13.6 pmol/mg, 7.7 ± 5.1 pmol/mg); β_2AR-$G_{16\alpha}$ (2.1–10.7 pmol/mg, 4.8 ± 4.0 pmol/mg). These data show that β_2AR-$G_{x\alpha}$ fusion proteins can be expressed at comparable levels, with β_2AR-$G_{s\alpha S}$ being the fusion protein that expresses the least well.

Analysis of the Expression of β_2AR-$G_{x\alpha}$ Fusion Proteins by Immunoblotting. In our previous studies we had already documented that structurally intact β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$ fusion proteins can be expressed in Sf9 cells (Seifert et al., 1998a,b). In immunoblots of membranes expressing β_2AR-$G_{i\alpha 2}$, β_2AR-$G_{i\alpha 3}$, β_2AR-$G_{16\alpha}$, and β_2AR-$G_{q\alpha}$, the M1 antibody recognized proteins with apparent molecular masses of ~90 kDa (Fig. 1A). These values correspond to the sum of the molecular mass of the β_2AR (~50 kDa) and $G_{x\alpha}$ (~40 kDa). Note that there were no immunoreactive bands of lower molecular mass than ~90 kDa, indicating that β_2AR-$G_{x\alpha}$ fusion proteins were not degraded by insect cell proteases. The anti-$G_{i\alpha 1/2}$ Ig reacted with β_2AR-$G_{i\alpha 2}$ but not with β_2AR-$G_{i\alpha 3}$ (Fig. 1B), whereas the anti-$G_{i\alpha 3}$ Ig recognized β_2AR-$G_{i\alpha 3}$ but not β_2AR-$G_{i\alpha 2}$ (Fig. 1C). However, the anti-$G_{i\alpha 3}$ Ig also reacted with proteins of lower molecular mass than ~90 kDa. Because the immunoblot with the M1 antibody clearly demonstrated that fusion proteins were not degraded (see Fig. 1A), the results obtained with the anti-$G_{i\alpha 3}$ Ig suggest that the additional bands recognized by this antibody represent nonspecific reactions.

We also quantified the expression of nonfused $G_{i\alpha 2}$ in membranes coexpressing β_2AR (2.3 pmol/mg) and $G_{i\alpha 2}$ (Fig. 1B). By using membranes expressing β_2AR-$G_{i\alpha 2}$ (100 μg of protein per lane) at a defined level (15 pmol/mg) as standard, we estimated that the expression level of nonfused $G_{i\alpha 2}$ is ~300 pmol/mg. Thus, the stoichiometry of the β_2AR to $G_{i\alpha 2}$ in the coexpression system is ~1:100. This ratio is similar to the ratio of the β_2AR to $G_{s\alpha}$ and of the FPR to $G_{i\alpha 2}$ expressed in Sf9 cells and most likely represents the in vivo β_2AR/$G_{i\alpha 2}$ ratio (Ransnäs and Insel, 1988; Gierschik et al., 1991; Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999).

We tried to detect β_2AR-$G_{16\alpha}$ with anti-$G_{16\alpha}$ Ig (AS 339) (Spicher et al., 1994) and β_2AR-$G_{q\alpha}$ with anti-$G_{q\alpha}$ Ig (AS 369) (Spicher et al., 1994). However, the signals detected in the 90-kDa area were quite weak, and there were numerous nonspecific reactions with proteins of molecular mass lower than 90 kDa (data not shown). In addition, we could not convincingly detect β_2AR-$G_{i\alpha}$ fusion proteins with the anti-$G_{i\alpha common}$ Ig (AS 266) (Leopoldt et al., 1997; data not shown). Evidently, the sensitivity of these antibodies is too low to detect the corresponding β_2AR-$G_{x\alpha}$ fusion proteins at the expression levels achievable. This interpretation is supported by the fact that anti-$G_{i\alpha common}$ Ig detected nonfused $G_{i\alpha 2}$ expressed at ~300 pmol/mg quite well (Wenzel-Seifert et al., 1998a) but β_2AR-$G_{i\alpha}$ fusion proteins are expressed at levels that are ~20 to 100 times lower than those of nonfused $G_{i\alpha 2}$.

Analysis of the Expression of β_2AR-$G_{x\alpha}$ Fusion Proteins by RT-PCR and Digestion with Restriction Enzymes. To overcome the difficulties associated with the use of certain G-protein antibodies, we analyzed the correct expression of fusion proteins by RT-PCR and restriction enzyme digestion using mRNA from infected Sf9 cells. The $G_{x\alpha}$ portions of fusion protein cDNAs were amplified by PCR and

digested with various restriction enzymes (Fig. 1D). As expected, $G_{i\alpha 2}$-, $G_{16\alpha}$-, and $G_{q\alpha}$ cDNAs were digested by *Pst*I. Because of its small size, the 83-bp fragment of $G_{q\alpha}$ is not visible. Digestion with *Bam*HI gave the expected fragments with $G_{i\alpha 2}$-, $G_{i\alpha 3}$-, and $G_{16\alpha}$-cDNA, and digestion with *Eco*RI resulted in cleavage of the cDNA of $G_{16\alpha}$ and $G_{q\alpha}$. Thus, the RT-PCR data confirm the specific expression of β_2AR-$G_{x\alpha}$ fusion proteins in Sf9 cell membranes.

Agonist-Competition Studies with β_2AR-$G_{x\alpha}$: Differential Ternary Complex Formation with Fusion Proteins. One of the most stringent tests of receptor/G-protein coupling is the formation of the ternary complex, i.e., the complex consisting of agonist-occupied receptor and guanine nucleotide-free $G_{x\alpha}$ (De Lean et al., 1980; Seifert et al., 1998b). This complex possesses high affinity for agonists and can be detected in radioligand binding studies in which unlabeled agonist competes with radiolabeled antagonist. Upon binding of GTP or its GTPase-resistant analog GTPγS, the ternary complex is disrupted, and the receptor converts into a state of low agonist affinity (De Lean et al., 1980; Seifert et al., 1998b).

In membranes expressing β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$ plus $\beta_1\gamma_2$-complex, 42.1 ± 3.4 and 51.2 ±4.9% of the receptors, respectively, displayed high affinity for the full agonist ISO (K_i values, 0.7 ± 0.1 and 1.3 ± 0.3 nM, respectively) (Fig. 2, A and B). GTPγS converted the β_2ARs into a single population of receptors displaying low agonist affinity (K_i values, 155 ± 19 and 182 ± 22 nM, respectively). These data are similar to our previous data on β_2AR-$G_{s\alpha}$ fusion proteins expressed without the $\beta_1\gamma_2$-complex (Seifert et al., 1998b) and demonstrate that mammalian βγ-complex is not required for efficient ternary complex formation in β_2AR-$G_{s\alpha}$ fusion proteins. In membranes expressing β_2AR-$G_{i\alpha 2}$ and β_2AR-$G_{q\alpha}$ there was no detectable high-affinity agonist bind-

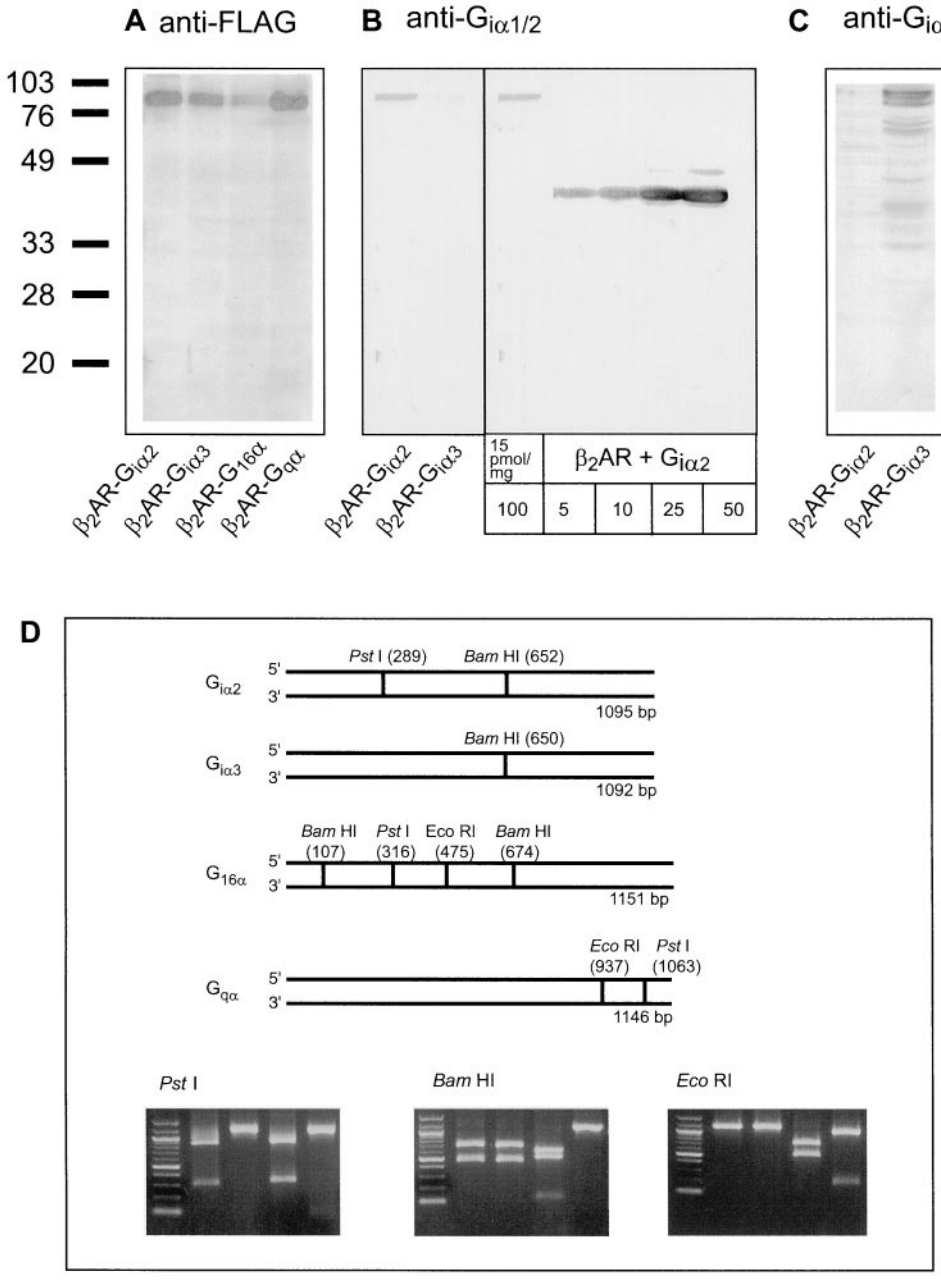

Fig. 1. Analysis of the expression of β_2AR-$G_{x\alpha}$ fusion proteins and of nonfused $G_{i\alpha 2}$ in Sf9 membranes. A, Sf9 membranes (50 μg of protein/lane) expressing β_2AR-$G_{i\alpha 2}$ (7.6 pmol/mg), β_2AR-$G_{i\alpha 3}$ (6.0 pmol/mg), β_2AR-$G_{16\alpha}$ (3.5 pmol/mg), or β_2AR-$G_{q\alpha}$ (8.5 pmol/mg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-FLAG Ig (M1 antibody). B, Sf9 membranes expressing β_2AR-$G_{i\alpha 2}$ (15.0 pmol/mg, 100 μg of protein/lane), β_2AR-$G_{i\alpha 3}$ (7.6 pmol/mg, 150 μg of protein/lane) or β_2AR (2.3 pmol/mg) plus nonfused $G_{i\alpha 2}$ (micrograms of protein loaded per lane given below the lanes) were separated by SDS-PAGE, transferred to Immobilon-P transfer membranes, and probed with anti-$G_{i\alpha 1/2}$-Ig. C, Sf9 membranes expressing β_2AR-$G_{i\alpha 2}$ (15.0 pmol/mg, 100 μg of protein/lane) or β_2AR-$G_{i\alpha 3}$ (7.6 pmol/mg, 150 μg of protein/lane) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-$G_{i\alpha 3}$ Ig. For further details, see *Experimental Procedures*. Numbers on the left indicate molecular masses of marker proteins. Shown are the horseradish-peroxidase-reacted membranes of gels containing 10% (w/v) acrylamide. D, mRNA from Sf9 cells infected with β_2AR-$G_{x\alpha}$ baculoviruses was isolated and reverse-transcribed as described under *Experimental Procedures*. The $G_{x\alpha}$ portions of fusion proteins were amplified by PCR and digested with *Pst*I, *Bam*HI, or *Eco*RI. Digested DNA was separated on gels containing 2% (w/v) agarose. DNA was stained with ethidium bromide. The left lanes of gels show the 100-bp DNA ladder. The broad calibration lanes represent the 100-, 500-, and 1000-bp standards. The scheme above the gels shows the relative positions of the relevant restriction sites in $G_{x\alpha}$.

ing (Fig. 2, C and F). $G_{16\alpha}$ conferred to 11.4 ± 2.9% of the β_2ARs the ability to bind agonist with high affinity (K_i 2.3 ± 0.4 nM) (Fig. 2E). The ternary complex formed with $G_{\alpha 16}$ was GTPγS sensitive. In membranes expressing β_2AR-$G_{i\alpha 3}$, some high-affinity binding of ISO (14.9 ± 3.5%; K_i, 4.0 nM) could be detected as well, but this high-affinity agonist binding was GTPγS-insensitive (Fig. 2D). These data show that, with regard to ternary complex formation, the β_2AR couples much more efficiently to G_s- than to G_i- and G_q-proteins and that the β_2AR discriminates between different members of the G_i- and G_q-family. The GTPγS insensitivity of the ternary complex formed with $G_{i\alpha 3}$ reflects the inability of GTPγS to promote dissociation of this G-protein from the β_2AR. Indeed, GTPγS-insensitive ternary complex formation has been repeatedly observed (Szele and Pritchett, 1993; Gürdal et al., 1997; Seifert et al., 1998a) and points to permanent physical interaction of the receptor with the G-protein during the entire G-protein cycle (see also discussion below on receptor agonist-regulated GTPγS dissociation from G-proteins).

General Considerations for GTPγS Binding Studies with β_2AR-$G_{x\alpha}$ Fusion Proteins and Advantages and Disadvantages of the Sf9 Cell System. Receptors catalyze GDP release from $G_{x\alpha}$ and subsequently promote the binding of GTP or its hydrolysis-resistant analog GTPγS to the G-protein (Gilman, 1987; Gierschik et al., 1991; Iiri et al., 1998; Wenzel-Seifert et al., 1998a, 1999). Because the sensitivity of the GTPγS binding assay surpasses the sensitivity of the steady-state GTPase assay (Gierschik et al., 1991; Seifert et al., 1998a), the GTPγS binding assay has become the most widely used assay to monitor receptor-G-protein coupling directly at the G-protein level. The GTPγS binding assay allows for the quantitative comparison of the coupling of a given receptor to different G-proteins and of different receptors to a given G-protein because the measurement of GTPγS binding is independent of an effector system.

A unique property of fusion proteins is the 1:1 stoichiometry of receptor and G-protein (Seifert et al., 1999c; Milligan, 2000). If each receptor interacts only with its fused $G_{x\alpha}$ partner, the B_{max} values of ligand-regulated GTPγS binding and radioligand antagonist binding should be similar. Ligand-regulated GTPγS binding is the difference between maximum agonist-stimulated GTPγS binding and minimum GTPγS binding in the presence of an inverse agonist (Wenzel-Seifert et al., 1998a, 1999). The ratio of the B_{max} of ligand-regulated GTPγS binding and the B_{max} of radioligand antagonist binding is defined as the coupling factor and should be approximately 1 for fusion proteins if the receptor interacts only with its fused $G_{x\alpha}$ partner (Wenzel-Seifert et al., 1999). In fact, in Sf9 insect cells, the coupling of the β_2AR and FPR to endogenous G-proteins is minimal or not detectable, rendering fusion proteins expressed in Sf9 cells a suitable system for analyzing the coupling of a given receptor to different G-proteins and of different receptors to a given G-protein in terms of GTPγS binding under defined conditions (Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999).

To quantitate β_2AR coupling to insect cell G-proteins, we performed GTPγS saturation binding studies with Sf9 cell

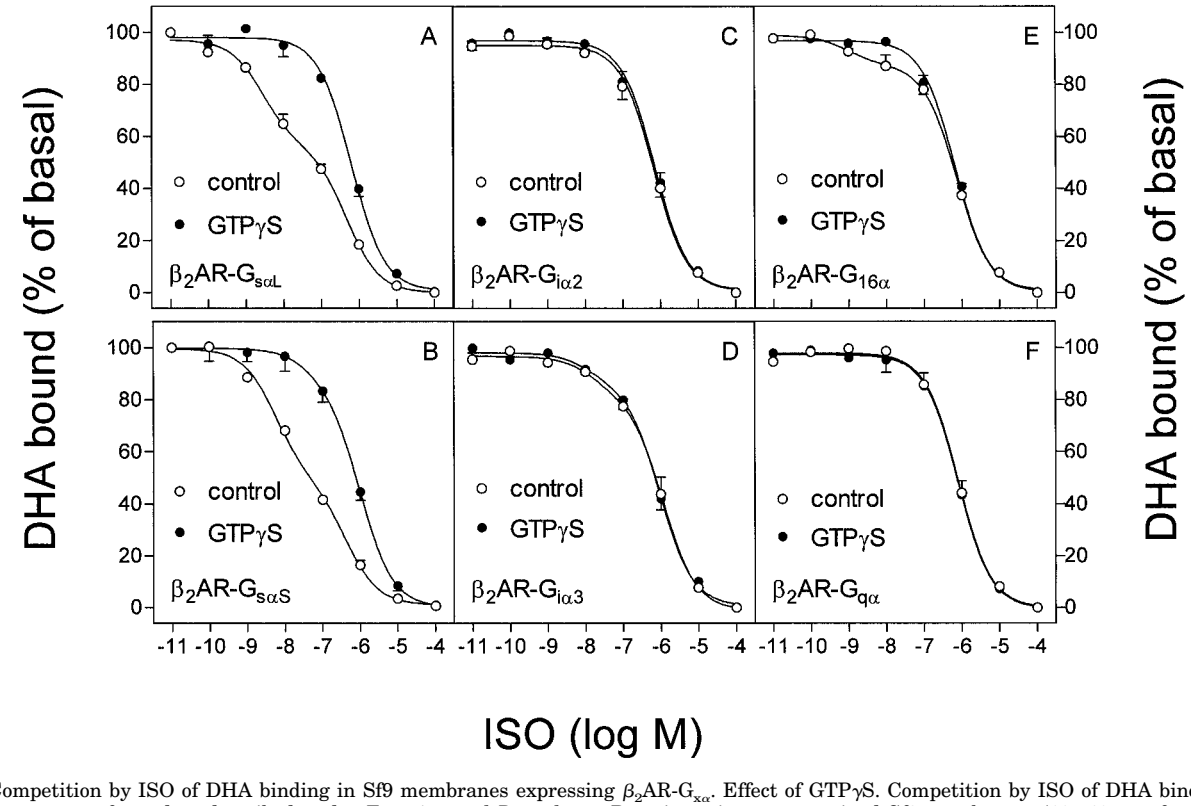

Fig. 2. Competition by ISO of DHA binding in Sf9 membranes expressing β_2AR-$G_{x\alpha}$. Effect of GTPγS. Competition by ISO of DHA binding in Sf9 membranes was performed as described under *Experimental Procedures*. Reaction mixtures contained Sf9 membranes (10–40 μg of protein/tube) expressing the fusion proteins identified in A to F at 2.1 to 18.6 pmol/mg, 1 nM DHA, and ISO at the concentration indicated on the abscissa. The DHA binding observed in the absence of competitor is referred to as basal binding and was set 100%. Reaction mixtures additionally contained distilled water (control) or GTPγS (10 μM). Data points shown are the means ± S.D. for three to five experiments performed in triplicate. Agonist competition curves were analyzed for best fit to single-site and two-site competitions.

membranes expressing nonfused β_2AR. The B_{max} of ISO-stimulated GTPγS binding in Sf9 membranes expressing β_2AR was extremely low. The coupling factor of 0.01 implies that only one G-protein per 100 expressed β_2AR molecules was activated upon agonist stimulation (Table 1). The poor coupling of the β_2AR to insect cell G-proteins underlines the feasibility of the Sf9 cell system for GTPγS binding studies.

However, it should be emphasized that, despite poor coupling of the β_2AR to insect cell G-proteins, there is a high concentration of as yet poorly defined GTPγS binding sites in Sf9 cell membranes. This results in high basal GTPγS binding rates (Fig. 3). Although those GTPγS binding sites are irrelevant with respect to G-protein coupling of the β_2AR (Table 1), these binding sites reduce, nonetheless, the sensitivity of the GTPγS binding assay. Grünewald et al. (1996) reported high basal GTPγS binding in Sf9 membranes, too. To eliminate this background GTPγS binding and to analyze GTPγS binding to the different G-proteins under comparable conditions, we focused our attention on ligand-regulated GTPγS binding to fusion proteins. The validity of this approach and the assumption that basal GTPγS binding is irrelevant to fusion proteins is substantiated by the finding that the coupling factor for most fusion proteins was, as expected, ~1 (Table 1). We realize that for determination of absolute K_d values for GTPγS binding to G-proteins, it would have been more appropriate not to subtract basal GTPγS binding values, but the considerable background (in particular for β_2AR-$G_{i\alpha}$ and β_2AR-$G_{q\alpha}$ fusion proteins) prevented us from doing so. To take into consideration this limitation of our studies, we use the term apparent K_d value where appropriate. We already adopted the background subtraction approach to the analysis of GTPγS binding to $G_{i\alpha}$-proteins coupled to the FPR in the fused and nonfused state (Wenzel-Seifert et al., 1998a, 1999). Thus, our approach allows for relative comparison of apparent K_d values for GTPγS binding to different G-proteins coupled to the β_2AR and to the same G-protein coupled to the β_2AR and FPR.

TABLE 1

Kinetic data of ligand-regulated GTPγS binding of β_2AR-$G_{x\alpha}$ fusion proteins: comparison with nonfused β_2AR, β_2AR coexpressed with $G_{i\alpha2}$, FPR-$G_{i\alpha2}$, and FPR coexpressed with $G_{i\alpha2}$

GTPγS binding was determined as described under *Experimental Procedures*. Sf9 membranes (10 μg protein/tube) expressing β_2AR-$G_{s\alpha}$ (3.7–8.6 pmol/mg) were incubated for 45 min in the presence of 0.1 to 1 nM [^{35}S]GTPγS plus unlabeled GTPγS at different concentrations to give the final GTPγS concentrations of 0.1 to 10 nM. Experiments with membranes expressing nonfused β_2AR (7.6–12.2 pmol/mg; 30 μg protein/tube) were performed under the same conditions as experiments with β_2AR-$G_{s\alpha}$ fusion proteins. GTPγS binding was stimulated with ISO (10 μM) or inhibited by ICI (1 μM). GTPγS saturation binding of β_2AR-$G_{i\alpha}$ was performed in membranes (42–71 μg protein/tube) expressing fusion proteins at 4.0 to 5.4 pmol/mg with GTPγS at 1 to 300 nM (1 nM [^{35}S]GTPγS plus various concentrations of unlabeled GTPγS). The incubation time was 60 min. GTPγS saturation with membranes (40 μg protein/tube) expressing β_2AR (2.3 pmol/mg) plus $G_{i\alpha2}$ (~300 pmol/mg) was performed as for membranes expressing β_2AR-$G_{i\alpha2}$. GTPγS saturation binding of β_2AR-$G_{16/q\alpha}$ was performed in membranes (41–81 μg/tube) expressing fusion proteins at 3.7–13.0 pmol/mg with GTPγS at 1–300 nM (1 nM [^{35}S]GTPγS plus various concentrations of unlabeled GTPγS). The incubation time was 3 h. β_2AR ligand-regulated GTPγS-binding saturation curves were analyzed by nonlinear regression and fit best to a one-site-binding hyperbola. The coupling factor is defined as the ratio of the B_{max} of ligand-regulated GTPγS binding and the B_{max} of receptor antagonist binding of a given membrane. Data shown are the means ± SD of two to three experiments performed in triplicates. The data for FPR-$G_{i\alpha2}$ and FPR + $G_{i\alpha2}$ were taken from Wenzel-Seifert et al. (1999). In case of the FPR, cyclosporin H was used as inverse agonist.

Construct	Apparent K_d of Agonist-Stimulated GTPγS Binding	Apparent K_d of Inverse Agonist-Inhibited GTPγS Binding	Coupling Factor
	nM		
β_2AR	2.4 ± 2.3	—[a]	0.01 ± 0.01
β_2AR-$G_{s\alpha L}$	0.4 ± 0.1	4.2 ± 0.5	1.12 ± 0.10
β_2AR-$G_{s\alpha S}$	0.7 ± 0.2	—	0.95 ± 0.08
β_2AR-$G_{i\alpha2}$	63.9 ± 5.7	—	1.05 ± 0.09
β_2AR + $G_{i\alpha2}$	18.9 ± 5.8	—	0.91 ± 0.35
β_2AR-$G_{i\alpha3}$	79.8 ± 9.0	—	0.95 ± 0.10
β_2AR-$G_{16\alpha}$	4.4 ± 2.1	—	0.53 ± 0.02
β_2AR-$G_{q\alpha}$	19.1 ± 1.4	—	0.08 ± 0.03
FPR-$G_{i\alpha2}$	0.9 ± 0.6	1.2 ± 0.3	~1[b]
FPR + $G_{i\alpha2}$	0.8 ± 0.2	1.8 ± 0.7	~1.0–1.5[b]

[a] —, ICI-inhibited GTPγS binding was minimal. Therefore, apparent K_d values could not be calculated.

[b] Coupling factors for FPR-$G_{i\alpha2}$ interaction can only be given approximately because FPR expression level cannot be precisely determined by receptor antagonist binding (Wenzel-Seifert et al., 1999).

Time Course of GTPγS Binding to β_2AR-$G_{x\alpha}$: Comparison with the FPR/$G_{i\alpha2}$ Pair and Possible Physiological Implications. We studied the time course of GTPγS binding to β_2AR$G_{X\alpha}$ fusion proteins in the presence and absence of a saturating concentration of ISO. The rate of GTPγS association to G-proteins is determined by the rate of GDP release (Gilman, 1987; Higashijima et al., 1988, 1990). In membranes expressing β_2AR-$G_{s\alpha L}$, ISO decreased the apparent $t_{1/2}$ of GTPγS association about 7-fold (Fig. 3A). However, GTPγS binding at late time points of the reaction was no longer stimulated by ISO. In our previous study we showed that the β_2AR coupled to $G_{s\alpha L}$ possesses constitutive activity, i.e., even the agonist-free receptor can efficiently promote GDP release from $G_{s\alpha L}$ (Seifert et al., 1998b). Thus, we assumed that the lack of agonist effect on GTPγS binding to β_2AR-$G_{s\alpha L}$ at late time points of the binding reaction reflects the ability of the agonist-free β_2AR to efficiently promote GTPγS binding to the G-protein. To validate this assumption, we studied the effect of the inverse agonist ICI on GTPγS binding. As expected, ICI substantially inhibited GTPγS binding to β_2AR-$G_{s\alpha L}$, particularly at late time points of the reaction (Fig. 3A).

ISO also accelerated GTPγS binding in membranes expressing β_2AR-$G_{s\alpha S}$, but in contrast to membranes expressing β_2AR-$G_{s\alpha L}$, a stimulatory effect of ISO was evident even at late time points of the binding reaction (Fig. 3B). We also noted that the $t_{1/2}$ of ISO-stimulated GTPγS binding to $G_{s\alpha S}$ was about twice as high as for $G_{s\alpha L}$. These data can be interpreted in that the agonist-occupied β_2AR catalyzes GDP release from $G_{s\alpha S}$ with a slower rate than from $G_{s\alpha L}$ (Seifert et al., 1998b). ICI had only a minimal inhibitory effect on GTPγS binding to β_2AR-$G_{s\alpha S}$ (data not shown).

With respect to β_2AR-$G_{i\alpha2}$, β_2AR-$G_{i\alpha3}$, β_2AR-$G_{q\alpha}$, and β_2AR-$G_{16\alpha}$, a significant stimulatory effect of ISO on GTPγS binding was detected at all time points of the reaction. In contrast to β_2AR-$G_{s\alpha}$ fusion proteins, there was no significant (β_2AR-$G_{i\alpha2}$, β_2AR-$G_{q\alpha}$; Fig. 3, C and F) or only a small decrease (β_2AR-$G_{i\alpha3}$, β_2AR-$G_{16\alpha}$; Fig. 3, D and E) of the $t_{1/2}$ of basal GTPγS association by ISO. In addition, the $t_{1/2}$ values for ISO-stimulated GTPγS binding to membranes expressing β_2AR-$G_{i\alpha}$ and β_2AR-$G_{q\alpha}$ fusion proteins were at least 3-fold higher than for β_2AR-$G_{s\alpha}$ fusion proteins. These data indicate that the β_2AR promotes guanine nucleotide exchange at G_i- and G_q-proteins much more slowly than at G_s-proteins.

The $t_{1/2}$ of FPR agonist-stimulated GTPγS binding to fused and nonfused $G_{i\alpha2}$ is much lower (~5 min) (Wenzel-Seifert et al., 1998a, 1999) than the $t_{1/2}$ of β_2AR-stimulated GTPγS binding to fused $G_{i\alpha2}$ (~45 min) (Fig. 3C), indicating that the FPR promotes guanine nucleotide exchange at $G_{i\alpha2}$ much more rapidly than the β_2AR. Based on these findings, one can assume that cellular responses mediated by the G_i- and G_q-

protein-coupled β_2AR are slower in onset than responses mediated by the G_s-coupled β_2AR and the G_i-coupled FPR. The different kinetics of receptor-G-protein interaction could thus generate intracellular signals in a timely, ordered fashion.

GTPγS Saturation Binding to β_2AR-$G_{s\alpha S}$ and β_2AR-$G_{s\alpha L}$: The β_2AR Bound to Inverse Agonist May Actively Reduce the Apparent GTPγS Affinity of $G_{s\alpha}$. Figure 4A shows a typical GTPγS saturation experiment for β_2AR-$G_{s\alpha L}$, and Table 1 provides a summary of the GTPγS saturation binding experiments for all fusion proteins studied. Because of the high constitutive activity of the β_2AR coupled to $G_{s\alpha L}$ (see Fig. 3A) (Seifert et al., 1998b), GTPγS saturation binding studies for β_2AR-$G_{s\alpha L}$ were performed in the presence of ISO and ICI. Moreover, reactions were conducted for 45 min only to detect both agonist and inverse agonist effects on GTPγS binding. We are aware of the fact that after an incubation time of 45 min, an equilibrium of the binding reaction is not yet reached, but at later time points, it becomes increasingly difficult to analyze the effect of an agonist on GTPγS binding to $G_{s\alpha L}$. The apparent K_d values of ISO-stimulated GTPγS binding to β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$ were in the subnanomolar range (0.4–0.7 nM). ICI reduced the apparent affinity of $G_{s\alpha L}$ for GTPγS by about 10-fold (apparent K_d, 4.2 nM). For both β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$, the coupling factor was ~1, indicating that the fused β_2AR interacts efficiently with its fused $G_{s\alpha}$ partner.

To address the question whether the differential regulation by agonist and inverse agonist of the apparent GTPγS affinity of $G_{s\alpha}$ was an artifact induced by the specific incubation time chosen (45 min), we also determined the apparent K_d values for GTPγS after a 3-h incubation, i.e., when the

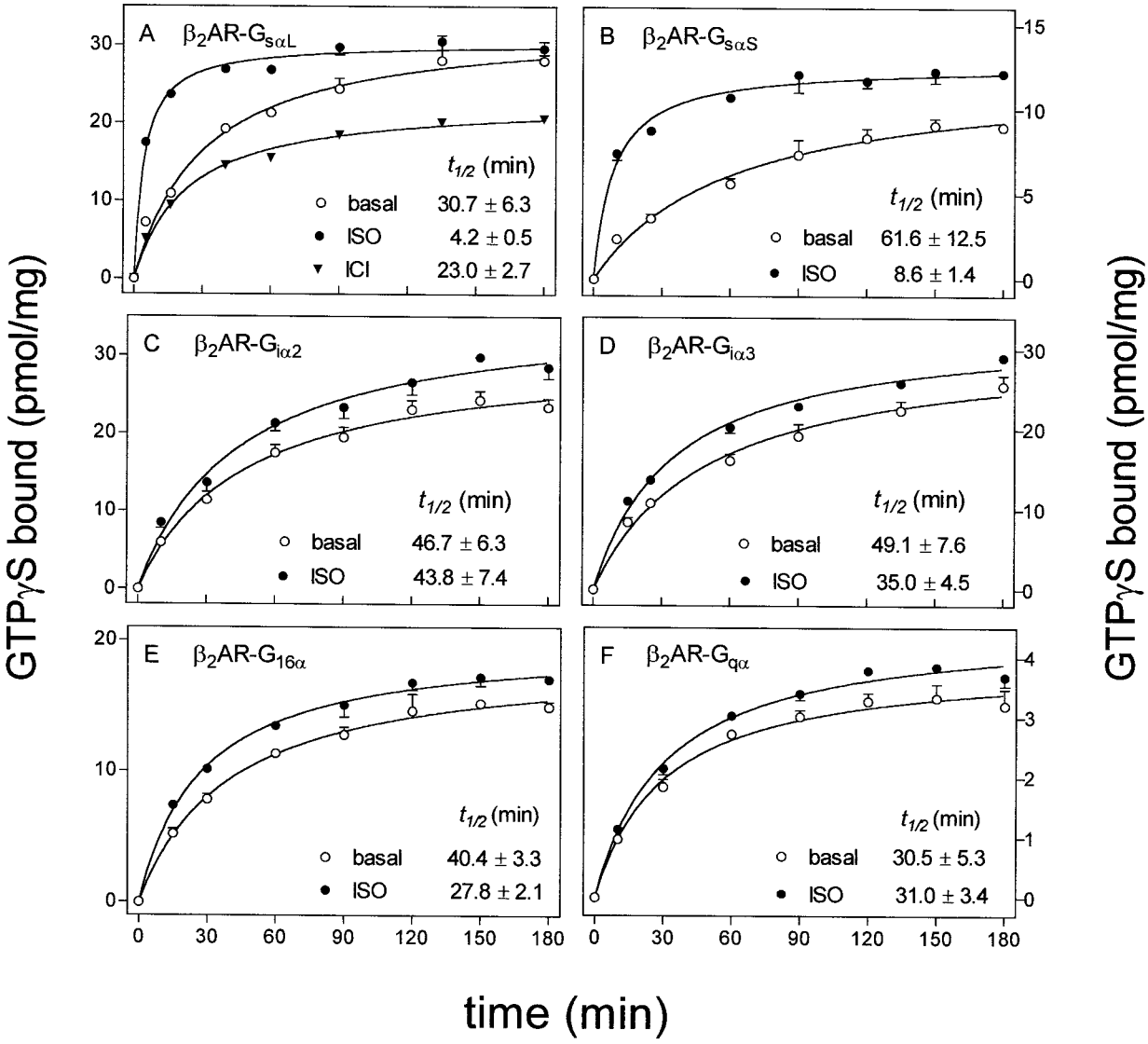

Fig. 3. Time course of GTPγS binding in Sf9 membranes expressing β_2AR-$G_{x\alpha}$. A, Sf9 membranes expressing β_2AR-$G_{s\alpha L}$ (10.6 pmol/mg) were incubated for the indicated periods in the presence of 1 nM [^{35}S]GTPγS plus 9 nM unlabeled GTPγS, 1 μM GDP, and distilled water (basal), ISO (10 μM), or ICI (1 μM). B, Sf9 membranes expressing β_2AR-$G_{s\alpha S}$ (4.6 pmol/mg) were incubated for the indicated periods in the presence of 1 nM [^{35}S]GTPγS plus 9 nM unlabeled GTPγS, 1 μM GDP, and distilled water (control) or ISO (10 μM). C to F, Sf9 membranes expressing β_2AR-Gi$\alpha_{2/3}$ (4.0–8.2 pmol/mg) or β_2AR-$G_{q/16\alpha}$ (2.1–3.4 pmol/mg) were incubated for the indicated periods with 2 nM [^{35}S]GTPγS plus 48 nM unlabeled GTPγS, 1 μM GDP, and distilled water (control) or ISO (10 μM). At the appropriate times, 200 μl-aliquots of reaction mixtures containing 15 to 55 μg of protein were removed and filtered. For further details, see *Experimental Procedures*. Data shown are the means ± S.D. of three experiments. Data were best fit to monophasic saturation hyperbolae.

binding reaction had reached a plateau. As already indicated above, ISO did not stimulate GTPγS binding to $G_{s\alpha L}$ under these conditions but ICI was still inhibitory (apparent K_d value of GTPγS binding, 4.9 ± 1.0 nM, mean ± S.D., $n = 3$). The apparent K_d value for ISO-stimulated GTPγS binding to $G_{s\alpha S}$ after a 3-h incubation was 0.5 ± 0.4 nM (mean ± S.D., $n = 3$). These values compare very favorably with the values obtained after a 45-min incubation (Table 1) and show that the differences in the apparent affinities of $G_{s\alpha}$ for GTPγS in the presence of the β_2AR bound to agonist and inverse agonist are observed at short and long incubation times. The differential regulation of the apparent GTPγS affinity of $G_{s\alpha}$ by the β_2AR bound to inverse agonist and agonist is intriguing because it indicates that the β_2AR bound to an inverse agonist actively reduces the apparent GTPγS affinity of $G_{s\alpha}$. The conclusion that the β_2AR bound to ICI actively regulates nucleotide affinities of $G_{s\alpha}$ is supported by the findings that ICI increases the K_m value of the steady-state GTPase of β_2AR-$G_{s\alpha L}$ (Seifert et al., 1998a) and that ICI stimulates the binding of xanthosine 5′-triphosphate to $G_{s\alpha L}$ (Seifert et al., 1999a). Thus, our data suggest that the inverse agonist stabilizes a specific conformation in the β_2AR that actively regulates nucleotide-affinities of G_s-proteins. Evidence for the existence of specific active states of receptors bound to inverse agonists was also obtained for cannabinoid receptors (Bouaboula et al., 1997, 1999).

Partial Agonists Reduce the Apparent GDP Affinity of $G_{s\alpha}$ Less Efficiently Than Full Agonists. The differential regulation of the apparent GTPγS affinity of $G_{s\alpha}$ by inverse agonists and full agonists raised the intriguing question of whether partial agonists increase the apparent GTPγS affinity of $G_{s\alpha}$ to a lesser extent than a full agonist. To address the question, we determined the apparent K_d value of GTPγS binding to $G_{s\alpha S}$ after a 3-h incubation, using the partial agonist dobutamine (DOB). The apparent K_d value of DOB-stimulated GTPγS binding to $G_{s\alpha S}$ was 0.7 ± 0.5 nM (mean ± SD, $n = 3$) and not significantly different from the apparent K_d value obtained for ISO (0.5 ± 0.4 nM). These data show that full and partial agonists do not differ from each other in their ability to alter the apparent GTPγS affinity of $G_{s\alpha}$. In agreement with our data, partial agonists of the α_2-adrenoceptor also do not differ from full agonists with respect to the K_m of high-affinity GTP hydrolysis, i.e., there is no differential regulation of GTP affinity of G-proteins by full versus partial agonists (Wise et al., 1997).

To identify differences in the regulation of GTPγS binding

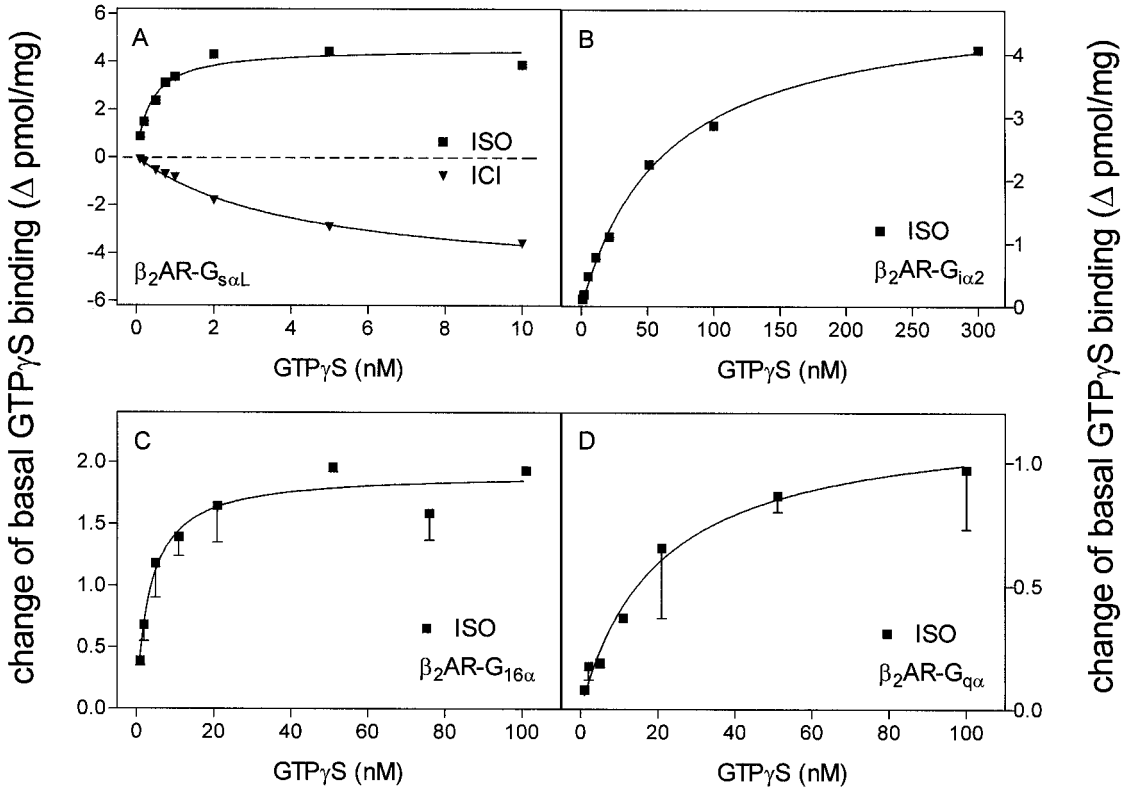

Fig. 4. Effects of ISO and ICI on GTPγS saturation binding in Sf9 membranes expressing β_2AR-$G_{x\alpha}$. A, Sf9 membranes (10 μg of protein/tube) expressing β_2AR-$G_{s\alpha L}$ (8.6 pmol/mg) were incubated for 45 min in the presence of 0.1 to 1 nM [^{35}S]GTPγS plus unlabeled GTPγS at different concentrations to give the final concentrations indicated on the abscissa, 1 μM GDP, and distilled water (control), ISO (10 μM), or ICI (1 μM). B, Sf9 membranes (50 μg of protein/tube) expressing β_2AR-$G_{i\alpha 2}$ (4.1 pmol/mg) were incubated for 60 min in the presence of 1 nM [^{35}S]GTPγS plus unlabeled GTPγS at different concentrations to give the final concentrations indicated on the abscissa, 1 μM GDP and distilled water (control) and ISO (10 μM). C and D, Sf9 membranes (55–70 μg of protein/tube) expressing β_2AR-$G_{16\alpha}$ (3.7 pmol/mg) or β_2AR-$G_{q\alpha}$ (13.6 pmol/mg) were incubated for 3 h in the presence of 1 nM [^{35}S]GTPγS plus unlabeled GTPγS at different concentrations to give the final concentrations indicated on the abscissa, 1 μM GDP, and distilled water (control) and ISO (10 μM). Nonspecific GTPγS binding was determined in the presence of 10 μM unlabeled GTPγS. For each GTPγS concentration, the basal GTPγS binding was subtracted from GTPγS binding observed in the presence of ISO to calculate the increase in GTPγS binding caused by ISO. In addition, for β_2AR-$G_{s\alpha L}$, GTPγS binding observed in the presence of ICI were subtracted from basal GTPγS binding values to calculate the decrease of GTPγS binding caused by ICI. The dashed line in A is the extrapolation of basal GTPγS binding. Data were best fit to a single-site saturation hyperbola. For further details, see *Experimental Procedures.* Data shown are the means ± S.D. of a representative experiments. Each experiment was performed at least three times. The kinetics of GTPγS binding are summarized in Table 1.

stimulated by full and partial agonists, we studied the effects of DOB and DCI (Seifert et al., 1998b) on the time course of GTPγS binding to β_2AR-$G_{s\alpha S}$. The rank order of efficacy of ligands at decreasing $t_{1/2}$ of GTPγS binding to $G_{s\alpha}$ and at saturating $G_{s\alpha}$ with GTPγS was ISO > DOB > DCI (Fig. 5A). Our data clearly show that partial agonists promote guanine nucleotide exchange less efficiently than full agonists, but differential effects of ligands on the apparent GTPγS affinity of $G_{s\alpha}$ do not explain these differences.

A recent study of cannabinoid receptors demonstrated that a major difference between full and partial agonists is that partial agonists decrease the apparent GDP affinity of G-proteins less efficiently than full agonists (Breivogel et al., 1998). In fact, with increasing GDP concentrations, the efficacy of partial agonists at promoting GTPγS binding decreases relative to the efficacy of a full agonist. Using a very similar experimental protocol as reported for cannabinoid receptors (Breivogel et al., 1998), we found that the efficacy of the partial agonists DOB and DCI at promoting GTPγS binding to $G_{s\alpha S}$ decreased with increasing GDP concentration

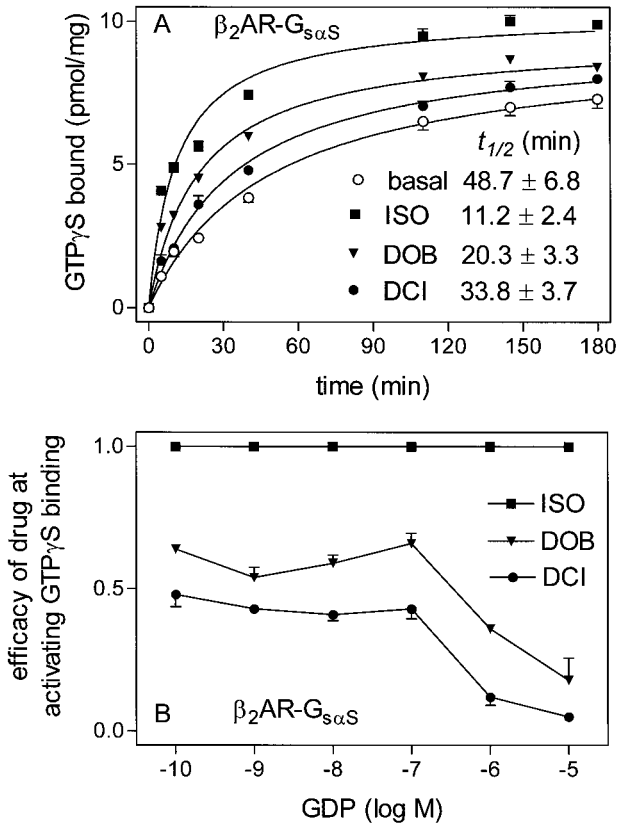

Fig. 5. Analysis of the effects of partial and full agonists on GTPγS binding in membranes expressing β_2AR-$G_{s\alpha S}$. A, Sf9 membranes (15 μg of protein/tube) expressing β_2AR-$G_{s\alpha S}$ (3.5 pmol/mg) were incubated for the indicated periods in the presence of 1 nM [^{35}S]GTPγS plus 9 nM unlabeled GTPγS, 1 μM GDP, and distilled water (basal), ISO (10 μM), DOB (100 μM), or DCI (10 μM). Data shown are the means ± S.D. of three experiments. Data were best fit to monophasic saturation hyperbolae. B, Sf9 membranes (15 μg of protein/tube) expressing β_2AR-$G_{s\alpha S}$ (3.5 pmol/mg) were incubated for 60 min in the presence of 0.4 nM [^{35}S]GTPγS, GDP at various concentrations and distilled water (basal), ISO (10 μM), DOB (100 μM) or DCI (10 μM). The efficacies of DOB and DCI at stimulating GTPγS binding at each GDP concentration were referred to the stimulatory effect of ISO which was set 1.0. Data shown are the means ± S. D. of three experiments. All experiments shown were performed with membranes expressing β_2AR-$G_{s\alpha S}$ without coexpressed $\beta_1\gamma_2$-complex.

(Fig. 5B). These data support the concept that agonist efficacy is related to the ability of ligands to reduce the apparent GDP affinity of $G_{x\alpha}$.

Differential Regulation of the Apparent GTPγS Affinity of G-Proteins by Receptors. The K_d value of GTPγS binding to purified $G_{s\alpha}$ is ~175 to 1750 higher (0.7 μM) (Northup et al., 1982) than the apparent K_d values of GTPγS binding to receptor-coupled $G_{s\alpha}$ (0.4–4.2 nM). These data suggest that a receptor can dramatically increase the GTPγS affinity of $G_{s\alpha}$. By analogy, the K_d value for GTPγS binding to purified G_i-proteins is ~50 to 100 nM (Carty et al., 1990), whereas the apparent K_d value for FPR-regulated GTPγS binding to G_i-proteins is ~0.7 to 1.8 nM (Table 1) (Wenzel-Seifert et al., 1998a, 1999). In addition, the β_2AR substantially increases the apparent affinity of $G_{q\alpha}$ for GTPγS compared with purified $G_{q\alpha}$ (Table 1) (Hepler et al., 1993; Chidiac et al., 1999). Thus, the data obtained for various receptors and classes of G-proteins suggest that receptors can induce a conformational change in the $G_{x\alpha}$ that increases the affinity of the G-protein for GTPγS (and presumably for the natural nucleotide GTP) considerably. Our data are in agreement with the concept that GTP/GTPγS binding does not passively follow GDP release but that receptors actively promote GTPγS binding to G-proteins (Iiri et al., 1998). Another factor that can contribute to the large differences in apparent GTPγS affinities in various systems is that purified G-proteins and G-proteins in membranes can exhibit quite different properties (Gierschik et al., 1991).

The above-discussed data raise the question whether different receptors coupled to the same G-protein alter its apparent GTPγS affinity in the same way. To address this question, we compared coupling of the β_2AR and the FPR, a prototypical G_i-protein-coupled receptor (Wenzel-Seifert et al., 1998a, 1999), to fused and nonfused $G_{i\alpha 2}$. The apparent K_d value of FPR agonist-stimulated GTPγS binding to fused and nonfused $G_{i\alpha 2}$ is ~1 nM (Table 1). The agonist-occupied β_2AR also catalyzed GTPγS binding to fused and nonfused $G_{i\alpha 2}$, but the apparent K_d values of agonist-stimulated GTPγS binding to $G_{i\alpha 2}$ were ~25 to 70 times higher for the β_2AR than for the FPR (Fig. 4B and Table 1). The apparent K_d values of ISO-stimulated GTPγS binding to β_2AR-$G_{i\alpha}$ fusion proteins are similar to the GTPγS affinity of purified G_i-proteins (Table 1) (~50–100 nM) (Carty et al., 1990). These data suggest that the FPR efficiently increases the GTPγS affinity of G_i-proteins, whereas the β_2AR does not. Thus, our data raise the intriguing hypothesis that receptor-specific G-protein conformational states exist that differ from each other in their GTPγS affinity. The molecular basis for such a receptor memory of G-proteins could be differences in the G-protein-coupling domains of various receptors. In fact, the G-protein-coupling domains of the β_2AR and FPR are quite different (Kobilka, 1992; Miettinen et al., 1999).

It is unknown why various receptors differ from each other with respect to regulation of the apparent GTPγS/GTP affinity of a given G-protein. One might assume that, because of the high intracellular GTP concentration (~50 μM) (Otero, 1990), GTP can readily saturate all G-proteins, even if they are in a state of low GTP affinity. There is, however, evidence for constrained access of GTP to $G_{x\alpha}$ in native membrane systems (Wieland and Jakobs, 1992; Klinker et al., 1994). Thus, it is possible that in vivo the GTP affinity of G-proteins critically determines their efficiency as signal transducers.

Stoichiometry of β_2AR/G_i-Protein Coupling. Inefficient β_2AR-induced increase in the apparent GTPγS affinity of G_i-proteins does not imply that the β_2AR is inefficient at activating G_i-proteins. Indeed, the coupling factor in β_2AR-$G_{i\alpha 2}$ and β_2AR-$G_{i\alpha 3}$ was ~1, indicating that all β_2AR molecules activated their fused $G_{i\alpha}$ partner (Table 1).

One nonfused FPR molecule activates ~1.0 to 1.5 G_i-proteins, i.e., there is rather linear signal transfer from the receptor to the G-protein (Wenzel-Seifert et al., 1999). These findings raise the question about the number of G_i-proteins activated by the nonfused β_2AR. The stoichiometry of receptor to G_i-proteins was ~1:100 for both the β_2AR- and FPR-Sf9 cell coexpression systems (Fig. 1B) (Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999). This stoichiometry reflects the in vivo expression stoichiometry of signaling components (Ransnäs and Insel, 1988; Gierschik et al., 1991; Seifert et al., 1998a; Wenzel-Seifert et al., 1998a, 1999). Of interest, one nonfused β_2AR molecule activated approximately one G_i-protein molecule (Table 1). These data show that the nonfused β_2AR and FPR activate a similar number of G_i-proteins, i.e., linear G_i-protein activation is not restricted to the FPR. Additionally, our data on β_2AR/G_i-protein coupling support the recent conclusion that the 1:1 stoichiometry of receptor and G-protein in fusion proteins reflects the in vivo stoichiometry of receptor-G-protein coupling more closely than was previously assumed (Seifert et al., 1999c; Milligan, 2000). These results also underline the usefulness and relevance of fusion proteins as systems to analyze receptor-G-protein coupling.

Incomplete GTPγS Saturation Binding to β_2AR-$G_{q\alpha}$ Fusion Proteins Can Be Explained by Rapid GTPγS Dissociation: Role of Guanine Nucleotide Dissociation as Mechanism of G-Protein Deactivation and Implications for Receptor/G-Protein Coupling. The coupling factor in β_2AR-$G_{16\alpha}$ and particularly in β_2AR-$G_{q\alpha}$ was much lower than ~1 (Table 1). An explanation for these findings could be that GTPγS dissociates from G_q-proteins much more rapidly than from G_s- and G_i-proteins. In fact, GTPγS and other GTPase-resistant guanine nucleotides can dissociate from various classes of G-proteins, including G_q-proteins (Cassel and Selinger, 1977; Higashijima et al., 1990; Berstein et al., 1992; Kupprion et al., 1993; Breivogel et al., 1998; Chidiac et al., 1999). To address this issue, fusion proteins were loaded with [^{35}S]GTPγS for 1 h in the absence of ISO to avoid interference with agonist-induced dissociation. [^{35}S]GTPγS dissociation was then stimulated by the addition of unlabeled GTPγS at a large molar excess in the absence or presence of ISO to reaction mixtures. Using this protocol, we could clearly detect time-dependent basal [^{35}S]GTPγS dissociation in membranes expressing β_2AR-$G_{s\alpha L}$, β_2AR-$G_{16\alpha}$, and β_2AR-$G_{16\alpha}$ (Fig. 6). Of interest, basal [^{35}S]GTPγS dissociation proceeded about three times faster in membranes expressing β_2AR-$G_{16\alpha}$ and β_2AR-$G_{q\alpha}$ (Fig. 6, B and C) than in membranes expressing β_2AR-$G_{s\alpha}$ (Fig. 6A). We did not observe any effect of ISO on [^{35}S]GTPγS dissociation in membranes expressing β_2AR-$G_{16\alpha}$ and β_2AR-$G_{q\alpha}$, presumably because the basal GTPγS dissociation rate from these fusion proteins is already high. These data support the notion that rapid dissociation of [^{35}S]GTPγS from β_2AR-$G_{16\alpha}$ and β_2AR-$G_{q\alpha}$ prevents these G-proteins from binding GTPγS in stoichiometric amounts.

It is generally assumed that GTPγS binding to G-proteins is quasi-irreversible (Gilman, 1987). Thus, on first glance, it may seem most unexpected that ISO decreased the $t_{1/2}$ of GTPγS dissociation from β_2AR-$G_{s\alpha L}$ by about 6-fold. Intriguingly, in very early studies it had already been observed that ISO induced dissociation of [^{3}H]guanylyl imidodiphosphate from $G_{s\alpha}$ in turkey erythrocyte membranes (Cassel and Selinger, 1977). However, those early studies were not followed up later. The findings that 1 mol of β_2AR-$G_{s\alpha}$ bound 1 mol of

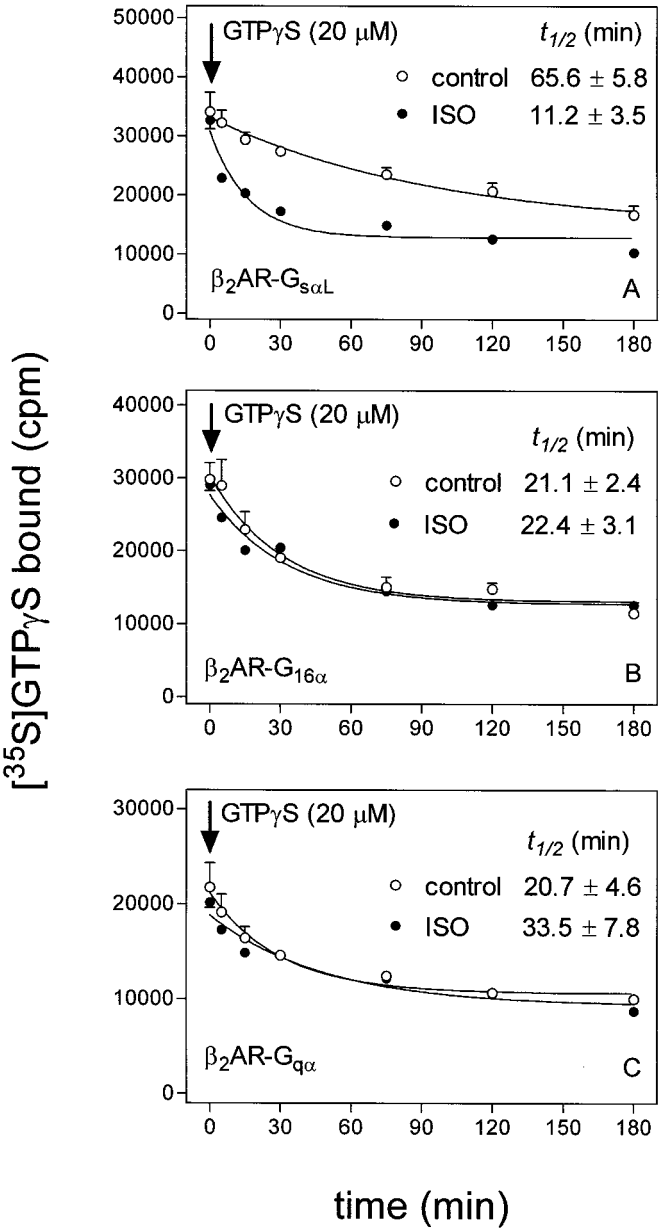

Fig. 6. GTPγS dissociation from β_2AR-$G_{x\alpha}$ fusion proteins in Sf9 membranes. Sf9 membranes expressing β_2AR-$G_{s\alpha L}$ (8.6 pmol/mg, 20 μg of protein/tube, A), β_2AR-$G_{16\alpha}$ (3.7 pmol/mg, 36 μg of protein/assay, B) or β_2AR-$G_{q\alpha}$ (12.4 pmol/mg, 36 μg of protein per tube, C) were loaded with [^{35}S]GTPγS in the absence of agonist as described under *Experimental Procedures*. Dissociation of [^{35}S]GTPγS was initiated by the addition of an excess of unlabeled GTPγS (20 μM) at t = 0 min in the presence of distilled water (control) or ISO (10 μM). Dissociation was allowed to proceed for the times indicated on the abscissa. At the appropriate times, 150 μl-aliquots of reaction mixtures containing 20 to 36 μg of protein were removed and filtered. Data were best fit to monophasic dissociation curves. Data shown are the means ± S.D. of three experiments.

GTPγS in the GTPγS saturation binding studies and that the affinity of β_2AR-coupled $G_{s\alpha}$ for GTPγS is very high (Fig. 4A and Table 1) are in stark contrast to the rapid agonist-induced GTPγS dissociation. The fact that the $t_{1/2}$ of ISO-stimulated GTPγS binding was only moderately lower than the $t_{1/2}$ of ISO-stimulated GTPγS dissociation suggests that GTPγS dissociation occurs already in the initial phase of the GTPγS association experiment and, therefore, delays net GTPγS association.

The GTPγS dissociation studies bear important implications for the mechanism by which G-proteins are deactivated. It is generally accepted that the hydrolysis of GTP to GDP and inorganic phosphate determines the transition of the G-protein from the active to the inactive state (Gilman, 1987; Iiri et al., 1998). However, in a recent study we observed dissociations in the efficacies of β_2AR agonists at supporting adenylyl cyclase activation in the presence of inosine 5′-triphosphate and their efficacy at hydrolyzing inosine 5′-triphosphate (Seifert et al., 1999a). In addition, xanthosine 5′-triphosphate supports β_2AR-mediated adenylyl cyclase activation, but xanthosine 5′-triphosphate is not hydrolyzed (Seifert et al., 1999a). Taken together, all these data indicate that nucleotide dissociation is an important mechanism of G-protein deactivation.

The observation of highly efficient β_2AR-stimulated GTPγS dissociation from $G_{s\alpha L}$ helps us understand the physical interaction of receptors and G-proteins. These data suggest that $G_{s\alpha}$ bound to GTPγS is in physical contact with the β_2AR, despite the reduction of agonist affinity of the β_2AR by GTPγS (Figs. 2 and 6). Evidence for continuous physical contact between receptor and G-protein during the entire G-protein cycle was already obtained for muscarinic acetylcholine receptors (Matesic et al., 1989). GTPγS-insensitive ternary complex formation also argues for contact of the β_2AR with its G-protein partner during the entire G-protein cycle (see Fig. 2) (Szele and Pritchett, 1993; Gürdal et al., 1997; Seifert et al., 1998a). Thus, in contrast to the generally held opinion (Gilman, 1987; Iiri et al., 1998), G-protein deactivation may be a step in the G-protein cycle that is under the direct control of the receptor. Evidence for direct regulation of nucleoside 5′-triphosphate dissociation and hydrolysis of $G_{s\alpha}$ by the β_2AR was also provided by two previous studies from our group (Wenzel-Seifert et al., 1998b; Seifert et al., 1999b).

Pharmacological Profile of β_2AR-$G_{x\alpha}$ Fusion Proteins: Evidence for Ligand-Specific Conformations of the β_2AR with Different G-Protein Coupling. It has been shown that in some systems the pharmacological profile of a receptor depends on the specific G-protein to which the receptor is coupled (Eason et al., 1994; Gettys et al., 1994; Gurwitz et al., 1994). These findings can be interpreted to mean that specific ligands stabilize ligand-specific receptor conformations that differ from each other in their ability to activate different G-proteins. To address this hypothesis, we determined the effects of the β_2AR agonists ISO, SAL, DOB, EPH, and DCI and the inverse agonist ICI on GTPγS binding to β_2AR-$G_{x\alpha}$ fusion proteins. We assessed both ligand efficacies and ligand potencies.

Efficacies of ligands at β_2AR-$G_{x\alpha}$ fusion proteins were analyzed in two ways (Table 2). First, we analyzed the efficacies of a given ligand at the various fusion proteins. Second, we analyzed the rank order of efficacies of agonists at the different fusion proteins. The efficacies of a given ligand varied largely at the different fusion proteins (0.55–1.03 for SAL; 0.40–0.98 for DOB; 0.26–0.98 for EPH; 0.08–0.67 for DCI and −0.15–0.00 for ICI). The efficacies of SAL and EPH at β_2AR-$G_{q\alpha}$ were considerably higher than at β_2AR-$G_{s\alpha S}$, β_2AR-$G_{i\alpha 2}$, and β_2AR-$G_{i\alpha 3}$, whereas the efficacy of DCI was lower at β_2AR-$G_{q\alpha}$ than at β_2AR-$G_{s\alpha S}$, β_2AR-$G_{i\alpha 2}$, and β_2AR-$G_{i\alpha 3}$. Moreover, the rank order of efficacy of ligands at activating GTPγS binding to β_2AR-$G_{i\alpha 2}$ was ISO ≫ SAL > DOB ≈ EPH ≈ DCI, whereas the rank order of efficacy at β_2AR-$G_{i\alpha 3}$ was ISO ≫ SAL ≈ DOB > EPH > DCI. We also observed

TABLE 2

Efficacies of β_2AR ligands at stimulating GTPγS binding to β_2AR-$G_{x\alpha}$ fusion proteins

For determination of the efficacies of ligands, GTPγS binding was measured in Sf9 membranes expressing β_2AR-$G_{x\alpha}$ at 3.7 to 13.0 pmol/mg with 0.4 nM [^{35}S]GTPγS as described under *Experimental Procedures*. The incubation time was 45 min. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. The concentration-response curves were generated by nonlinear regression analysis, and the plateau values for each ligand were divided by the (maximal) plateau value of ISO to calculate the efficacies. The inhibitory effects of ICI are referred to the stimulatory effects of ISO. Data shown are the means ± S.D. values of three independent experiments performed in triplicates.

Construct	ISO	SAL	DOB	EPH	DCI	ICI
β_2AR-$G_{s\alpha L}$	1.00	1.03 ± 0.03	0.98 ± 0.06	0.87 ± 0.07	0.67 ± 0.08	−0.15 ± 0.01
β_2AR-$G_{s\alpha S}$	1.00	0.81 ± 0.04	0.68 ± 0.02	0.33 ± 0.10	0.19 ± 0.04	−0.05 ± 0.02
β_2AR-$G_{i\alpha 2}$	1.00	0.64 ± 0.10	0.40 ± 0.02	0.35 ± 0.02	0.29 ± 0.07	−0.02 ± 0.01
β_2AR-$G_{i\alpha 3}$	1.00	0.55 ± 0.03	0.52 ± 0.08	0.31 ± 0.02	0.15 ± 0.03	−0.05 ± 0.02
β_2AR-$G_{16\alpha}$	1.00	0.64 ± 0.13	0.35 ± 0.11	0.26 ± 0.08	0.08 ± 0.03	0.00 ± 0.01
β_2AR-$G_{q\alpha}$	1.00	1.00 ± 0.06	0.48 ± 0.03	0.48 ± 0.11	0.08 ± 0.04	−0.04 ± 0.04

TABLE 3

Potencies of β_2AR ligands at stimulating GTPγS binding to β_2AR-$G_{x\alpha}$ fusion proteins

For determination of the potencies of ligands, GTPγS binding was measured in Sf9 membranes expressing β_2AR-$G_{x\alpha}$ at 3.7 to 13.0 pmol/mg with 0.4 nM [^{35}S]GTPγS as described under *Experimental Procedures*. The incubation time was 45 min. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. The EC_{50} values are given in nM and were calculated by nonlinear regression. Data shown are the means ± S.D. values of three independent experiments performed in triplicates.

Construct	ISO	SAL	DOB	EPH	DCI
β_2AR-$G_{s\alpha L}$	1.7 ± 0.5	39 ± 23	90 ± 16	923 ± 270	13 ± 12
β_2AR-$G_{s\alpha S}$	6.2 ± 3.3	167 ± 78	310 ± 108	3600 ± 498	36 ± 15
β_2AR-$G_{i\alpha 2}$	31 ± 16	712 ± 401	1860 ± 890	1850 ± 500	15 ± 8
β_2AR-$G_{i\alpha 3}$	42 ± 18	809 ± 241	155 ± 90	917 ± 10	—[a]
β_2AR-$G_{16\alpha}$	143 ± 69	587 ± 311	595 ± 109	5990 ± 2130	—
β_2AR-$G_{q\alpha}$	23 ± 13	310 ± 132	540 ± 211	4670 ± 1110	—

[a] —, DCI-stimulated GTPγS binding was too small to calculate an EC_{50} value.

differences in agonist efficacies of the β_2AR coupled to G_q-proteins. Specifically, at β_2AR-$G_{16\alpha}$, ligands activated GTPγS binding in the order of efficacy ISO > SAL > DOB ≈ EPH > DCI. In contrast, the order of efficacy of ligands at β_2AR-$G_{q\alpha}$ was ISO ≈ SAL ≫ DOB ≈ EPH ≫ DCI. These data show that the efficacies of typical β_2AR ligands differ from each other, depending on to which G-protein the receptor is coupled. There are even differences in the pharmacological profile of the β_2AR coupled to different members of the same G-protein family, be it G_s-, G_i-, or G_q-proteins.

Differences in the pharmacological profile of the β_2AR coupled to different G-proteins were also evident upon analysis of ligand potencies (Table 3). We observed large variations in the potencies of a given ligand at stimulating GTPγS binding to the different fusion proteins. The variation of the EC_{50} values was 1.7 to 143 nM for ISO, 39 to 809 nM for SAL, 90 to 1860 nM for DOB, and 917 to 5990 nM for EPH. As was observed for agonist efficacies, the potencies of ligands did not vary systematically for different G-proteins. For example, at β_2AR-$G_{16\alpha}$, ISO and EPH exhibited a particularly low potency, whereas at β_2AR-$G_{i\alpha2}$, DOB showed a very low potency. We also observed variations in the rank order of potency of ligands at the different fusion proteins. At β_2AR-$G_{s\alpha}$ fusion proteins, the rank order of potency was ISO > SAL > DOB ≫ EPH. Intriguingly, the rank order of potency of ligands at β_2AR-$G_{i\alpha3}$ was different from the corresponding rank order at β_2AR-$G_{i\alpha2}$ (ISO > DOB ≫ SAL ≈ EPH at β_2AR-$G_{i\alpha3}$ versus ISO ≫ SAL > DOB ≈ EPH at β_2AR-$G_{i\alpha2}$). Taken together, our data clearly show that the pharmacological properties of the β_2AR depend on to what G-protein the β_2AR is coupled. These data are compatible with a model in which ligand-specific receptor conformations exist. Those ligand-specific receptor conformations differ from each other in their ability to activate different G-proteins. Our conclusions for the β_2AR are supported by data regarding the pharmacological profiles of the 5-hydroxytryptamine$_{1A}$ receptor, α_2-adrenoceptor, and muscarinic acetylcholine receptors coupled to different G-proteins (Eason et al., 1994; Gettys et al., 1994; Gurwitz et al., 1994).

Pertussis toxin uncouples agonist-free and agonist-occupied receptors from G_i-proteins (Gierschik et al., 1991; Wenzel-Seifert et al., 1998a). By analyzing the effect of pertussis toxin on the coupling of the β_2AR to G_i-proteins in cardiac myocytes, Xiao et al. (1999) concluded that, in this coupling situation, the β_2AR is not constitutively active. At β_2AR-$G_{i\alpha2}$ and β_2AR-$G_{i\alpha3}$, the efficacies and potencies of partial agonists were, in general, considerably smaller than at β_2AR-$G_{s\alpha L}$ (Tables 1 and 2). These findings corroborate the conclusion by Xiao et al. (1999) that the G_i-protein-coupled β_2AR is not constitutively active.

The analysis of the effects of agonists and inverse agonists at β_2AR-$G_{16\alpha}$ did also not provide evidence for constitutive activity of the β_2AR in this coupling setting (Tables 2 and 3). For β_2AR-$G_{q\alpha}$, we observed an unexpectedly high efficacy (but not potency) of SAL at activating GTPγS binding. However, this finding does not allow the conclusion that the β_2AR coupled to $G_{q\alpha}$ is constitutively active, because the analysis of the effects of partial agonists and inverse agonists did not reveal additional evidence for constitutive activity of the β_2AR in this coupling situation.

Conclusions

The fusion protein technique provides a rigorous approach for comparing the coupling of a given receptor to various G-protein α-subunits (Seifert et al., 1999c). It ensures a defined 1:1 stoichiometry of receptor and G-protein and promotes efficient coupling. By using this approach, we have shown that the β_2AR couples to G_s-, G_i-, and G_q-proteins as assessed by ternary complex formation and ligand-regulated GTPγS binding. The combined analysis of ternary complex formation, the kinetics of GTPγS binding, and agonist potencies and agonist efficacies revealed substantial differences in the interaction of the β_2AR with the various classes of G-proteins. Our data suggest the existence of ligand-specific receptor conformations that differ from each other in their ability to activate various G-proteins. In addition, our data on differential coupling of the β_2AR and FPR to G_i-proteins suggest that G-proteins can adopt receptor-specific conformations, i.e., G-proteins possess a receptor-memory. The 1:1 stoichiometry of receptor to G-protein in fusion proteins reflects the in vivo stoichiometry of receptor/G-protein coupling more closely than was previously assumed. Finally, GTPγS dissociation may be a much more important factor in G-protein deactivation than is generally appreciated.

Acknowledgments

We are most indebted to Dr. Brian Kobilka for numerous stimulating discussions, critical review of the paper, and generously providing laboratory space, equipment, and supplies. We thank the reviewers of this paper for very helpful suggestions, Drs. Hui-Yu Liu and Tae Weon Lee for help with the immunoblots, and Dr. George Traiger for critical reading of the manuscript. We also acknowledge the assistance of Maria Bakk with the cell culture.

References

Amatruda TT, Steele DA, Slepak VZ and Simon MI (1991) Gα16, a G protein α subunit specifically expressed in hematopoietic cells. *Proc Natl Acad Sci USA* **88:**5587–5591.

Asano T, Katada T, Gilman AG and Ross EM (1984) Activation of the inhibitory GTP-binding protein of adenylate cyclase, G_i, by β-adrenergic receptors in reconstituted phospholipid vesicles. *J Biol Chem* **259:**9351–9354.

Berstein G, Blank JL, Smrcka AV, Higashijima T, Sternweis PC, Exton JH and Ross EM (1992) Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, $G_{q/11}$, and phospholipase C-β1. *J Biol Chem* **267:**8081–8088.

Biddlecome GH, Berstein G and Ross EM (1996) Regulation of phospholipase C-β1 by G_q and m_1 muscarinic cholinergic receptor: Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. *J Biol Chem* **271:**7999–8007.

Bouaboula M, Desnoyer N, Carayon P, Combes T and Casellas P (1999) G_i protein modulation induced by a selective inverse agonist for the peripheral cannabinoid receptor CB2: Implication for intracellular signalization cross-regulation. *Mol Pharmacol* **55:**473–480.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand J-P, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1: Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.

Breivogel CS, Selley DE and Childers SR (1998) Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. *J Biol Chem* **273:**16865–16873.

Carty DJ, Padrell E, Codina J, Birnbaumer L, Hildebrandt JD and Iyengar R (1990) Distinct guanine nucleotide binding and release properties of the three G_i proteins. *J Biol Chem* **265:**6268–6273.

Cassel D and Selinger Z (1977) Catecholamine-induced release of [^{3}H]-Gpp(NH)p from turkey erythrocyte adenylate cyclase. *J Cyclic Nucleotide Res* **3:**11–22.

Chidiac P, Markin VS and Ross EM (1999) Kinetic control of guanine nucleotide binding to $G\alpha_q$. *Biochem Pharmacol* **58:**39–48.

Daaka Y, Luttrell LM and Lefkowitz RJ (1997) Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature (Lond)* **390:**88–91.

De Lean A, Stadel JM and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255:**7108–7117.

Eason MG, Jacinto MT and Liggett SB (1994) Contribution of ligand structure to activation of α_2-adrenergic receptor subtype coupling to G_s. *Mol Pharmacol* **45:** 696–702.
Gettys TW, Fields TA and Raymond JR (1994) Selective activation of inhibitory G-protein α-subunits by partial agonists of the human 5-HT_{1A} receptor. *Biochemistry* **33:**4283–4290.
Gierschik P, Moghtader R, Straub C, Dietrich K and Jakobs KH (1991) Signal amplification in HL-60 granulocytes: Evidence that the chemotactic peptide receptor catalytically activates guanine-nucleotide-binding regulatory proteins in native plasma membranes. *Eur J Biochem* **197:**725–732.
Gilman AG (1987) G proteins: Transducers of receptor-generated signals. *Annu Rev Biochem* **56:**615–649.
Grünewald S, Reiländer H and Michel H (1996) *In vivo* reconstitution of dopamine D_{2S} receptor-mediated G protein activation in baculovirus-infected insect cells: Preferred coupling to G_{i1} versus G_{i2}. *Biochemistry* **35:**15162–15173.
Gürdal H, Bond RA, Johnson MD, Friedman E and Onaran HO (1997) An efficacy-dependent effect of cardiac overexpression of β_2-adrenoceptor on ligand affinity in transgenic mice. *Mol Pharmacol* **52:**187–194.
Gurwitz D, Haring R, Heldman E, Fraser CM, Manor D and Fisher A (1994) Discrete activation of transduction pathways associated with acetylcholine m1 receptor by several muscarinic ligands. *Eur J Pharmacol* **267:**21–31.
Hepler JR, Kozasa T, Smrcka AV, Simon MI, Rhee SG, Sternweis PC and Gilman AG (1993) Purification from Sf9 cells and characterization of recombinant $G_{q\alpha}$ and $G_{11\alpha}$: Activation of purified phospholipase C isozymes by G_α subunits. *J Biol Chem* **268:**14367–14375.
Higashijima T, Burnier J and Ross EM (1990) Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines: Mechanism and structural determinants of activity. *J Biol Chem* **265:**14176–14186.
Higashijima T, Uzu S, Nakajima T and Ross EM (1988) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding proteins (G proteins) *J Biol Chem* **263:**6491–6494.
Iiri T, Farfel Z and Bourne HR (1998) G-protein diseases furnish a model for the turn-on switch. *Nature (Lond)* **394:**35–38.
Jones DT and Reed RR (1987) Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J Biol Chem* **262:**14241–14249.
Katada T, Amano T and Ui M (1982) Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. *J Biol Chem* **257:**3739–3746.
Klinker JF, Hagelüken A, Grünbaum L, Heilmann I, Nürnberg B, Harhammer R, Offermanns S, Schwaner I, Ervens J, Wenzel-Seifert K, Müller T and Seifert R (1994) Mastoparan may activate GTP hydrolysis by G_i-proteins in HL-60 membranes indirectly through interaction with nucleoside diphosphate kinase. *Biochem J* **304:**377–383.
Kobilka BK (1992) Adrenergic receptors as models for G protein-coupled receptors. *Annu Rev Neurosci* **15:**87–114.
Kupprion C, Wieland T and Jakobs KH (1993) Receptor-stimulated dissociation of GTP[S] from G_i-proteins in membranes of HL-60 cells. *Cell Signal* **5:**425–433.
Leopoldt D, Harteneck C and Nürnberg B (1997) G proteins endogenously expressed in Sf9 cells: Interactions with mammalian histamine receptors. *Naunyn Schmiedeberg's Arch Pharmacol* **356:**216–224.
Matesic DF, Manning DR, Wolfe BB and Luthin GR (1989) Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein. *J Biol Chem* **264:**21638–21645.
Miettinen HM, Gripentrog JM, Mason MM and Jesaitis AJ (1999) Identification of putative sites of interaction between the human formyl peptide receptor and G-protein. *J Biol Chem* **274:**27934–27942.
Milligan G (2000) Insights into ligand pharmacology using receptor-G-protein fusion proteins. *Trends Pharmacol Sci* **21:**24–28.
Northup JK, Smigel MD and Gilman AG (1982) The guanine nucleotide activating site of the regulatory component of adenylate cyclase: Identification by ligand binding. *J Biol Chem* **257:**11416–11423.
Offermanns S and Simon MI (1995) $G_{\alpha 15}$ and $G_{\alpha 16}$ couple a wide variety of receptors to phospholipase C. *J Biol Chem* **270:**15175–15180.
Otero AS (1990) Transphosphorylation and G protein activation. *Biochem Pharmacol* **39:**1399–1404.
Pavoine C, Magne S, Sauvadet A and Pecker F (1999) Evidence for a β_2-adrenergic/arachidonic acid pathway in ventricular cardiomyocytes: Regulation by the β_1-adrenergic/cAMP pathway. *J Biol Chem* **274:**628–637.
Ransnäs LA and Insel PA (1988) Quantitation of the guanine nucleotide binding regulatory protein G_s in S49 cell membranes using antipeptide antibodies to α_s. *J Biol Chem* **263:**9482–9485.
Seifert R, Gether U, Wenzel-Seifert K and Kobilka BK (1999a) Effects of guanine, inosine, and xanthine nucleotides on β_2-adrenergic receptor/G_s interactions: Evidence for multiple receptor conformations. *Mol Pharmacol* **56:**348–358.
Seifert R, Lee TW, Lam VT and Kobilka BK (1998a) Reconstitution of β_2-adrenoceptor-GTP-binding-protein interaction in Sf9 cells: High coupling efficiency in a β_2-adrenoceptor-$G_{s\alpha}$ fusion protein. *Eur J Biochem* **255:**369–382.
Seifert R, Wenzel-Seifert K, Gether U, Lam VT and Kobilka BK (1999b) Examining the efficiency of receptor/G-protein coupling with a cleavable β_2-adrenoceptor-$G_{s\alpha}$ fusion protein. *Eur J Biochem* **260:**661–666.
Seifert R, Wenzel-Seifert K and Kobilka BK (1999c) GPCR-Gα fusion proteins: Molecular analysis of receptor-G-protein coupling. *Trends Pharmacol Sci* **20:**383–389.
Seifert R, Wenzel-Seifert K, Lee TW, Gether U, Sanders-Bush E and Kobilka BK (1998b) Different effects of $G_s\alpha$ splice variants on β_2-adrenoreceptor-mediated signaling: The β_2-adrenoreceptor coupled to the long splice variant of $G_s\alpha$ has properties of a constitutively active receptor. *J Biol Chem* **273:**5109–5116.
Spicher K, Kalkbrenner F, Zobel A, Harhammer R, Nürnberg B, Söling A and Schultz G (1994) G_{12} and G_{13} α-subunits are immunochemically detectable in most membranes of various mammalian cells and tissues. *Biochem Biophys Res Commun* **198:**906–914.
Szele FG and Pritchett DB (1993) High affinity agonist binding to cloned 5-hydroxytryptamine$_2$ receptor is not sensitive to GTP analogs. *Mol Pharmacol* **43:**915–920.
Wenzel-Seifert K, Arthur J, Liu H-Y and Seifert R (1999) Quantiative analysis of formyl peptide receptor coupling to $G_i\alpha_1$, $G_i\alpha_2$, and $G_i\alpha_3$. *J Biol Chem* **274:**33259–33266.
Wenzel-Seifert K, Hurt CM and Seifert R (1998a) High constitutive activity of the human formyl peptide receptor. *J Biol Chem* **273:**24181–24189.
Wenzel-Seifert K, Lee TW, Seifert R and Kobilka BK (1998b) Restricting mobility of $G_s\alpha$ relative to the β_2-adrenoceptor enhances adenylate cyclase activity by reducing $G_s\alpha$ GTPase activity. *Biochem J* **334:**519–524.
Wieland T and Jakobs KH (1992) Evidence for nucleoside diphosphokinase-dependent channeling of guanosine 5′-(gamma-thio)triphosphate to guanine nucleotide-binding proteins. *Mol Pharmacol* **42:**731–735.
Wise A, Carr IC, Groarke DA and Milligan G (1997) Measurement of agonist efficacy using an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein. *FEBS Lett* **419:**141–146.
Wu D, Kuang Y, Wu Y and Jiang H (1995) Selective coupling of β_2-adrenergic receptor to hematopoietic-specific G proteins. *J Biol Chem* **270:**16008–16010.
Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ and Lakatta EG (1999) Coupling of the β_2-adrenoceptor to G_i-proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* **84:**43–52.
Xiao RP, Ji X and Lakatta EG (1995) Functional coupling of the β_2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol Pharmacol* **47:**322–329.
Zhu X, Gilbert S, Birnbaumer M and Birnbaumer L (1994) Dual signaling potential is common among G_s-coupled receptors and dependent on receptor density. *Mol Pharmacol* **46:**460–469.

Send reprint requests to: Dr. Roland Seifert, Department of Pharmacology and Toxicology, The University of Kansas, 5064 Malott Hall, Lawrence, KS 66045. E-mail: rseifert@falcon.cc.ukans.edu