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Cellular signaling by an agonist-activated receptor/$G_s\alpha$ fusion protein

(β_2-adrenergic receptor/adenylyl cyclase/receptor desensitization)

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ABSTRACT **The consequences of agonist-dependent activation of guanine nucleotide-binding protein (G protein)-coupled receptors vary from cell to cell, depending on a complex network of regulations between components of the signaling cascade. Specific interactions between receptors, G proteins, and effectors are difficult to analyze in intact cells. Engineering of receptor-transducer fusion proteins might be an effective strategy to target cellular effectors more efficiently and specifically. As a model, we evaluated the ability of a fusion protein of β_2-adrenergic receptor bound to the α subunit of adenylyl cyclase-stimulatory G protein ($G_s\alpha$) to restore the defective activation of adenylyl cyclase in S49 cyc$^-$ cells that lack endogenous $G_s\alpha$. The coupling between the two partners of the fusion protein was functional, and the agonist-dependent activation of the effector was more potent and more productive in transfected than in wild-type S49 cells. The covalent link between receptor and $G_s\alpha$ could thus convey an advantage over freely interacting components. Such receptor-Gα fusion proteins may help to elucidate the complex interactions between members of signaling pathways and may also constitute a useful tool for studying the effects of single effector activation.**

Guanine nucleotide-binding protein (G protein)-coupled receptors regulate the function of cellular effectors through modulation of heterotrimeric G proteins. The signal resulting from the activation of a given receptor with an agonist depends on a complex network of interactions between receptors, α and $\beta\gamma$ subunits of G proteins, and effectors, which may vary from cell to cell. The number of activated receptors (1), the availability of specific G-protein subunits, and the activation of their respective regulatory pathways (2, 3) contribute to this variability.

In addition, ligand-dependent activation of single receptors may elicit dual signaling (reviewed in refs. 4 and 5). The branching points that lead to the modulation of multiple effectors after activation of a single receptor have not been completely elucidated. It has been shown that, beside Gα subunits, $\beta\gamma$ subunits may modulate independently cellular effectors (6, 7). Alternatively, receptors may activate multiple effector pathways by interacting with multiple Gα subunits at the same time (8, 9). The functional consequences of the specific interaction between a receptor and a given G protein subtype are difficult to analyze in intact cells, since most G proteins are ubiquitous. We reasoned that, in a cell, precoupling of a receptor to a Gα subunit by a physical link might focus the receptor-mediated signal toward a more potent and/or a more selective targeting of a single cellular effector. To test this hypothesis, we have evaluated the ability of a recombinant fusion protein of the β_2-adrenergic receptor (β_2AR) bound to the α subunit of adenylyl cyclase-stimulatory G protein ($G_s\alpha$) to mediate the activation of adenylyl cyclase in mutant S49 cyc$^-$ cells, which are devoid of endogenous $G_s\alpha$ subunits and are thus insensitive to catecholamine stimulation.

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MATERIALS AND METHODS

Construction of the Expression Vector and Transfection. A *Nco* I site was created by site-directed mutagenesis at position 2486 of the β_2AR nucleotide sequence (10) and was used for subcloning in phase the *Nco* I–*Sal* I fragment of the $G_s\alpha$ short splice variant ($G_s\alpha$-S) coding region (11). Fused β_2AR-$G_s\alpha$ coding regions were subcloned in the eukaryotic expression vector pRc/CMV (Invitrogen). S49 cyc$^-$ cells were transfected with this construct and grown as described (12).

Photoaffinity Labeling. Intact cells (≈200 pM β_2AR) were incubated in the dark for 90 min at room temperature with 500 pM (−)-[^{125}I]iodocyanopindolol (ICYP)-diazirine (Amersham) in 0.5 ml of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. Nonspecific binding was determined in the presence of 10 μM (±)-propranolol (Sigma). After centrifugation for 10 min at 500 × *g* at 4°C, pellets were resuspended in 3 ml of cold PBS and exposed to UV for 5 min. After additional centrifugation, cells were lysed in 5% SDS/5% 2-mercaptoethanol/10% (vol/vol) glycerol/0.05% bromophenol blue at room temperature for 2 hr. Proteins were separated by SDS/PAGE and transferred onto a nitrocellulose membrane; strips corresponding to labeled samples were submitted to autoradiography. Nonlabeled samples run in parallel were used for immunoblots.

Immunoblots. Nitrocellulose strips were incubated for 3 hr at room temperature with PBS containing 5% milk powder and 0.1% Tween 20 and then incubated overnight at 4°C with purified anti-$G_s\alpha$ C-terminal decapeptide antibodies (Gramsch Laboratories, Schwabhausen, Germany) or with anti-β_2AR peptide antibodies (13). After washings, strips were incubated for 1 hr at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG. Immunoreactive material was visualized by chemiluminescence (Amersham ECL kit).

Membrane Preparations. Cells (2–5 × 10^6 per ml) were pelleted at 500 × *g* for 10 min, resuspended in 0.5–1.5 ml of 25 mM Tris·HCl, pH 7.5/1 mM EDTA/10% sucrose/0.5 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted with a

Abbreviations: G protein, guanine nucleotide-binding protein; G_s protein, adenylyl cyclase-stimulatory G protein; $G_s\alpha$, α subunit of G_s; β_2AR, β_2-adrenergic receptor; ICYP, (−)-[^{125}I]iodocyanopindolol; S49 wt cells, lymphoma S49 wild-type cells; S49 cyc$^-$, S49 cells deficient in $G_s\alpha$; S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, S49 cyc$^-$ cells expressing β_2AR-$G_s\alpha$ fusion protein; Guo*PP*[NH]*P*, guanosine 5′-[β,γ-imido]triphosphate; $G_s\alpha$-S, short splice variant of $G_s\alpha$.

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Dounce homogenizer (20 strokes). Nuclei and cellular debris were removed by centrifugation at 1500 × *g* for 5 min. Pools of supernatants from two cycles of lysis were ultracentrifuged in a SW-41 rotor (Beckman) at 110,000 × *g* for 1 hr. Pellets were resuspended in Tris·HCl, pH 7.5/1 mM EDTA/5 mM $MgCl_2$ (TEM buffer) up to 1 mg/ml and frozen immediately in liquid nitrogen. Protein concentration was determined by the method of Bradford.

Binding Assays. Isoproterenol was from Sigma; guanosine 5′-[γ-thio]triphosphate and guanosine 5′-[β,γ-imido]triphosphate (Guo*PP*[NH]*P*) were from Boehringer Mannheim. Membrane proteins (2–10 μg) were incubated for 1.5 hr at room temperature with appropriate ligands in TEM buffer supplemented with 0.5 mM PMSF and 10 μg of benzamidine per ml in a total volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM (±)-propranolol. Data from competition experiments were subjected to a computer-assisted (GRAPHPAD) curvilinear regression analysis.

Intracellular cAMP Accumulation Assays. Cells (2×10^6 per ml) were centrifuged for 10 min at 500 × *g*, and pellets were resuspended in PBS containing 1 mM isobutylmethylxanthine (Sigma) and 1 mM ascorbic acid and were incubated for 15 min at 37°C in a final volume of 0.5 ml with agonists or buffer alone. cAMP concentrations were measured in cell lysates with a [^{3}H]cAMP assay kit (TRK432, Amersham).

Cholera Toxin-Mediated ADP-Ribosylation of $G_s\alpha$-S. Recombinant $G_s\alpha$-S purification (11) and ADP-ribosylation (14) were carried out as described. Briefly, 1 μg (≈20 pmol) of purified recombinant $G_s\alpha$-S was combined with 2 pmol of purified brain ADP-ribosylation factor (provided by R. A. Kahn, National Institutes of Health, Bethesda, MD) and 0.5 μg of preactivated cholera toxin (Calbiochem), and the reaction was carried out in the presence of 0.1 mM [^{32}P]NAD (NEN; specific activity, 100 cpm/pmol) with or without 1 μg (20 pmol) of purified bovine brain βγ subunit complex (15) in a final volume of 20 μl. At the time points indicated in Fig. 5*B*, the reaction mixture was subjected to SDS/PAGE; protein bands were visualized by staining with Coomassie Blue, and autoradiography was performed on the dried gel.

RESULTS

Expression of the β_2AR-$G_s\alpha$ Fusion Protein in S49 cyc$^-$ Cells. The 3′ end of the β_2AR coding region was fused in phase with the initiation codon of that of the $G_s\alpha$ subunit (Fig. 1). S49 cyc$^-$ cells, devoid of endogenous $G_s\alpha$ subunits, were transfected with this construct. Among neomycin-resistant clones, which expressed an increased number of ICYP-binding sites compared with nontransfected S49 cyc$^-$ cells, one clone (named S49 β_2AR-$G_s\alpha$ cyc$^-$) was selected for further studies.

Visualization of the β_2AR-$G_s\alpha$ Fusion Protein. Labeling experiments with the photoactivable β-adrenergic ligand ICYP-diazirine showed that S49 β_2AR-$G_s\alpha$ cyc$^-$ cells expressed two molecular forms of β_2AR-binding sites. The more abundant was absent in control S49 cyc$^-$ cells and displayed an apparent molecular weight of ≈110 kDa, consistent with that expected for the β_2AR-$G_s\alpha$ fusion protein (Fig. 2). The second, of ≈67 kDa, was present in both S49 β_2AR-$G_s\alpha$ and control S49 cyc$^-$ cells and corresponded to endogenous β_2AR. Immunoblots with anti-$G_s\alpha$ and anti-β_2AR peptide antibodies confirmed that the 110-kDa protein was indeed formed by the association of a β_2AR with a $G_s\alpha$ subunit (Fig. 2). The C terminus of the $G_s\alpha$ subunit, known to be critical for the coupling with receptor (19), was present in the fusion protein, since the antibodies used in the experiments were raised against the last 10 amino acid residues of $G_s\alpha$.

Binding Properties of the Fusion β_2AR-$G_s\alpha$ Protein. The K_d value of ICYP for β-adrenergic binding sites of S49 β_2AR-$G_s\alpha$ cyc$^-$ cells was 60 ± 20 pM, close to that measured for endogenous β_2AR in S49 cyc$^-$ and wild-type (wt) S49 cells (Table 1). The number of receptors in these cell lines was 900 ± 110, 300 ± 50, and 120 ± 40 fmol/mg, respectively.

In S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, the ICYP competition binding curve with isoproterenol fitted better with a two-site model curve: 54 ± 10% of the isoproterenol binding sites were of high affinity (K_H = 13 ± 9 nM), while 46 ± 12% were of low affinity (K_L = 230 ± 100 nM). The GTP hydrolysis-resistant analogue Guo*PP*[NH]*P* caused a complete shift of binding sites toward the low-affinity state. In S49 cyc$^-$ cells, only low-affinity isoproterenol binding sites were present, even in the absence of Guo*PP*[NH]*P* (Fig. 3).

Agonist-Induced Stimulation of cAMP Accumulation in S49 wt Cells and in S49 β_2AR-$G_s\alpha$ cyc$^-$ Cells. In S49 wt cells, isoproterenol increased intracellular cAMP levels up to 10-fold over basal values (Table 1), while in S49 cyc$^-$ cells, isoproterenol had no effect on cAMP accumulation (data not shown). In S49 wt and S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, forskolin-stimulated accumulation of cAMP reached similar values (Table 1). Dose–response experiments of intracellular cAMP accumulation were performed with isoproterenol (Fig. 4).

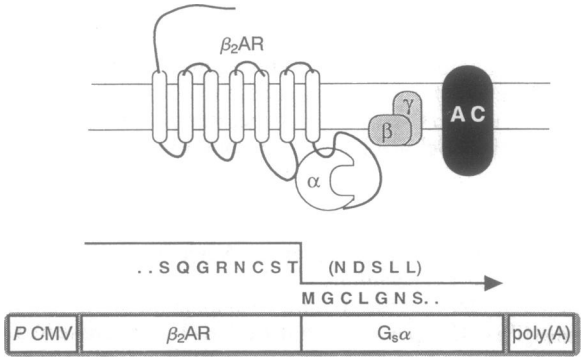

FIG. 1. Expression of the β_2AR-$G_s\alpha$ subunit fusion gene in S49 cyc$^-$ cells. The 3′ end of the coding region for β_2AR was fused in phase with the initiation codon of that of $G_s\alpha$. This construct was placed under the control of the cytomegalovirus promoter (*P* CMV) and 5′ to the bovine growth hormone polyadenylylation signal [poly(A)]. The last 5 amino acid residues of β_2AR (in brackets) were deleted during the construction of the fusion gene. S49 cyc$^-$ cells are devoid of endogenous $G_s\alpha$ subunits but synthesize βγ subunits and adenylyl cyclase (AC).

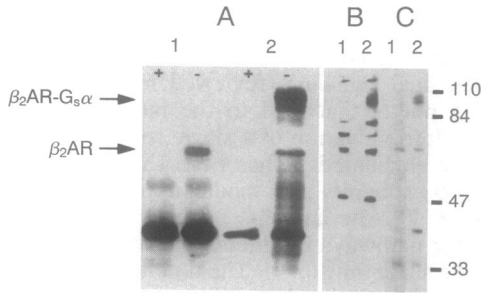

FIG. 2. Visualization of the β_2AR-$G_s\alpha$ fusion protein. (*A*) Photoaffinity labeling of control (lane 1) and transfected S49 cyc$^-$ cells (lane 2) in the presence (lanes +) or in the absence (lanes −) of the βAR antagonist propranolol. Fusion protein (β_2AR-$G_s\alpha$) and β_2AR are indicated by arrows. Molecular mass markers in kDa are shown on the right side. (*B* and *C*) Immunoblots with proteins from control (lanes 1) and transfected S49 cyc$^-$ cells (lanes 2), with specific anti-β_2AR peptide (*B*) and anti-$G_s\alpha$ (*C*) antibodies. In *C*, lane 2, the protein of about 40 kDa labeled by anti-$G_s\alpha$ antibodies did not comigrate with $G_s\alpha$-S or the long splice variant form of $G_s\alpha$ (not shown). It might be an N-terminal truncated and thus presumably inactive (16–18) product of the $G_s\alpha$ moiety.

Table 1. Pharmacological parameters of the β_2AR-$G_s\alpha$ fusion protein

S49 lymphoma cells	ICYP saturation		Isoproterenol inhibition			cAMP accumulation		
			K_i, nM				B_{max}, pmol per 10^7 cells	
			− Guo*PP*(NH)*P*					
	K_d, pM	B_{max}, fmol/mg	K_H	K_L	+ Guo*PP*(NH)*P*	EC_{50}, nM	iso	FK
β_2AR/$G_s\alpha$	60 ± 20	900 ± 110	13 ± 9 (54 ± 10%)	230 ± 100 (46 ± 12%)	128 ± 65	45 ± 30	565 ± 15	1100 ± 500
cyc⁻	70 ± 30	300 ± 50	—	123 ± 50	144 ± 56	—	—	100 ± 25
wt	60 ± 30	120 ± 40	14 ± 4 (21 ± 8%)	224 ± 36 (80 ± 7%)	210 ± 110	650 ± 150	330 ± 30	990 ± 330

Membranes were assayed for ICYP saturation isotherms (n = 2) and isoproterenol inhibition binding (n = 5). Binding parameters were estimated by computer-assisted nonlinear least-squares analyses. K_d is the equilibrium dissociation constant. The inhibition constant K_i values were determined in the presence or absence of Guo*PP*[NH]*P*. K_H and K_L are the high- and low-affinity equilibrium dissociation constants for isoproterenol, respectively. The percentage of receptors in the high- or low-affinity agonist binding form is in parentheses. Intracellular cAMP accumulation assays (n = 3) were performed after stimulating intact cells with either isoproterenol (iso) or forskolin (FK). EC_{50} indicates the concentration of isoproterenol inducing half-maximal cAMP levels. Basal cAMP levels ranged from 20 to 30 pmol per 10^7 cells in the three cell lines. Mean values ± SEM are shown.

The concentration inducing half-maximal (EC_{50}) cAMP accumulation in S49 β_2AR-$G_s\alpha$ cyc⁻ cells (45 ± 30 nM) was significantly lower than that in S49 wt cells (650 ± 150 nM). Moreover, maximal intracellular cAMP values were respectively 565 ± 15 and 330 ± 30 pmol per 10^7 cells. These results showed that the $G_s\alpha$ partner of the fusion protein was still active and capable of interacting with both the β_2AR and with adenylyl cyclase.

Effects of Cholera Toxin Treatment on Recombinant $G_s\alpha$. The incubation with cholera toxin induced a significant increase of intracellular cAMP in both S49 β_2AR-$G_s\alpha$ cyc⁻ and S49 wt cells, while it was ineffective on S49 cyc⁻ cells (Fig. 5*A*). Previous experiments have shown that, after reconstitution of $G_s\alpha$-deficient membranes of S49 cyc⁻ with recombinant $G_s\alpha$, only a modest amount of ADP-ribose could be incorporated in the presence of cholera toxin (11). Stoichiometric labeling required the exogenous addition of $\beta\gamma$ dimers, suggesting that the reaction was limited by the availability of $\beta\gamma$ subunits present in the membranes (20). This hypothesis was verified by carrying out the ADP-ribosylation reaction with purified components: ADP-ribosylation of purified recombinant $G_s\alpha$-S was dependent on the presence of the $\beta\gamma$ dimer, since no radioactivity was incorporated into the protein in the absence of the dimer even after an incubation period of 2 hr (Fig. 5*B*).

Long-Term Desensitization of the Fusion Protein. After a 24-hr treatment with isoproterenol, the basal level of cAMP in S49 β_2AR-$G_s\alpha$ cyc⁻ cells was 10-fold higher than in cells not treated with the agonist. A further 15-min incubation of washed cells with the agonist increased even more intracellular cAMP ($P < 0.05$), which reached about 50% of the maximal level obtained in nonpretreated cells (Fig. 6 *Upper*). In the same experimental conditions, β_2AR of S49 wt cells were completely desensitized (Fig. 6 *Lower*).

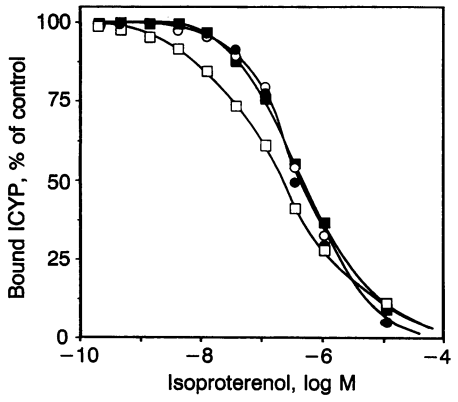

FIG. 3. Binding properties of the fusion β_2AR-$G_s\alpha$ protein. Competition binding study of ICYP with isoproterenol on membranes from S49 β_2AR-$G_s\alpha$ cyc⁻ (□,■) and cyc⁻ cells (●,○) in the absence (□,○) or presence (■,●) of 0.1 mM Guo*PP*[NH]*P*. ICYP was used at fixed concentration (200 pM). Data are representative of four or five individual experiments performed in duplicate. The calculated K_i values and total binding sites are indicated in Table 1.

DISCUSSION

Dual signaling by G protein-coupled receptors raises the questions of the cellular effects that may be specifically attributable to each activated effector. We have developed a strategy, based on the expression of a fusion receptor–transducer protein, that would bypass endogenous transducing proteins and that would directly target a cellular effector.

The signaling properties of a fusion β_2AR-$G_s\alpha$ protein were studied in S49 cyc⁻ cells, which are devoid of endogenous $G_s\alpha$ and thus unable to produce cAMP upon β_2AR activation. Immunochemical studies and β_2AR radiolabeling experiments showed that S49 β_2AR-$G_s\alpha$ cells expressed a protein of 110 kDa, capable of labeling by the β_2AR photoactivable ligand ICYP-diazirine and recognized by an antiserum directed against the C terminus of $G_s\alpha$ (Fig. 2). Several observations demonstrate that this fusion protein is functional. (*i*) Binding experiments showed that the number of β_2AR binding sites was >3-fold higher in S49 β_2AR-$G_s\alpha$ cyc⁻ cells, compared with untransfected controls (Table 1) and

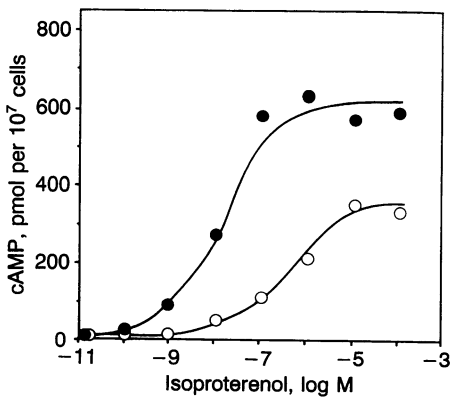

FIG. 4. Stimulation of cAMP accumulation in S49 wt cells (○) and in S49 β_2AR-$G_s\alpha$ cyc⁻ cells (●), showing dose–response curves of isoproterenol-dependent cAMP accumulation. B_{max} and EC_{50} values are indicated in Table 1. Data are representative of three individual experiments in duplicate.

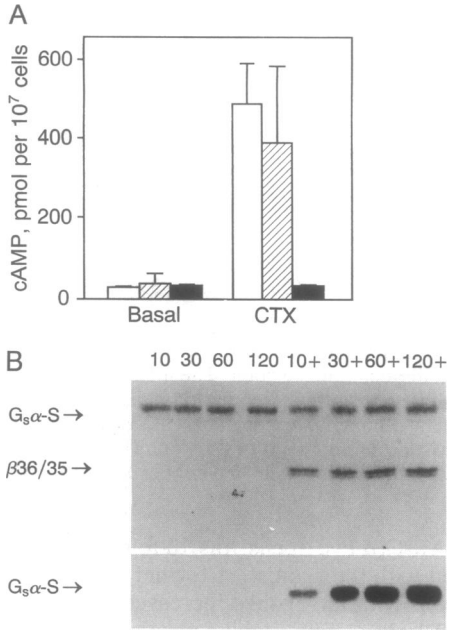

FIG. 5. Effects of cholera toxin treatment on intact cells and on recombinant $G_s\alpha$. (*A*) Cholera toxin (CTX) action on S49 β_2AR-$G_s\alpha$ cyc$^-$ cells (▨) was tested by measuring the intracellular cAMP level after a 90-min incubation period with 250 ng of CTX per ml of culture medium. S49 wt (□) and cyc$^-$ (■) cells were used as controls. Data are means ± SEM of three individual experiments in duplicate. (*B*) CTX-induced ADP-ribosylation of purified $G_s\alpha$-S in the presence or absence of the $\beta\gamma$ dimer. The amount of α and β subunits used in each test was controlled by Coomassie blue (*B Upper*). The incorporation of [^{32}P]ADP-ribose into $G_s\alpha$-S was determined at four time points (in min) in the absence (*Left*) or in the presence (*Right*) of $\beta\gamma$ and visualized by autoradiography after SDS/PAGE (*B Lower*). β36/35 corresponds to the mixture of β subunits that migrate with apparent molecular masses of 36 and 35 kDa.

that the pharmacological specificity of the receptor moiety for β_2AR agonists and antagonists was maintained (not shown). (*ii*) In membranes from S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, the

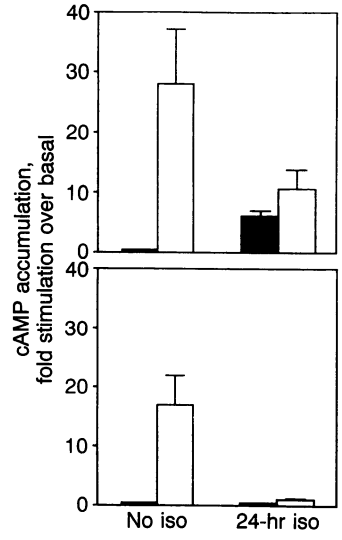

FIG. 6. Agonist-promoted long-term desensitization of β_2AR in S49 β_2AR-$G_s\alpha$ cyc$^-$ cells (*Upper*) and S49 wt cells (*Lower*). After a 24-hr pretreatment with 10 μM isoproterenol (24-hr iso) or medium alone (No iso), cells were washed and then submitted (open bars) or not (filled bars) to further stimulation for 15 min with 10 μM isoproterenol. Intracellular cAMP accumulation levels were measured in each condition. Data are means ± SEM of three individual experiments performed in duplicate.

binding of the agonist isoproterenol is sensitive to the modulation by guanine nucleotides (Table 1 and Fig. 3), indicating that the β_2AR and the $G_s\alpha$ moiety form, in the presence of the agonist, a ternary complex that is functionally dissociated by the addition of GTP analogues (21). (*iii*) The fusion protein is capable of efficient signaling in intact S49 β_2AR-$G_s\alpha$ cyc$^-$ cells as shown by the isoproterenol-induced cAMP accumulation.

Basal levels of cAMP were comparable in S49 β_2AR-$G_s\alpha$ cyc$^-$ and wt S49 cells, indicating that, even though the $G_s\alpha$ is covalently linked to the receptor, there is no constitutive activation of the transducing moiety in the absence of agonist. This observation is in good agreement with the "allosteric ternary complex model" of G protein activation (22), which introduces an isomerization step that regulates the agonist-dependent formation of an "activated state" of the receptor. Only receptors in the activated state are capable of binding productively to the G protein. Accordingly, in the fusion protein, the receptor moiety still requires to be activated by the agonist before interacting with the $G_s\alpha$ moiety.

The coupling between native receptors and G proteins requires that the G protein is under its $\alpha\beta\gamma$ oligomeric form, since the $\beta\gamma$-subunit complex contributes to the interaction with the receptor (23, 24). To show that the interaction between $\beta\gamma$ dimers and the $G_s\alpha$ moiety indeed occur in S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, we have exploited the fact that ADP-ribosylation of $G_s\alpha$ by cholera toxin displays a stringent requirement for the $\beta\gamma$-subunit complex (11, 20). Following treatment with cholera toxin, increased cAMP levels were measured in S49 β_2AR-$G_s\alpha$ cells (Fig. 5*A*). Moreover, we confirmed that the cholera toxin-dependent ADP-ribosylation of the purified recombinant $G_s\alpha$-S (the splice variant used for the construction of the fusion protein) is strictly dependent on the presence of $\beta\gamma$ subunits (Fig. 5*B*). These observations provide strong evidence that the $G_s\alpha$-moiety of the fusion protein interacts with $\beta\gamma$ dimers in the membranes of S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, although the N terminus of $G_s\alpha$, which represents an important contact site between the α subunit and the $\beta\gamma$ dimers (16–18), was linked to the C terminus of the receptor.

Upon agonist activation, S49 β_2AR-$G_s\alpha$ cyc$^-$ cells accumulated cAMP to a level that exceeded by a factor of 2 that observed in wt S49 cells and was almost equivalent to that obtained with forskolin. In addition, in S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, the potency of isoproterenol was increased 15-fold compared with that in S49 wt cells. The apparently more productive signaling in cells transfected with the fusion protein may either result from differences in the relative levels of receptors and $G_s\alpha$ or from some advantage provided by the covalent link between the two components of the signaling pathway. S49 wt cells contain ≈16–20 pmol of $G_s\alpha$ per mg of membrane proteins (25). In S49 β_2AR-$G_s\alpha$ cyc$^-$ cells, the maximal number of $G_s\alpha$ moieties was evaluated from our binding studies (one functional $G_s\alpha$ is associated with one intact receptor). Since two-thirds of the 900 fmol of ICYP-binding sites per mg of membrane proteins—i.e., 600 fmol/mg corresponds to the amount of the fusion protein (the remaining ICYP binding sites being endogenous βAR)—wt cells express about 30 times more $G_s\alpha$ than β_2AR-$G_s\alpha$ cyc$^-$ cells. It has been shown that S49 cyc$^-$ cells, reconstituted by transfection with "wt" levels of $G_s\alpha$, displayed the same isoproterenol-induced cAMP signal as S49 wt cells (12). Thus, if cell signaling is proportional to the number of expressed $G_s\alpha$ proteins, we would expect a lower signal in β_2AR-$G_s\alpha$ cyc$^-$ cells. The number of β_2ARs (including those that are not covalently coupled to the $G_s\alpha$ moiety) is 7.5 times higher in β_2AR-$G_s\alpha$ cyc$^-$ cells than in S49 wt cells. In transfected hamster CHW cells, the isoproterenol-stimulated maximal adenylyl cyclase activity increases with the number of exogenous β_2ARs (26). However, in CHW cells, $G_s\alpha$

proteins are in large excess over receptors and are catalytically activated. Thus, it is difficult from these results to evaluate the part attributable to the higher number of receptors in the increased isoproterenol-mediated signaling observed in β_2AR-$G_s\alpha$ cyc$^-$ cells. In addition, in another β_2AR-$G_s\alpha$ cyc$^-$ clone, which expressed approximately half of the ICYP-binding sites per mg of protein, we measured an identical increase of isoproterenol potency (data not shown). Taken together, all of these results suggest that the covalent link between β_2AR and $G_s\alpha$, by itself, may increase the signaling efficiency. The reasons for this particular effect have not been addressed in the present study but two hypotheses may be proposed: (*i*) the physical linkage of the two fusion partners may favor a rapid cycling between the agonist-dependent receptor-mediated activation of the $G_s\alpha$ moiety and deactivation by the intrinsic GTPase, and (*ii*) the close physical contact in the fusion protein might impede early regulatory mechanisms (such as receptor uncoupling) that in intact cells initiate the desensitization process after a few minutes of incubation with agonists (27). Receptor uncoupling is potentially relevant under our experimental conditions—i.e., a 15-min incubation period. After a longer period of incubation with the agonist, a pronounced desensitization of β-adrenergic signaling occurred in wt S49 cells, while it was partially prevented in S49 β_2AR-$G_s\alpha$ cyc$^-$ cells (Fig. 6). This observation strengthens the hypothesis that the covalent link between the C terminus of β_2AR and $G_s\alpha$ impairs the regulation process that normally follows receptor activation. Molecular determinants involved in receptor desensitization (28, 29) may be masked. In addition, it has been shown that activation of $G_s\alpha$ loosens its attachment to membrane and increases its degradation rate (3); by fixing $G_s\alpha$ to the membrane, β_2AR could impede its normal regulation.

The results reported here show that receptor $G_s\alpha$ fusion proteins may reconstitute a functional intramolecular coupling, as shown by the restoration of adenylyl cyclase activation in S49 cyc$^-$ cells. Apart from controlling the activity of adenylyl cyclase, β_2AR regulates NHE1 Na^+/H^+-exchangers, presumably via $G\alpha$13 (30, 31). Many other receptors share with β_2AR the ability to regulate the activity of multiple $G\alpha$ subunits and of multiple effectors. Receptor–transducer fusion proteins, which reduce signal diversification by targeting a single class (or a limited set) of cellular effectors, offer a different approach to study the physiological importance of signaling pathways.

A molecular connection exists between cAMP signaling and the control of cell growth (32) that could be exploited to revert the malignant phenotype of *ras*-transformed cells (33). Because of its strong signaling and to its resistance to long-term desensitization, the β_2AR-$G_s\alpha$ fusion protein is the appropriate tool to evaluate this possibility in animal models.

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