Restricting mobility of $G_s\alpha$ relative to the β_2 -adrenoceptor enhances adenylate cyclase activity by reducing $G_s\alpha$ GTPase activity

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The β_2 -adrenoceptor (β_2 AR) activates the G-protein $G_s\alpha$ to stimulate adenylate cyclase (AC). Fusion of the β_2 AR C-terminus to the N-terminus of $G_s\alpha$ (producing β_2 ARG α) markedly increases the efficiency of receptor/G-protein coupling compared with the non-fused state. This increase in coupling efficiency can be attributed to the physical proximity of receptor and G-protein. To determine the optimal length for the tether between receptor and G-protein we constructed fusion proteins from which 26 [β_2 AR(Δ 26) $G_s\alpha$] or 70 [β_2 AR(Δ 70) $G_s\alpha$] residues of the β_2 AR C-terminus had been deleted and compared the properties of these fusion proteins with the previously described β_2 ARG α . Compared with β_2 ARG α , basal and agonist-stimulated GTP

hydrolysis was markedly decreased in β_2 AR(Δ 70) $G_s\alpha$, whereas the effect of the deletion on binding of guanosine 5'-[γ -thio]triphosphate (GTP[S]) was relatively small. Surprisingly, deletions did not alter the efficiency of coupling of the β_2 AR to $G_s\alpha$ as assessed by GTP[S]-sensitive high-affinity agonist binding. Moreover, basal and ligand-regulated AC activities in membranes expressing β_2 AR(Δ 70) $G_s\alpha$ and β_2 AR(Δ 26) $G_s\alpha$ were higher than in membranes expressing β_2 ARG α . These findings suggest that restricting the mobility of $G_s\alpha$ relative to the β_2 AR results in a decrease in G-protein inactivation by GTP hydrolysis and thereby enhanced activation of AC.

INTRODUCTION

The β_2 -adrenoceptor (β_2 AR) couples to the heterotrimeric G-protein G_s to mediate the activation of adenylate cyclase (AC) [1,2]. Interaction of the agonist-occupied β_2 AR with G_s causes the release of GDP from, and GTP binding to, the α -subunit of G_s [1]. β_2 AR coupling to $G_s\alpha$ can be monitored by guanine nucleotide-sensitive high-affinity agonist binding [3,4] as well as ligand-regulated GTPase activity, guanosine 5'-[γ -thio]triphosphate (GTP[S]) binding and AC activity [4–8]. We and others have observed that fusion of the β_2 AR C-terminus to the N-terminus of $G_s\alpha$ (producing β_2 ARG α) markedly increases the efficiency of receptor/G-protein coupling compared with the non-fused state without altering the fundamental properties of either protein [4,5,9,10]. This increase in coupling efficiency can be explained by the physical proximity of the two proteins. However, one can imagine that, depending on the length of the physical link between receptor and G-protein, the fusion might impair coupling by preventing the optimal orientation of the G-protein relative to the receptor. Moreover, one might predict that a very short link between receptor and G-protein could impair interactions between $G_s\alpha$ and AC.

To determine the optimal length for the tether between β_2 AR and $G_s\alpha$ we constructed fusion proteins from which 26 [β_2 AR(Δ 26) $G_s\alpha$] or 70 [β_2 AR(Δ 70) $G_s\alpha$] residues of the β_2 AR C-terminus had been deleted and compared the properties of these fusion proteins with the previously described β_2 ARG α . C-terminal deletions from the β_2 AR change the desensitization

properties of the receptor but not receptor/G-protein coupling itself [11,12]. Here we report that restricting the mobility of $G_s\alpha$ relative to the β_2 AR decreases GTP hydrolysis and thereby increases AC activation.

MATERIALS AND METHODS

Materials

Materials for DNA construction, cell culture and functional assays were as described [4,5,7,8].

Construction of β_2 ARG α fusion protein DNA species

The construction of a fusion protein consisting of the β_2 -adrenoceptor and the long splice variant of $G_s\alpha$ (β_2 ARG α_L) DNA was described recently [4,5]. In the new fusion proteins, 26 and 70 residues respectively of the β_2 AR C-terminus were deleted by overlap-extension PCR to give β_2 AR(Δ 26) $G_s\alpha_L$ and β_2 AR(Δ 70) $G_s\alpha_L$ DNA species. Sets of the appropriate deletion primers (sense and anti-sense) were synthesized. In PCR 1A, the sequence between a primer 5' of the *EcoRV* site in the β_2 AR and the anti-sense deletion primer for β_2 AR(Δ 26) $G_s\alpha_L$ was amplified with β_2 ARG α_L DNA in pGEM-3Z as template. For generation of β_2 AR(Δ 70) $G_s\alpha_L$ (PCR 2A), a primer 5' of the *HpaI* site of the β_2 AR was used. In PCRs 1B and 2B, the sequences between the sense deletion primers and the anti-sense primer 3' of the *EcoRI*

Abbreviations used: AC, adenylate cyclase; β_2 AR, β_2 -adrenoceptor; β_2 ARG α_L , fusion protein consisting of the β_2 -adrenoceptor and the long splice variant of $G_s\alpha$; β_2 AR(Δ 26/ Δ 70) $G_s\alpha_L$, fusion protein consisting of the β_2 -adrenoceptor and $G_s\alpha_L$ from which the last 26 and 70 residues respectively of the β_2 AR C-terminus were deleted; [3 H]DHA, [3 H]dihydroalprenolol; $G_s\alpha_L$, long splice variant of $G_s\alpha$; GTP[S], guanosine 5'-[γ -thio]triphosphate; ISO, (–)-isoprenaline; ICI 118,551, [*erythro*-D,L-1-(7-methylindan-4-yl)-3-(propan-2-yl)aminobutan-2-ol].

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site of $G_s\alpha$ were amplified with pGEM-3Z- $\beta_2G_s\alpha_L$ as template. In PCR 3, the products of PCRs 1A and 1B were annealed and the sense primer 5' of the *EcoRV* site of the β_2AR and the anti-sense primer 3' of the *EcoRI* site of $G_s\alpha$ were used for amplification. In PCR 4, the products of PCRs 2A and 2B were annealed and the sense primer 5' of the *HpaI* site of the β_2AR and the anti-sense primer 3' of the *EcoRI* site of $G_s\alpha$ were used for amplification. In this way, DNA fragments encoding the C-terminus of the β_2AR lacking the last 26 and 70 residues respectively of the β_2AR , but containing the hexahistidine tag, and the N-terminal portion of $G_s\alpha$ were generated. The PCR fragment encoding for $\beta_2AR(\Delta 26)G_s\alpha_L$ was digested with *EcoRV* and *EcoRI* and cloned into pGEM-3Z- $\beta_2ARG_s\alpha_L$ digested with *EcoRV* and *EcoRI*. The PCR fragment encoding $\beta_2AR(\Delta 70)G_s\alpha_L$ was digested with *HpaI* and *EcoRI* and cloned into pGEM-3Z- $\beta_2ARG_s\alpha_L$ digested with *HpaI* and *EcoRI*. PCR-generated DNA sequences were confirmed by sequencing. The $\beta_2ARG_s\alpha$ DNA species with deletions and pVL1392- β_2AR plasmid were digested with *SalI* and *BamHI* respectively and the 3'-recessed ends were refilled with DNA polymerase I (large Klenow fragment). Thereafter both linearized vectors were digested with *NcoI*. The *NcoI/SalI* (filled) fragments encoding fusion proteins with deletions were ligated into the *NcoI/BamHI* (filled)-digested pVL 1392- β_2AR vector.

Generation of recombinant baculoviruses, cell culture and membrane preparation

Recombinant baculoviruses were generated in Sf9 cells by using the BaculoGOLD transfection kit (Pharmlingen, San Diego, CA, U.S.A.) in accordance with the manufacturer's instructions. High-titre virus stocks were generated by three sequential amplifications of virus colonies. Sf9 cells were cultured under the conditions described recently [4,5]. For infection, cells were seeded at 3.0×10^6 cells/ml and infected with 1:50 dilutions of high-titre recombinant virus stocks. Cells were cultured for 48 h before membrane preparation [4]. Recombination of baculoviruses was confirmed by reverse transcriptase-mediated PCR.

Binding of [3H]dihydroalprenolol ([3H]DHA)

[3H]DHA saturation binding and agonist-competition binding were performed in accordance with the recently described protocols [4,5].

GTP[S] binding

GTP[S] binding was determined as described [5] with 15 μ g of membrane protein per tube, 1 μ M GDP, 0.4 nM [^{35}S]GTP[S] (0.1 μ Ci per tube) and ligands at various concentrations.

Steady-state GTPase activity

GTP hydrolysis was determined as described, with 10 μ g of membrane protein per tube and 100 nM [γ - ^{32}P]GTP (0.5 μ Ci per tube) [4,5].

AC activity

cAMP formation in Sf9 membranes was assessed as described [4,5] in the presence of 1 μ M GTP and 40 μ M [α - ^{32}P]ATP (2.0 μ Ci per tube).

SDS/PAGE and immunoblot analysis

The conditions for SDS/PAGE and immunoblot analysis were as specified [4,5]. Immunoreactive bands were visualized by

sheep anti-(mouse IgG) (M1 antibody) and donkey anti-(rabbit IgG) (anti- $G_s\alpha$ antibody) respectively coupled to peroxidase, with *o*-dianisidine and H_2O_2 as substrates.

Protein assay

Protein was determined with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

Data reproducibility and calculations

The number of experiments performed and S.D. values are given in the legends to Figures 3 and 4. Relative changes of GTP[S] binding, GTPase activity and AC activity given in the text refer to the mean values shown in Figure 4. GTP turnover numbers and nucleotide affinities of fusion proteins are given only in the text without reference to a figure. These results represent the means $\pm$ S.D. for three or four experiments performed in duplicate or triplicate.

The absolute magnitude of signals recorded in GTP[S] binding, GTPase and AC studies depend on the expression level of the fusion proteins. To permit a direct comparison of the constructs for all three parameters, we used membranes expressing the three fusion proteins at similar levels (5.9–7.0 pmol/mg). Because high-affinity agonist binding is independent of expression level [4,5], we could pool data obtained with fusion proteins expressed at levels varying from 3.0 to 7.4 pmol/mg (see Figure 3).

The defined 1:1 stoichiometry of receptor to $G\alpha$ in fusion proteins permits the precise determination of G-protein expression level by antagonist binding [4,5,13]. This property of fusion proteins, together with a defined background activity of endogenous G-proteins, provides the basis for calculation of agonist-stimulated GTP turnover of the fused G-protein in native membranes [4,5,13]. Thus, by calculating GTP turnover, we could also directly compare $\beta_2ARG_s\alpha_L$, $\beta_2AR(\Delta 26)G_s\alpha_L$ and $\beta_2AR(\Delta 70)G_s\alpha_L$ when expressed at different levels. For GTP turnover studies we used three different membrane preparations for each fusion protein expressing constructs at 3.0–7.4 pmol/mg.

Concentration/response curves were analysed by non-linear regression, with the PRISM program (GraphPad; Prism, San Diego, CA, U.S.A.).

RESULTS

The C-terminus of the β_2AR between the palmitoylation site and the N-terminus of $G_s\alpha_L$ in $\beta_2ARG_s\alpha_L$ is 84 residues in length (including the hexahistidine tag) (Figure 1A). We deleted the distal 26 residues of the receptor C-terminus to produce $\beta_2AR(\Delta 26)G_s\alpha_L$. This deletion eliminated all G-protein-coupled receptor kinase (GRK) phosphorylation sites and shortened the linker between receptor core and $G_s\alpha$ to 58 residues (Figure 1B). In $\beta_2AR(\Delta 70)G_s\alpha_L$, all except two residues of the β_2AR C-terminus were deleted, and the linker between the C-terminal palmitoylation site of the β_2AR and the N-terminus of $G_s\alpha$ comprised only eight amino acids (Figure 1C). This deletion also led to the loss of the protein kinase A phosphorylation site.

Membranes from Sf9 cells expressing $\beta_2ARG_s\alpha$ fusion proteins (5.9–7.0 pmol/mg) were probed with the M1 monoclonal antibody, permitting the detection of the N-terminal FLAG epitope modification of the β_2AR , and with an antibody against the C-terminus of $G_s\alpha$ [4,5]. As reported [4,5], the apparent molecular mass of $\beta_2ARG_s\alpha_L$ is 104 kDa (Figure 2). The differentially glycosylated forms of $\beta_2ARG_s\alpha$ are more easily distinguished with the anti- $G_s\alpha$ antibody than with the M1 antibody. The expected molecular masses of $\beta_2AR(\Delta 26)G_s\alpha_L$ and $\beta_2AR(\Delta 70)G_s\alpha_L$ are 100 and 95 kDa respectively. Whereas for

Figure 1 Schematic presentation of $\beta_2\text{ARG}_s\alpha_L$ fusion proteins with different tether lengths between receptor and $G_s\alpha_L$

(A) $\beta_2\text{ARG}_s\alpha_L$: in this fusion protein the C-terminus of the $\beta_2\text{AR}$ is linked to the N-terminus of $G_s\alpha_L$ via a hexahistidine tag. The length of the tether between the palmitoylation site of the receptor and $G_s\alpha_L$ (including the hexahistidine tag) is 84 residues. The long linker ensures the high mobility of receptor and the fused G-protein. (B) $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$: in this fusion protein the 26 C-terminal residues of the $\beta_2\text{AR}$ have been deleted. Compared with $\beta_2\text{ARG}_s\alpha_L$, the mobility of receptor and G-protein in this fusion protein is moderately decreased. (C) $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$: in this fusion protein the tether between the palmitoylation site of the receptor and G-protein is eight residues. Therefore the mobility of G-protein relative to receptor is severely decreased. In all panels amino acids are given in the single-letter code, the hexahistidine tag is marked with white letters on black background and the arrows indicate potential phosphorylation sites for βAR kinase (βARK) and protein kinase A (PKA).

Figure 2 Immunological characterization of $\beta_2\text{ARG}_s\alpha_L$, $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ and $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$

Sf9 membranes expressing fusion proteins (5.9–7.0 pmol/mg) were separated by SDS/PAGE, transferred to nitrocellulose membranes and probed with M1 antibody (1:1000; 12 μg of protein per lane) (A) or anti- $G_s\alpha$ antibody (1:1000; 24 μg of protein per lane) (B) as described [4,5]. The molecular masses of marker proteins are indicated (in kDa) at the left. Shown are the horseradish-peroxidase-reacted nitrocellulose membranes of gels containing 10% (w/v) polyacrylamide.

$\beta_2\text{AR}(\Delta 26)G_s\alpha_L$, the expected and actual molecular masses on SDS/polyacrylamide gels corresponded, the molecular mass of $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$ was lower than expected. Differences between expected and actual molecular masses on SDS/polyacrylamide gels have been reported for other receptors [14]. Our results show

that even extreme C-terminal receptor deletions do not impair membrane targeting and expression of $\beta_2\text{ARG}_s\alpha$ fusion proteins.

The ligand-binding properties of $\beta_2\text{ARG}_s\alpha$ fusion proteins were studied. The inverse agonist [*erythro*-D,L-1-(7-methylindan-4-yloxy)-3-(propan-2-yl)aminobutan-2-ol] (ICI 118,551) inhibited [^3H]DHA binding in membranes expressing $\beta_2\text{ARG}_s\alpha_L$, $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ and $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$ with K_i values of 1.4 ± 0.2 , 1.4 ± 0.4 and 2.8 ± 1.5 nM respectively. These values are in agreement with the values obtained for non-fused $\beta_2\text{AR}$ [4,5,10,15,16]. As previously reported for $\beta_2\text{ARG}_s\alpha_L$ [4], the agonist (–)-isoprenaline (ISO) inhibited [^3H]DHA binding in membranes expressing $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ and $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$ in accordance with biphasic functions (Figure 3). The fraction of $\beta_2\text{AR}$ s displaying high affinity for agonist was $44.3 \pm 3.8\%$ for membranes expressing $\beta_2\text{ARG}_s\alpha_L$, $33.7 \pm 4.5\%$ for membranes expressing $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ and $39.6 \pm 3.5\%$ for membranes expressing $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$. The high-affinity K_i values of ISO were 1.0 ± 0.6 nM for $\beta_2\text{ARG}_s\alpha_L$, 0.6 ± 0.3 nM for $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ and 0.6 ± 0.2 nM for $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$. GTP[S] abolished high-affinity binding of agonist in membranes expressing $\beta_2\text{ARG}_s\alpha_L$ (K_i value for ISO, 189 ± 20 nM) and $\beta_2\text{AR}(\Delta 70)G_s\alpha_L$ (K_i for ISO, 165 ± 22 nM). In membranes expressing $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$, GTP[S] decreased the fraction of $\beta_2\text{AR}$ s displaying high affinity for agonist from $33.7 \pm 4.5\%$ to $12.0 \pm 3.1\%$. The high-affinity K_i for ISO under these conditions was 3.6 ± 1.1 nM; the low-affinity K_i for ISO was 264 ± 34 nM.

In our previous studies we have shown that in $\beta_2\text{ARG}_s\alpha$ fusion proteins, receptor/G-protein coupling can also be assessed by measuring GTPase activity [4,5]. In membranes expressing $\beta_2\text{ARG}_s\alpha_L$, the stimulatory effect of ISO (10 μM) on GTPase activity was 245%, whereas ICI 118,551 (1 μM) reduced GTP hydrolysis by 50% (Figure 4D). In membranes expressing $\beta_2\text{AR}(\Delta 26)G_s\alpha_L$ (5.9 pmol/mg) the basal GTPase activity was 48% lower than in membranes expressing $\beta_2\text{ARG}_s\alpha_L$ at

Figure 3 Competition by ISO of [3 H]DHA binding in Sf9 membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$, $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ and $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$: effect of GTP[S]

[3 H]DHA binding in Sf9 membranes was performed as described [4,5]. Reaction mixtures contained Sf9 membranes (15–40 μg of protein per tube) expressing fusion proteins (3.0–7.4 pmol/mg), 1 nM [3 H]DHA and ISO at the concentrations indicated on the abscissa. Reaction mixtures additionally contained distilled water (control) or GTP[S] (10 μM). Data points shown are the means $\pm$ S.D. for four to seven experiments performed in triplicate. Competition isotherms were generated by non-linear regression analysis.

7.0 pmol/mg (compare Figures 4D and 4E) and the ligand-regulated GTPase activity was decreased by 62 % (ligand-regulated activity is the difference between maximum ISO-stimulated and maximum ICI 118,551-inhibited activity). With $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ even more drastic changes in GTP hydrolysis were observed. Specifically, the basal GTPase activity in membranes expressing $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ (6.0 pmol/mg) was 73 % lower than in membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$, and the ligand-regulated GTPase activity was decreased by 83 % (Figure 4F).

We also calculated the ISO-stimulated GTP turnover of fusion proteins expressed at levels between 3.0 and 7.4 pmol/mg. This procedure permits a comparison of fusion proteins even when expressed at different levels (see the Materials and methods section) [4,5,13]. With 100 nM GTP as substrate, the GTP turnover of $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ stimulated by ISO (10 μM) was $0.96 \pm 0.15 \text{ min}^{-1}$. The corresponding numbers for $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ and $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ were 0.34 ± 0.05 and $0.16 \pm 0.08 \text{ min}^{-1}$ respectively. These results show that the decreased GTP-hydrolysing activity of fusion proteins with C-terminal receptor deletions is an intrinsic property of the constructs rather than a property related to changes in expression level.

The GTP[S] binding assay monitors an initial event of the G-protein activation/inactivation cycle, i.e. GDP/GTP exchange, whereas the GTPase assay monitors the overall velocity of the cycle [1,4–6]. In membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ (7.0 pmol/mg), ISO (10 μM) increased GTP[S] binding by 296 %, and ICI 118,551 (1 μM) decreased GTP[S] binding by 32 % (Figure 4A). In contrast with GTPase regulation, basal GTP[S] binding in membranes expressing $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ was increased by 50 % compared with membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$. In membranes expressing $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$, basal GTP[S] binding was 34 % lower than in membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ (Figures 4A–4C). Compared with membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$, ligand-regulated GTP[S] binding in membranes expressing $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ was decreased by 39 % and in membranes expressing $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ ligand-regulated GTP[S] binding was decreased by 36 %.

In membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$, ISO (10 μM) increased AC activity by 34 %, whereas ICI 118,551 (1 μM) decreased AC activity by 40 % (Figure 4G). Membranes expressing $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ had a 27 % higher basal AC activity than membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ despite the slightly lower

expression level of $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ (5.9 pmol/mg) than of $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ (7.0 pmol/mg) (Figure 4H). In addition, the ligand-regulated AC activity was 61 % higher than in membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$. As a result of these changes, the maximum agonist-stimulated AC activity in membranes expressing $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ was 50 % higher than in membranes expressing $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$. In membranes expressing $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ (6.0 pmol/mg), basal AC activity was of similar magnitude to that in membranes expressing $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ (5.9 pmol/mg) (Figure 4I) but the increase in ligand-regulated AC activity compared with $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ was less pronounced [14 % increase with $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ compared with 61 % increase with $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$].

DISCUSSION

In an effort to determine the optimal tether length between receptor and G-protein in $\beta_2\text{ARG}_{\text{s}\alpha}$ fusion proteins, we deleted 26 and 70 residues of the receptor C-terminus. In $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ approx. one-third of the receptor C-terminus has been deleted, whereas in $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ most of the C-terminus has been removed (Figure 1). Thus in $\beta_2\text{AR}(\Delta 26)\text{G}_{\text{s}\alpha\text{L}}$ the mobility of $\text{G}_{\text{s}\alpha}$ relative to the $\beta_2\text{AR}$ is moderately decreased compared with that of $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$, whereas in $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ the mobility of $\text{G}_{\text{s}\alpha}$ relative to the $\beta_2\text{AR}$ is highly restricted.

It is of interest that all three fusion proteins can be expressed at similarly high levels in Sf9 membranes, indicating that the large deletion in $\beta_2\text{AR}(\Delta 70)\text{G}_{\text{s}\alpha\text{L}}$ did not interfere with correct folding and membrane-targeting of the fusion protein (Figure 2). C-terminal receptor deletions did not change affinities for inverse agonist or impair the ability of the $\beta_2\text{ARG}_{\text{s}\alpha\text{L}}$ fusion protein to efficiently form ternary complexes (Figure 3). The potencies of agonist and inverse agonist were not changed by deletions (Figures 3 and 4), and the pharmacological profile of a series of $\beta_2\text{AR}$ agonists with different intrinsic activities was not altered by C-terminal $\beta_2\text{AR}$ deletions from fusion proteins (results not shown). When coupled to $\text{G}_{\text{s}\alpha\text{L}}$ the $\beta_2\text{AR}$ shows properties of constitutive activity as demonstrated by the high inhibitory efficacy of the inverse agonist ICI 118,551 in AC assays [4,5]. This property is preserved in fusion proteins with C-terminal receptor deletions (Figure 4G–4I). Collectively these results demonstrate that C-terminal receptor deletions do not compromise receptor/G-protein coupling.

Figure 4 Effects of ISO (●) and ICI 118,551 (○) on GTP[S] binding, GTPase activity and AC activity in Sf9 membranes expressing $\beta_2\text{ARG}_{s\alpha_L}$, $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$

(A–C) GTP[S] binding was determined as described [5]. Reaction mixtures contained 0.4 nM [^{35}S]GTP[S] (0.1 μCi per tube), 1 μM GDP and ISO or ICI 118,551 (ICI) at the indicated concentrations. (D–F) GTPase activity was determined as described [4,5]. Reaction mixtures contained 100 nM GTP (0.5 μCi per tube) and ligands at the indicated concentrations. (G–I) AC activity was determined as described [4]. Reaction mixtures contained 1 μM GTP, 40 μM [α - ^{32}P]ATP (2.0 μCi per tube) and ligands at the indicated concentrations. The expression levels of constructs were as follows: $\beta_2\text{ARG}_{s\alpha_L}$, 7.0 pmol/mg; $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$, 5.9 pmol/mg; $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$, 6.0 pmol/mg. Data shown are the means of three to five independent experiments performed in duplicate.

The most unexpected result of our study was that C-terminal receptor deletions enhance signalling efficiency in $\beta_2\text{ARG}_{s\alpha_L}$ fusion proteins in terms of basal and receptor-regulated AC activity (Figures 4G–4I). The $\beta_2\text{AR}$ C-terminus contains numerous phosphorylation sites that are involved in the desensitization processes [11,12] and are eliminated in $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ (Figure 1). Additionally, Sf9 cells contain kinases that can desensitize the $\beta_2\text{AR}$ [15,17]. Thus the enhanced AC activity in $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ might be explained by the removal of an inhibitory constraint, i.e. the loss of constitutive desensitization normally occurring in non-truncated $\beta_2\text{ARG}_{s\alpha_L}$. Desensitization is manifested by the decreased ability of agonist to stimulate GTPase and AC, as well as by the loss of high-affinity agonist binding [9,10]. However, the fraction of high-affinity binding

sites in membranes expressing $\beta_2\text{ARG}_{s\alpha_L}$ was slightly higher and not lower than in membranes expressing $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ (Figure 3). In addition, C-terminal deletions decreased basal and ISO-stimulated GTP hydrolysis (Figures 4D–4F). Moreover, treatment of Sf9 cells with the inverse agonist ICI 118,551 or the protein kinase A inhibitor K252b during infection with $\beta_2\text{ARG}_{s\alpha_L}$ -encoding baculovirus did not increase the fraction of $\beta_2\text{AR}$ s displaying high affinity for agonist (results not shown). Taken together these results suggest that there is no significant constitutive desensitization in $\beta_2\text{ARG}_{s\alpha_L}$ and that the enhanced AC activity in membranes expressing $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ cannot be explained by the removal of regulatory phosphorylation sites by the C-terminal deletions.

We also examined the effect of C-terminal receptor truncation on the affinity of fused $G_{s\alpha}$ for nucleotides. An increase in affinity for GTP relative to the affinity for GDP of fused $G_{s\alpha}$ could enhance its efficacy of AC activation. We measured affinity of $G_{s\alpha_L}$ in $\beta_2\text{ARG}_{s\alpha_L}$, $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ for GTP by assessing the potency of GTP at disrupting high-affinity agonist binding [4]. We found similar affinities of $\beta_2\text{ARG}_{s\alpha_L}$, $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ for GTP (49 ± 20 , 179 ± 78 and 65 ± 22 nM respectively). The affinities of $\beta_2\text{ARG}_{s\alpha_L}$, $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ for GDP were also similar (1.8 ± 0.2 , 1.6 ± 0.4 and 1.6 ± 0.3 μM respectively). These findings rule out the possibility that gross changes in the affinity of $G_{s\alpha_L}$ for GTP and GDP account for the enhanced AC activity in membranes expressing $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$.

Another possible explanation for the enhanced AC activation by $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ is that GDP/GTP exchange proceeds more efficiently in these fusion proteins than in $\beta_2\text{ARG}_{s\alpha_L}$. However, there was a moderate decrease in ligand-regulated GTP[S] binding in membranes expressing $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ compared with those expressing $\beta_2\text{ARG}_{s\alpha_L}$ (Figures 4A–4C). Intriguingly, compared with the modest effect of C-terminal receptor deletions on GTP[S] binding, the effect on GTPase activity, which measures the overall velocity of the G-protein activation/inactivation cycle, was much more pronounced (Figures 4D–4F). Thus C-terminal receptor deletions from $\beta_2\text{ARG}_{s\alpha_L}$ fusion proteins slow down GTP hydrolysis more efficiently than GTP binding. As a result of these kinetic changes, the fraction of $G_{s\alpha_L}$ molecules in the active GTP-liganded state in membranes expressing $\beta_2\text{AR}(\Delta 26)G_{s\alpha_L}$ and $\beta_2\text{AR}(\Delta 70)G_{s\alpha_L}$ would be expected to be higher than in membranes expressing $\beta_2\text{ARG}_{s\alpha_L}$. The final consequence of these changes would be more efficient AC activation in $\beta_2\text{ARG}_{s\alpha}$ fusion proteins with C-terminal receptor deletions compared with non-truncated $\beta_2\text{ARG}_{s\alpha_L}$. The experimental results are in accordance with this model (Figures 4G–4I).

The relatively selective inhibitory effect of C-terminal $\beta_2\text{AR}$ deletions on GTP hydrolysis by $G_{s\alpha_L}$ was somewhat surprising. We expected that C-terminal deletions would have a drastic effect on more direct indicators of receptor/G-protein coupling, such as high-affinity agonist binding, or on the ability of activated $G_{s\alpha}$ to access and stimulate AC. One might envisage that a shorter tether between $\beta_2\text{AR}$ and $G_{s\alpha}$ could prevent the optimal orientation of the receptor relative to the G-protein. However, receptor/G-protein coupling as assessed by GTP[S]-sensitive high-affinity agonist binding was not greatly altered (Figure 3), and receptor-regulated GTP[S] binding was only modestly impaired (Figures 4A–4C). In addition one could assume that a short tether between receptor and G-protein might prevent effective interaction between $G_{s\alpha}$ and AC. However, receptor-regulated AC activity was enhanced by truncations (Figures 4G–4I). The effect of the C-terminal deletions on GTPase activity might be due to more stable (or forced) interactions between the

β_2 AR and $G_s\alpha$, or between $G_s\alpha$ and AC. The forced interactions of $G_s\alpha$ with β_2 AR (or with AC) might restrict the conformational change in $G_s\alpha$ required for efficient GTP hydrolysis. Our finding that the GTPase step of the G-protein activation/inactivation cycle can be regulated is not without published precedent. Specifically, certain G-protein-regulated effector systems accelerate GTP hydrolysis [18–20] and a novel class of proteins, Regulators of G-protein signalling (RGS proteins), act as GTPase-activating proteins [21].

It should be noted that there is no linear relation between linker length and construct efficacy at activating AC. Specifically, β_2 AR($\Delta 70$) $G_s\alpha_L$ was not more but rather less efficient in this respect than β_2 AR($\Delta 26$) $G_s\alpha_L$. Possibly, in β_2 AR($\Delta 70$) $G_s\alpha_L$, the mobility of the tethered $G_s\alpha$ is restricted to such a great extent that the efficiency of G-protein interaction with effector system is impaired, i.e. $G_s\alpha$ might no longer be able to expose its AC-activating domain optimally when tethered too tightly to the receptor. Thus in β_2 AR($\Delta 26$) $G_s\alpha_L$ there might be an optimum mobility restriction with respect to both GTPase inhibition and AC activation.

Are there any physiological implications of our studies with β_2 ARG $s\alpha_L$ fusion proteins? Receptors and G-proteins cannot freely diffuse and interact with each other in the plasma membrane. Rather, membranes are likely to consist of multiple cytoskeleton-created microcompartments that constrain the mobility of signalling components [22–26]. Unfortunately the impact of protein mobility on signalling efficiency is virtually impossible to study in membranes because membrane preparation destroys the cytoskeleton and greatly increases protein mobility [27]. Our findings suggest that the cytoskeleton, by changing the relative mobility of signalling proteins relative to each other, could fine-tune signal transduction efficiency. Such an idea is supported by the fact that receptors and G-proteins do interact with cytoskeletal proteins [28–31]. Of particular interest is the fact that microtubule-disrupting substances potentiate β_2 AR-mediated AC activity in S49 lymphoma cells [32]. Thus β_2 ARG $s\alpha$ fusion proteins with increasing C-terminal receptor deletions decrease the mobility of receptor and G-protein in a graded manner and therefore could provide a model for the impact of protein mobility on signalling efficiency *in vivo*.

In conclusion, deletions of the receptor C-terminus in β_2 ARG $s\alpha$ fusion proteins decrease the mobility of $G_s\alpha$ relative to receptor and enhance basal and agonist-regulated AC activity by slowing down G-protein inactivation. Restricting the mobility of G-proteins relative to receptors represents a potential mechanism by which signalling efficiency can be modulated *in vivo*.

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