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Distinct interactions of $G_{s\alpha\text{-long}}$, $G_{s\alpha\text{-short}}$, and $G_{\alpha olf}$ with GTP, ITP, and XTP

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Abstract

The G_s-proteins $G_{s\alpha\text{-short}}$ ($G_{s\alpha S}$) and $G_{s\alpha\text{-long}}$ ($G_{s\alpha L}$), and the olfactory G_s protein ($G_{\alpha olf}$) mediate activation of adenylyl cyclase by the β_2-adrenoceptor (β_2AR). Early studies showed that the purine nucleotides GTP, ITP, and XTP differentially support receptor-mediated adenylyl cyclase activation in various native membrane systems, but those findings have remained unexplained thus far. We systematically analyzed the effects of GTP, ITP, and XTP on the coupling of the β_2AR to $G_{s\alpha S}$, $G_{s\alpha L}$, and $G_{\alpha olf}$, respectively, using fusion proteins expressed in Sf9 insect cells. Fusion proteins ensure defined receptor/G-protein stoichiometry and efficient coupling. At all three fusion proteins, GTP, ITP, and XTP exhibited unique profiles with respect to their potency and efficacy at disrupting high-affinity agonist binding and supporting adenylyl cyclase activation by partial and full agonists. Our data can be interpreted in two ways: (i) GTP, ITP, and XTP may stabilize different active conformations in various G_s-proteins, or (ii) GTP, ITP, and XTP may differ from one another in the kinetics of interaction with various G_s-proteins. Regardless of which of the two explanations is correct, our present data demonstrate that GTP, ITP, and XTP are highly efficient regulators of signal transduction mediated through a specific G-protein. Also discussed is the possibility that G-protein activation by ITP and XTP may be of relevance in Lesch–Nyhan syndrome, a defect of the purine salvage pathway associated with abnormalities in various neurotransmitter systems.

Keywords: β_2-Adrenoceptor; G_s-proteins; Adenylyl cyclase; Purine nucleotides; Fusion proteins; Sf9 insect cells

1. Introduction

Upon agonist binding, GPCRs undergo a conformational change that enables them to interact with G-proteins [1,2]. G-proteins are heterotrimeric and serve as signal transducers between GPCRs and effector systems [3–5]. The extended ternary complex model of GPCR activation assumes that GPCRs isomerize from an inactive (R) state to an active (R^*) state and that agonists stabilize the R^* state [2,6]. In the R^* state, GPCRs promote the dissociation of GDP from G_α and subsequently form a TC with guanine nucleotide-free G_α. The TC is characterized by high agonist affinity. In the next step, GPCRs actively promote binding of GTP to G_α. GTP binding to G_α uncouples GPCRs from G_α and disrupts the TC. $G_{\alpha\text{-GTP}}$ dissociates from the G-protein $\beta\gamma$-complex, and both $G_{\alpha\text{-GTP}}$ and the $\beta\gamma$-complex regulate the activity of effector systems.

The β_2AR is a prototypical GPCR that interacts with G_s-proteins to mediate AC activation [7]. The G-protein $G_{s\alpha}$ exists in three isoforms, $G_{s\alpha S}$, $G_{s\alpha L}$, and $G_{\alpha olf}$, respectively [8–11]. The establishment of fusion protein technology greatly facilitated the analysis of GPCR-G_s-protein interactions. Fusion proteins provide a defined 1:1 stoichiometry of GPCR and $G_{s\alpha}$, promote efficient coupling, and allow for the analysis of the coupling of a given GPCR to various G_s-proteins under precisely defined experimental conditions

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Abbreviations: AC, adenylyl cyclase; β_2AR, β_2-adrenoceptor; β_2AR-$G_{\alpha olf}$, fusion protein containing the β_2AR and $G_{\alpha olf}$; β_2AR-$G_{s\alpha L}$, fusion protein containing the β_2AR and the long splice variant of $G_{s\alpha}$; β_2AR-$G_{s\alpha S}$, fusion protein containing the β_2AR and the short splice variant of $G_{s\alpha}$; cAMP, cyclic AMP; DCI, dichloroisoproterenol; DOB, dobutamine; [^{3}H]DHA, [^{3}H]dihydroalprenolol; EPH, (−)-ephedrine; G_α, unspecified G-protein α-subunit; $G_{\alpha olf}$, G-protein of the G_s-family that is expressed in several cell types including olfactory sensory cells; GPCR, G-protein-coupled receptor; $G_{s\alpha L}$, long splice variant of the G_s-protein $G_{s\alpha}$; $G_{s\alpha}$, unspecified G-protein of the G_s-family; $G_{s\alpha S}$, short splice variant of the G_s-protein $G_{s\alpha}$; ISO, (−)-isoproterenol; NTP, nucleoside 5′-triphosphate; PRO, (−)-propranolol; SAL, salbutamol; and TC, ternary complex.

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[12–16]. Studies with fusion proteins showed that $G_{s\alpha S}$, $G_{s\alpha L}$, and $G_{\alpha olf}$ differ from each other in their interactions with the β_2AR and AC [13,14,16]. In a recent study, we used the purine nucleotides GTP, ITP, and XTP as experimental probes to analyze the interactions of the β_2AR with $G_{s\alpha L}$ and AC (EC 4.6.1.1) [17]. The three NTPs differ from each other in the substitution of C_2 of the purine ring, causing differences in the interaction of the guanine, xanthine, and inosine ring with a highly conserved aspartate residue in G_α (Asp295 in $G_{s\alpha L}$). In the β_2AR-$G_{s\alpha L}$ fusion protein, NTPs disrupt the TC and support AC activation by agonists in the following order of potency: GTP > ITP > XTP [17]. Moreover, ITP and XTP are less efficient than GTP at disrupting the TC between the β_2AR and $G_{s\alpha L}$ and supporting AC activation. Furthermore, the pharmacological profile of the β_2AR coupled to $G_{s\alpha L}$ in terms of agonist potencies and efficacies is different for GTP, ITP, and XTP. We explained these findings by a model in which different agonists stabilize unique β_2AR conformations. Those GPCR states, in turn, stabilize distinct $G_{s\alpha L}$ conformations with unique characteristics for interaction with GTP, ITP, and XTP.

Early studies showed that GTP, ITP, and XTP differ substantially from each other in their efficacies at supporting AC activation in various native membrane systems [18–21]. However, those findings have remained unexplained thus far. Therefore, in our present study we tested the hypothesis that GTP, ITP, and XTP interact differentially with $G_{s\alpha L}$, $G_{s\alpha S}$, and $G_{\alpha olf}$. As a model system, we analyzed fusion proteins of the β_2AR with the individual G_s-proteins expressed in Sf9 insect cells.

2. Materials and methods

2.1. Materials

The construction of the cDNAs for β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ and the generation of recombinant baculoviruses encoding the three fusion proteins have been described elsewhere [13,16,22]. [α-^{32}P]ATP (3000 Ci/mmol) was from Perkin-Elmer. [^{3}H]DHA (85–90 Ci/mmol) was from Amersham Pharmacia Biotech. Unlabeled ATP [special quality, catalogue No. 519 979; <0.01% (w/w) GTP contamination as assessed by HPLC analysis] and GTP were obtained from Roche Diagnostics. ISO, SAL, EPH, DOB, (±)-alprenolol, ITP, and XTP were from the Sigma Chemical Co. DCI was from the Aldrich Chemical Co. Glass fiber filters (GF/C) were from Schleicher & Schuell.

2.2. Cell culture and membrane preparation

Sf9 cells were cultured in 250-mL disposable Erlenmeyer flasks at 28° under rotation at 125 rpm in SF 900 II medium (Life Technology) supplemented with 5% (v/v) fetal bovine serum (BioWhittaker) and 0.1 mg/mL of gentamicin (BioWhittaker). Cells were maintained at a density of 1.0 to 6.0 × 10^6/mL. For infection, Sf9 cells were sedimented by centrifugation (5 min at 500 *g*, 4°) and suspended in fresh medium. Cells were seeded at 3.0 × 10^6/mL and infected with 1:100 or 1:1000 dilutions of high-titer baculovirus stocks encoding fusion proteins. In the present study, fusion proteins were exclusively analyzed without co-expressed mammalian βγ-complexes. Mammalian βγ-complexes are not required for the efficient function of β_2AR-$G_{s\alpha}$ fusion proteins and reduce the signal-to-noise ratio for the analysis of agonist effects in the AC assay [22]. Sf9 cells were cultured for 48 hr before membrane preparation. Sf9 membranes were prepared as described [22], using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL of benzamidine, and 10 μg/mL of leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA, and 75 mM Tris/HCl, pH 7.4), and stored at −80° for periods of up to 3 years (longer periods of time were not analyzed) without loss of functional activity in the various assays employed.

2.3. [^{3}H]DHA binding assay

Sf9 membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous nucleotides as far as possible, and resuspended in binding buffer. Expression levels of fusion proteins were determined by incubating Sf9 membranes (10 μg of protein/tube) in the presence of 10 nM [^{3}H]DHA and 0.05% (w/v) bovine serum albumin. Non-specific binding was determined in the presence of 10 μM (±)-alprenolol. The total volume of the binding reaction was 500 μL. Incubations were conducted for 90 min at 25° with shaking at 250 rpm. For the determination of the potencies and efficacies of NTPs at TC disruption, reaction mixtures contained Sf9 membranes (20–25 μg of protein/tube), 1 nM [^{3}H]DHA, 1 μM SAL, and NTPs at increasing concentrations. Bound [^{3}H]DHA was separated from free [^{3}H]DHA by filtration through GF/C filters using a 48-well harvester (model M-48R, Brandel), followed by three washes with 2 mL of binding buffer (4°). Filter-bound radioactivity was determined by liquid scintillation counting using Cytoscint fluid from ICN. The experimental conditions chosen ensured that not more than 10% of the total amount of [^{3}H]DHA added to the binding tubes bound to filters.

2.4. AC activity assay

Sf9 membranes were thawed, sedimented by a 15-min centrifugation at 4° and 15,000 *g* to remove residual endogenous nucleotides as far as possible, and resuspended in binding buffer. AC activity in Sf9 membranes was determined as described [13,22]. Tubes contained Sf9 membranes (15–30 μg of protein/tube), 5 mM $MgCl_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, and NTPs at

various concentrations without or with various β_2AR ligands at different concentrations. Assay tubes containing membranes, NTPs, and β_2AR ligands in a total volume of 30 μL were incubated for 3 min at 37° before initiating reactions by the addition of 20 μL of reaction mixture containing (final) [α-^{32}P]ATP (1.0 to 1.5 μCi/tube) plus 40 μM unlabeled ATP, 0.1 mM cAMP, and an NTP-regenerating system consisting of 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, and 1 IU of myokinase. We used only the special quality ATP from Roche (catalogue No. 519 979; <0.01% GTP content as assessed by HPLC analysis) to minimize exogenous GTP contamination. Reactions were conducted for 20 min at 37°. For all experiments reported in this study we used the endogenous AC of Sf9 cell membranes as effector. Reactions were terminated by the addition of 20 μL of 2.2 N HCl. Denatured protein was sedimented by a 3-min centrifugation at 25° and 15,000 *g*. Sixty-five microliters of the supernatant fluid was applied onto disposable columns filled with 1.3 g of neutral alumina (Sigma A-1522, super I, WN-6). [^{32}P]cAMP was separated from [α-^{32}P]ATP by elution of [^{32}P]cAMP with 4 mL of 0.1 M ammonium acetate, pH 7.0. Recovery of [^{32}P]cAMP was ∼80%. Blank values were routinely ∼0.01% of the total amount of [α-^{32}P]ATP added. [^{32}P]cAMP was determined by liquid scintillation counting using ScintiSafe Econo2 scintillation fluid from Fisher Scientific. The experimental conditions chosen ensured that not more than 1–3% of the total amount of [α-^{32}P]ATP added was converted to [^{32}P]cAMP.

2.5. Miscellaneous

Protein concentrations were determined using a DC protein assay kit from Bio-Rad. Data shown were subjected to linear and/or non-linear regression analysis using the Prism III program (GraphPad, Prism). Statistical comparisons of the effects of GTP versus ITP, GTP versus XTP, and ITP versus XTP were made using the *t*-test.

3. Results

3.1. Differential disruption of the TC by GTP, ITP, and XTP in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$

One of the earliest steps of the G-protein cycle is the formation of the TC consisting of agonist-occupied GPCR and nucleotide-free G-protein. We examined binding of a fixed concentration of the β_2AR antagonist [^{3}H]DHA to β_2AR-$G_{s\alpha}$ fusion proteins in the presence of an agonist (SAL) at a fixed sub-saturating concentration (1 μM) and NTPs at increasing concentrations. NTPs decrease the affinity of the β_2AR for SAL and, as a result, increase [^{3}H]DHA binding [13,17]. As reported before [17], at β_2AR-$G_{s\alpha L}$ NTPs inhibited TC formation in the order of potency GTP > ITP > XTP and in the order of efficacy GTP > ITP ∼ XTP (Fig. 1B and Table 1). At β_2AR-$G_{s\alpha S}$ the order of potency of NTPs for TC disruption was GTP > XTP ∼ ITP, and the order of efficacy was GTP > ITP > XTP (Fig. 1A and Table 1). Relative to GTP, ITP was more efficacious at β_2AR-$G_{s\alpha S}$ than at β_2AR-$G_{s\alpha L}$. Moreover, at β_2AR-$G_{s\alpha L}$, XTP was ∼120-fold less potent than GTP, whereas at β_2AR-$G_{s\alpha S}$, XTP was only ∼6-fold less potent than GTP. At β_2AR-$G_{\alpha olf}$, NTPs disrupted TC in the order of efficacy GTP ∼ ITP > XTP (Fig. 1C and Table 1). At β_2AR-$G_{\alpha olf}$ XTP was only ∼3-fold less potent than GTP and even ∼4-fold more potent than ITP. Collectively, these data show that the efficacies

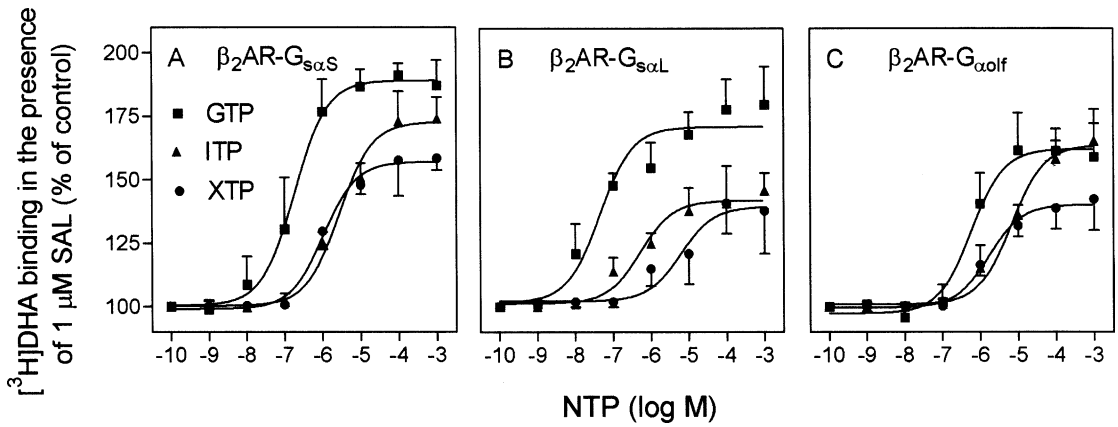

Fig. 1. Effects of GTP, ITP, and XTP on TC formation in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, or β_2AR-$G_{\alpha olf}$. [^{3}H]DHA binding in Sf9 membranes was performed as described in Section 2. Reaction mixtures contained Sf9 membranes (20–25 μg of protein/tube) expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, or β_2AR-$G_{\alpha olf}$, 1 nM [^{3}H]DHA, 1 μM SAL, and NTPs (■, GTP; ▲, ITP; ●, XTP) at the concentration indicated on the abscissa. Note that "−10" designates the absence of added GTP, ITP, or XTP. (A) Membranes expressing β_2AR-$G_{s\alpha S}$ at 2.6 to 4.4 pmol/mg; (B) membranes expressing β_2AR-$G_{s\alpha L}$ at 5.0 to 7.5 pmol/mg; (C) membranes expressing β_2AR-$G_{\alpha olf}$ at 3.3 to 6.2 pmol/mg. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 3–5 independent experiments performed in triplicate. Data shown in (B) were taken from Fig. 1C in [17]. The data shown in (A) and (C) for GTP were from Fig. 3 in [16].

Table 1
Comparison of the effects of GTP, ITP, and XTP on TC disruption and AC activation in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$

Parameter	GTP	ITP	XTP
β_2AR-$G_{s\alpha S}$			
TC disruption			
Efficacy	1.00 (0.96–1.04)	0.82 (0.73–0.90)*	0.65 (0.59–0.69)**,***
EC_{50} (μM)	0.18 (0.13–0.26)	2.7 (1.4–5.3)*	1.1 (063–1.7)**
AC activation			
Efficacy	1.00 (0.93–1.07)	0.98 (0.90–1.02)	0.67 (0.59–0.76)**,***
EC_{50} (μM)	0.30 (0.19–0.48)	8.0 (6.0–11)*	14 (8.4–22)**
β_2AR-$G_{s\alpha L}$			
TC disruption			
Efficacy	1.00 (0.84–1.16)	0.59 (0.50–0.67)*	0.53 (0.41–0.67)**
EC_{50} (μM)	0.05 (0.01–0.20)	0.52 (0.18–1.6)*	6.1(2.5–25.3)**,***
AC activation			
Efficacy	1.00 (0.77–1.23)	0.68 (0.49–0.76)*	0.51 (0.42–0.56)**
EC_{50} (μM)	0.14 (0.02–0.68)	4.6 (0.74–15)*	21 (9.3–46)**
β_2AR-$G_{\alpha olf}$			
TC disruption			
Efficacy	1.00 (0.91–1.10)	0.97 (0.86–1.07)	0.62 (0.58–0.68)**,***
EC_{50} (μM)	0.58 (0.29–1.2)	6.6 (3.6–12)*	1.7 (0.98–2.9)***
AC activation			
Efficacy	1.00 (0.95–1.06)	0.77 (0.70–0.91)*	0.64 (0.58–0.72)**
EC_{50} (μM)	1.1 (0.72–1.8)	53 (28–100)*	18 (13–25)**,***

The efficacies (maximum effects) and EC_{50} values (potencies) of NTPs at disrupting the TC in membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ were derived from non-linear regression analysis of the data shown in Fig. 1. The efficacies (maximum effects) and EC_{50} values (potencies) of NTPs at supporting ISO-stimulated AC activity in membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ were derived from non-linear regression analysis of the data shown in Fig. 2. The efficacies of GTP were set 1.00, and the efficacies of ITP and XTP were referred to the effect of GTP. Shown are the mean values and, in parentheses, the 95% confidence intervals.

* $P < 0.05$ for comparison of GTP versus ITP.

** $P < 0.05$ for comparison of GTP versus XTP.

*** $P < 0.05$ for comparison of ITP versus XTP.

and/or potencies of GTP, ITP, and XTP for TC disruption at β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ differ considerably from each other.

3.2. Differential effects of GTP, ITP, and XTP on basal and ISO-stimulated AC activity in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$

NTPs increased basal AC activity in membranes expressing β_2AR-$G_{s\alpha L}$ in the order of potency and efficacy GTP > ITP > XTP (Fig. 2D–F). In membranes expressing β_2AR-$G_{s\alpha S}$, NTPs had only small stimulatory effects on basal AC activity (Fig. 2A–C). In membranes expressing β_2AR-$G_{\alpha olf}$, the maximum stimulatory effects of GTP and ITP on basal AC activity (Fig. 2G and H) were much smaller than in membranes expressing β_2AR-$G_{s\alpha L}$. However, in contrast to membranes expressing β_2AR-$G_{s\alpha L}$, XTP had a robust stimulatory effect on basal AC activity in membranes expressing β_2AR-$G_{\alpha olf}$ (Fig. 2I).

As reported before [13,16], membranes expressing β_2AR-$G_{s\alpha S}$ exhibited a much higher basal AC activity in the absence of NTPs than membranes expressing β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{\alpha olf}$ (Fig. 2). Additionally, ISO efficiently reduced AC activity in the absence of NTPs or in the presence of NTPs at low concentrations in membranes expressing β_2AR-$G_{s\alpha S}$ but not in membranes expressing β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{\alpha olf}$. These differences between the fusion proteins are explained by the fact that $G_{s\alpha S}$ possesses a higher GDP affinity than $G_{s\alpha L}$ and $G_{\alpha olf}$ and that $G_{s\alpha\text{-GDP}}$ activates AC more efficiently than nucleotide-free $G_{s\alpha}$ [13,16]. Accordingly, in membranes expressing β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{\alpha olf}$, the majority of the $G_{s\alpha}$ molecules are GDP-free in the absence of NTP so that ISO is inefficient at promoting additional GDP dissociation from these G-proteins. In contrast, the majority of the $G_{s\alpha S}$ molecules are GDP-liganded in the absence of NTP so that ISO efficiently induces GDP dissociation from $G_{s\alpha S}$ and, thereby, decreases AC activity. With increasing NTP concentration, the β_2AR becomes capable of stimulating NTP binding to $G_{s\alpha}$-proteins, thereby reverting the agonist-inhibition of AC activity into an agonist-stimulation of AC. Based on the extent of TC formation of the β_2AR with $G_{s\alpha S}$ and $G_{s\alpha L}$, it is unlikely that the affinity of the β_2AR for these two $G_{s\alpha}$ proteins differs greatly [13]. Thus, differences in the affinity of β_2AR-$G_{s\alpha}$ interactions are unlikely to provide an explanation for the differential inhibitory effects of ISO on AC activity in membranes expressing various β_2AR-$G_{s\alpha}$ fusion proteins. Inhibitory effects of

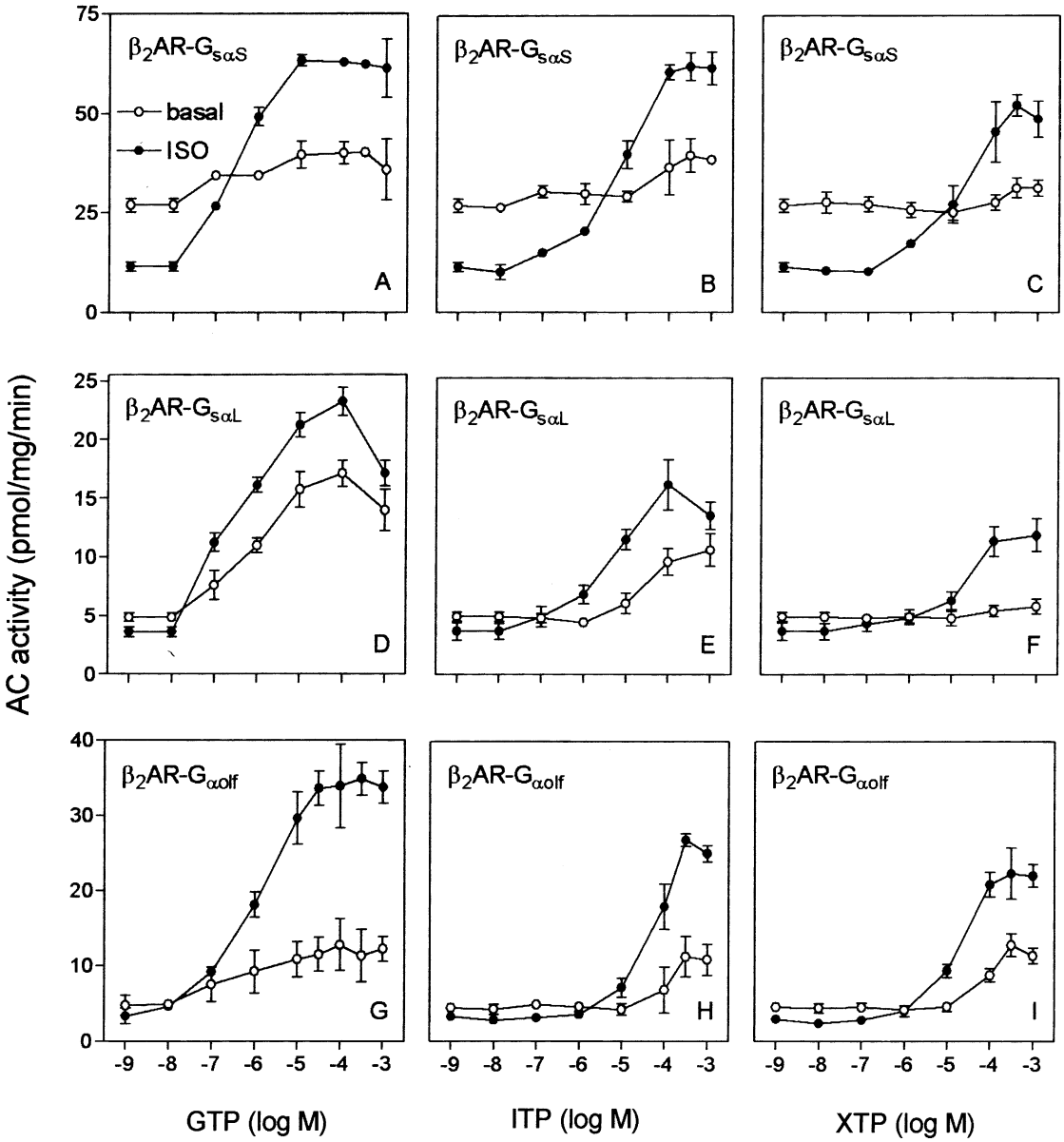

Fig. 2. Effects of GTP, ITP, and XTP on AC activity in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, or β_2AR-$G_{\alpha olf}$. AC activity in Sf9 membranes was determined as described in Section 2. Reaction mixtures contained Sf9 membranes (15–30 μg protein per tube) expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, or β_2AR-$G_{\alpha olf}$, and NTPs at the concentrations indicated on the abscissa with solvent (basal) (○) or with 10 μM ISO (●). Note that "−9" designates the absence of GTP, ITP, or XTP. (A–C) membranes expressing β_2AR-$G_{s\alpha S}$ at 2.6 to 3.0 pmol/mg; (D–F) membranes expressing β_2AR-$G_{s\alpha L}$ at 2.3 to 2.7 pmol/mg; (G–I) membranes expressing β_2AR-$G_{\alpha olf}$ at 3.1 to 3.5 pmol/mg. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. The figure shows point-to-point connections to allow for better comparison with previously published data on the effects of GTP, ITP, and XTP on AC regulation in membranes expressing β_2AR-$G_{s\alpha L}$ [17]. The results of the non-linear regression analyses are summarized in Table 1. Data shown are the means ± SD of 3–5 independent experiments performed in duplicate. Data shown in (D–F) were taken from panels A–C of Fig. 3 in [17]. Data shown in (A) and (G) were from Fig. 6 in [16]. Note that the scale of the y-axis is different for the three fusion proteins, reflecting their different maximum efficacies at activating AC [13,16]. The different scales of the y-axis were chosen to facilitate comparison of the relative stimulatory effects of NTPs at the three fusion proteins.

agonists on AC activity in the absence of GTP were also observed in non-fused systems [23], indicating that our observations are not an artifact of the fusion.

In membranes expressing β_2AR-$G_{s\alpha L}$, NTPs supported ISO-stimulated AC activity in the order of potency and efficacy GTP > ITP ∼ XTP (Fig. 2D–F and Table 1). At β_2AR-$G_{s\alpha S}$, the order of potency of NTPs at supporting ISO-stimulated AC activity was the same as at β_2AR-$G_{s\alpha L}$, but at β_2AR-$G_{s\alpha S}$ the order of efficacy was GTP ∼ ITP > XTP (Fig. 2A–C and Table 1). In membranes expressing β_2AR-$G_{\alpha olf}$, the order of efficacy of NTPs at supporting ISO-stimulated AC activity was GTP > ITP ∼ XTP, but the order of potency was GTP > XTP > ITP (Fig. 2G–I and Table 1).

3.3. Differences in agonist potencies and efficacies for AC activation in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ in the presence of GTP, ITP, and XTP

In a previous study, we showed that the efficacies of various β_2AR agonists at activating AC by $G_{s\alpha L}$ in the presence of GTP, ITP, and XTP differ considerably from each other [17]. This is reflected by the non-linear relations between the ligand efficacies with GTP versus ITP, GTP versus XTP, and ITP versus XTP (Fig. 3D–F). In addition, the potencies of the individual β_2AR agonists at activating AC by $G_{s\alpha L}$ in the presence of GTP, ITP, and XTP varied considerably from each other (see ratios EC_{50} NTP_1/EC_{50} NTP_2 in Table 2). In contrast to the data obtained for β_2AR-$G_{s\alpha L}$, the efficacies of β_2AR agonists at activating AC in membranes expressing β_2AR-$G_{s\alpha S}$ in the presence of GTP, ITP, and XTP were similar (Table 2). This is reflected by linear correlations of the efficacies of agonists with GTP versus ITP, GTP versus XTP, and ITP versus XTP (Fig. 3A–C). Moreover, the ratios of EC_{50} NTP_1/EC_{50} NTP_2 for agonists at β_2AR-$G_{s\alpha S}$ differed substantially from the corresponding values for β_2AR-$G_{s\alpha L}$. For AC activation by β_2AR-$G_{\alpha olf}$, we observed linear correlations between the efficacies of agonists with GTP versus ITP and GTP versus XTP (Fig. 3G and H). For the comparison of ligand efficacies with ITP versus XTP, a non-linear relation emerged (Fig. 3I). The ratios of EC_{50} NTP_1/EC_{50} NTP_2 for agonists at β_2AR-$G_{\alpha olf}$ differed

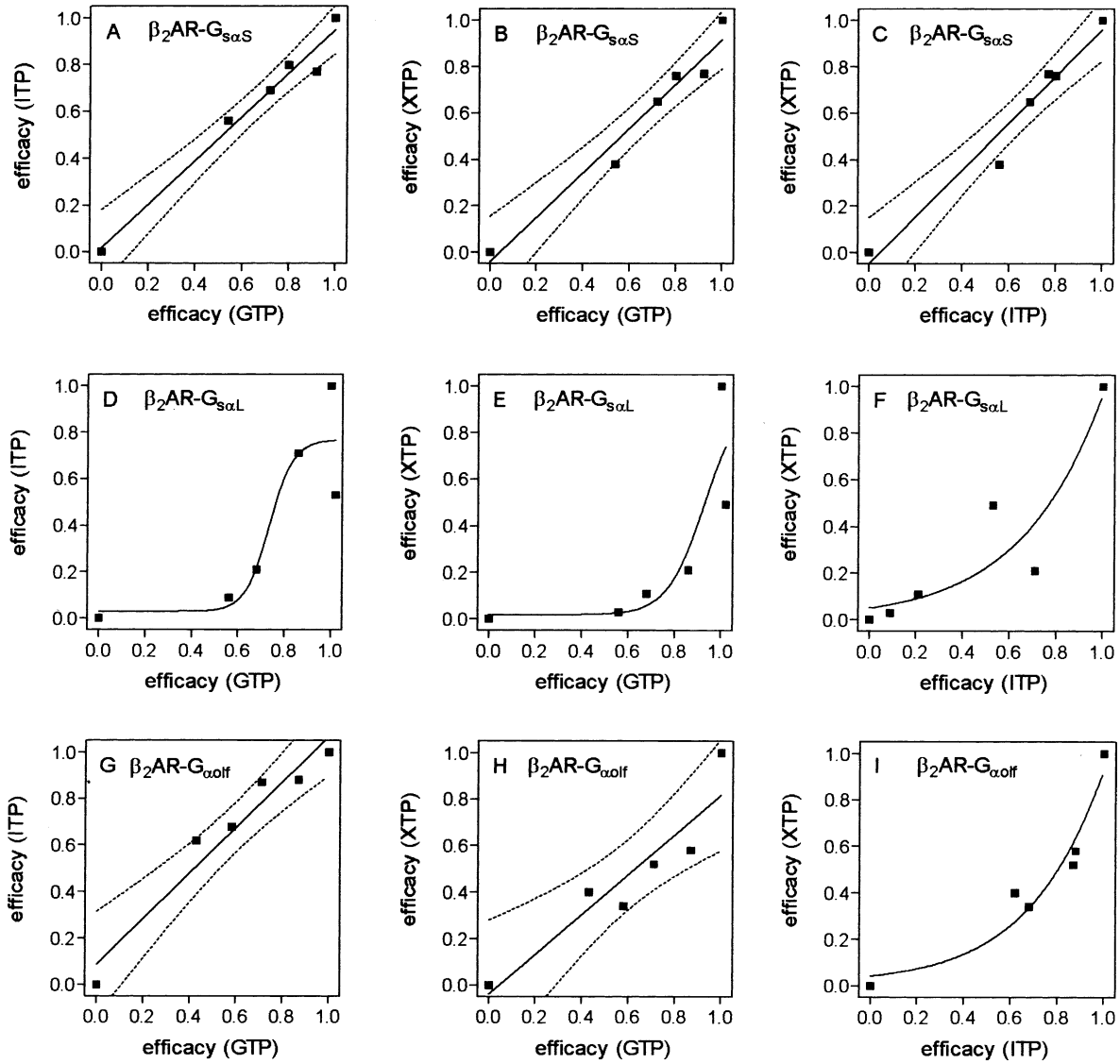

Fig. 3. Relations of the efficacies of agonists at activating AC in Sf9 membranes expressing a given β_2AR-$G_{s\alpha}$ fusion protein in the presence of various NTPs. The efficacies of PRO, DCI, EPH, DOB, SAL, and ISO at activating AC in membranes expressing β_2AR-$G_{s\alpha S}$ (A–C), β_2AR-$G_{s\alpha L}$ (D–F), and β_2AR-$G_{\alpha olf}$ (G–I) in the presence of various NTPs were taken from Table 2. For each fusion protein, ligand efficacies in the presence of GTP, ITP, and XTP were plotted against each other and analyzed for best fit to linear or non-linear regression functions (F-test). In the case of best fit to linear regressions, the 95% confidence intervals of the regression lines are shown as dotted lines. A: $r^2 = 0.97$; $P = 0.0003$. B: $r^2 = 0.96$; $P = 0.0006$. C: $r^2 = 0.96$; $P = 0.0006$. G: $r^2 = 0.93$; $P = 0.0012$. H: $r^2 = 0.93$; $P = 0.0012$.

Table 2
Efficacies and potencies of β_2AR agonists at activating AC in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ in the presence of GTP, ITP, and XTP

Ligand	Efficacy			EC_{50} (nM)			Ratio EC_{50} NTP_1/EC_{50} NTP_2		
	GTP	ITP	XTP	GTP	ITP	XTP	ITP/GTP	XTP/GTP	XTP/ITP
β_2AR-$G_{s\alpha S}$									
ISO	1.00 (6)	1.00 (5)	1.00 (4)	30 ± 8	17 ± 5*	870 ± 310**	0.57	29.0	51.2
SAL	0.92 ± 0.02 (3)	0.77 ± 0.07* (5)	0.77 ± 0.06** (4)	280 ± 150	450 ± 90	2400 ± 660**,***	1.61	8.57	5.33
DOB	0.80 ± 0.08 (4)	0.80 ± 0.11 (5)	0.76 ± 0.07 (4)	160 ± 40	480 ± 170*	420 ± 100**	3.00	2.63	0.88
EPH	0.72 ± 0.09 (4)	0.69 ± 0.10 (5)	0.65 ± 0.07 (4)	410 ± 130	1200 ± 240*	17000 ± 1300**	2.93	41.5	14.2
DCI	0.54 ± 0.03 (3)	0.56 ± 0.05 (5)	0.38 ± 0.04**,*** (4)	170 ± 20	60 ± 40*	300 ± 150***	0.35	1.77	5.00
β_2AR-$G_{s\alpha L}$									
ISO	1.00 (4)	1.00 (4)	1.00 (4)	18 ± 8	233 ± 34*	416 ± 44**,***	12.9	23.1	1.79
SAL	1.02 ± 0.14 (4)	0.53 ± 0.15* (4)	0.49 ± 0.20** (4)	63 ± 20	1700 ± 200*	900 ± 300**,***	27.0	14.3	0.53
DOB	0.86 ± 0.16 (4)	0.71 ± 0.03 (4)	0.21 ± 0.10**,*** (4)	300 ± 110	850 ± 200*	3500 ± 800**,***	2.83	11.7	4.12
EPH	0.68 ± 0.16 (4)	0.21 ± 0.13* (4)	0.11 ± 0.11** (4)	8400 ± 110	10000 ± 2000	NA	1.19	NA	NA
DCI	0.56 ± 0.19 (4)	0.09 ± 0.07* (4)	0.03 ± 0.04** (4)	38 ± 12	48 ± 10	NA	1.26	NA	NA
β_2AR-$G_{\alpha olf}$									
ISO	1.00 (4)	1.00 (4)	1.00 (4)	6 ± 3	6 ± 1	100 ± 20**,***	1.00	16.7	16.7
SAL	0.87 ± 0.02 (4)	0.88 ± 0.08 (4)	0.58 ± 0.08**,*** (4)	100 ± 30	90 ± 30	660 ± 300**,***	0.90	6.60	7.33
DOB	0.71 ± 0.06 (4)	0.87 ± 0.09* (4)	0.52 ± 0.15**,*** (4)	250 ± 60	190 ± 70	1600 ± 890**,***	0.76	6.40	8.42
EPH	0.58 ± 0.04 (4)	0.68 ± 0.05* (4)	0.34 ± 0.03**,*** (4)	2900 ± 420	2500 ± 1300	4900 ± 1200**	0.86	1.69	1.96
DCI	0.43 ± 0.04 (4)	0.62 ± 0.12* (4)	0.40 ± 0.14 (4)	72 ± 10	48 ± 8	230 ± 10**,***	0.77	3.19	4.79

AC activity was determined as described in Section 2 using Sf9 membranes expressing β_2AR-$G_{s\alpha S}$ (2.6 to 3.0 pmol/mg), β_2AR-$G_{s\alpha L}$ (2.3 to 2.7 pmol/mg), and β_2AR-$G_{\alpha olf}$ (3.3 to 4.0 pmol/mg). Reaction mixtures contained GTP (100 μM), ITP (1 mM), or XTP (1 mM) and β_2AR agonists at concentrations from 1 nM–1 mM as appropriate to obtain saturated concentration/response curves. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. The maximum effects of ligands (efficacies) in the presence of a given NTP were referred to the maximum effect of ISO (set 1.00). Similar normalization procedures were used in a previous study [17]. Agonist potencies (EC_{50} values) are given in nM. The efficacy of PRO was 0 for all constructs and conditions and, for the sake of clarity, is not included in the table. Data shown are the means ± SD of the number of experiments given in parentheses in the second, third, and fourth columns. Data for β_2AR-$G_{s\alpha L}$ were taken from Tables 1 and 2 in [17]. NA: not applicable since the effects of agonists were too small to calculate precise EC_{50} values.

* $P < 0.05$ for comparison of GTP versus ITP.

** $P < 0.05$ for comparison of GTP versus XTP.

*** $P < 0.05$ for comparison of ITP versus XTP.

substantially from the corresponding values for β_2AR-$G_{s\alpha S}$ and β_2AR-$G_{s\alpha L}$ (Table 2).

We also correlated the efficacies of agonists at activating AC in membranes expressing different fusion proteins in the presence of a given NTP. For GTP, we found linear correlations between the efficacies of agonists at β_2AR-$G_{s\alpha S}$ versus β_2AR-$G_{s\alpha L}$, β_2AR-$G_{s\alpha S}$ versus β_2AR-$G_{\alpha olf}$, and β_2AR-$G_{s\alpha L}$ versus β_2AR-$G_{\alpha olf}$ (Fig. 4A–C). There was also a linear correlation between the efficacies of agonists in the presence of ITP at β_2AR-$G_{s\alpha S}$ versus β_2AR-$G_{\alpha olf}$ (Fig. 4E), but for the other two comparisons of fusion proteins with ITP and for all combinations of fusion proteins with XTP, the relations of agonist efficacies were non-linear and differed from each other (Fig. 4D and F–I).

4. Discussion

4.1. Different G-protein states

It is generally accepted that G-proteins exist in the GDP-liganded off-state, the nucleotide-free transitory state that forms the TC with GPCR, and the GTP-liganded on-state [3–5]. In the GTP-liganded state, $G_{s\alpha}$ disrupts the TC with the β_2AR (Fig. 1) and activates AC (Fig. 2). In a recent study, we observed that the purine nucleotides GTP, ITP, and XTP exhibit quite different effects on the G-protein $G_{s\alpha L}$ with respect to TC disruption and AC activation [17]. We explained those data by a model in which GTP, ITP, and XTP stabilize distinct conformations in $G_{s\alpha L}$. Previous studies showed that GTP, ITP, and XTP differ substantially in their efficacies at supporting AC activation in various native membrane systems [18–21]. An explanation for these data could be that different G_s-proteins are differentially expressed in various systems [24,25], and that GTP, ITP, and XTP stabilize distinct conformations in various G_s-proteins. Therefore, we studied the effects of GTP, ITP, and XTP on various β_2AR-$G_{s\alpha}$ fusion proteins expressed in Sf9 cells.

4.2. Hydrogen bonding of GTP, ITP, and XTP with G_α

Based on the hydrogen bonding of the purine ring with a highly conserved aspartate residue in G_α, we expected that the order of potency of NTPs at all G_s-proteins would be GTP > ITP > XTP [17]. If GTP, ITP, and XTP stabilized similar conformations in all G_s-proteins, we would also predict the efficacies of NTPs at β_2AR-$G_{s\alpha S}$ and β_2AR-$G_{\alpha olf}$ to be similar to the previously observed efficacies of NTPs at β_2AR-$G_{s\alpha L}$ [17]. In agreement with our prediction, the order of potencies of NTPs for TC disruption and AC activation at β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$ was GTP > ITP ≥ XTP

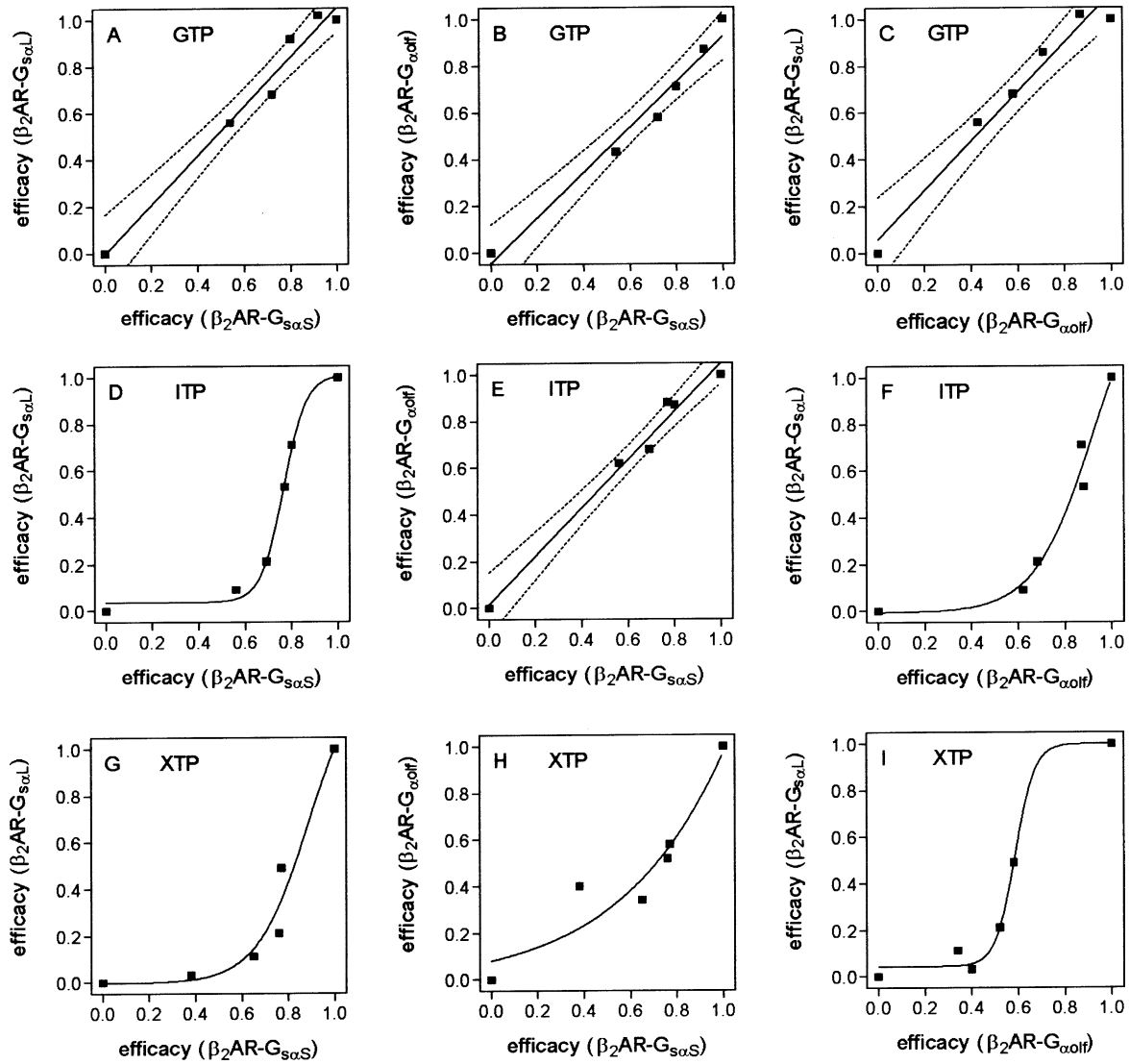

Fig. 4. Relations of the efficacies of agonists at activating AC in Sf9 membranes expressing various β_2AR-$G_{s\alpha}$ fusion proteins in the presence of a given NTP. The efficacies of PRO, DCI, EPH, DOB, SAL, and ISO at activating AC in membranes expressing various β_2AR-$G_{s\alpha}$ fusion proteins in the presence of GTP (A–C), ITP (D–F), and XTP (G–I) were taken from Table 2. For each NTP, ligand efficacies with β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ were plotted against each other and analyzed for best fit to linear or non-linear regression functions (F-test). In the case of best fit to linear regressions, the 95% confidence intervals of the regression lines are shown as dotted lines. A: $r^2 = 0.98$; $P = 0.0002$. B: $r^2 = 0.97$; $P = 0.0003$. C: $r^2 = 0.97$; $P = 0.0004$. E: $r^2 = 0.98$; $P = 0.0001$.

(Table 1). In contrast, at β_2AR-$G_{\alpha olf}$ the order of potency of NTPs for TC disruption and AC activation was GTP > XTP > ITP. The differences in NTP potencies indicate that GTP, ITP, and XTP interact differently with $G_{s\alpha S}$ and $G_{s\alpha L}$ relative to $G_{\alpha olf}$. The fact that at β_2AR-$G_{\alpha olf}$, XTP was more potent than ITP cannot be explained with our current knowledge on hydrogen bonding of the purine ring with a highly conserved aspartate residue in G-proteins and low molecular mass GTP-binding proteins [13,26–28]. However, it is also unknown why mutation of the highly conserved aspartate residue to asparagine in G_α, unlike in low-molecular mass GTP-binding proteins, increases the affinity of G_α for XTP only together with the mutation of another amino acid that abolishes the GTPase activity of G_α [26,28]. Of interest, $G_{\alpha olf}$ significantly differs from $G_{s\alpha S}$ and $G_{s\alpha L}$ in the α-helical domain [11], and such structural differences could have an impact on nucleotide-binding properties [29,30].

4.3. Differential interactions of GTP, ITP, and XTP with various β_2AR-$G_{s\alpha}$ fusion proteins. Link to previous studies

We observed distinct effects of GTP, ITP, and XTP on G_s-proteins with respect to the efficacies of NTPs at disrupting the TC and activating AC (Table 1). Specifically, at β_2AR-$G_{s\alpha L}$, NTPs disrupted the TC and supported ISO-stimulated AC activity in the order of efficacy GTP > ITP ∼ XTP. For TC disruption, the order of efficacy with β_2AR-$G_{s\alpha S}$ was GTP > ITP > XTP, and for AC activation the order of efficacy was GTP ∼ ITP > XTP. The order of

efficacy of NTPs for TC disruption at β_2AR-$G_{\alpha olf}$ was GTP ∼ ITP > XTP, but for AC activation in the presence of ISO the order was GTP > ITP ∼ XTP. The differences in the potencies and efficacies of GTP, ITP, and XTP concerning TC disruption and AC activation at β_2AR-$G_{s\alpha}$ fusion proteins could provide an explanation for the differential AC activation by GTP, ITP, and XTP in native systems [18–21]. Specifically, different native systems differentially express $G_{s\alpha S}$, $G_{s\alpha L}$, and $G_{\alpha olf}$ [24,25], and these G-proteins, in turn, differ from each other in their interactions with GTP, ITP, and XTP.

4.4. Non-linear and linear correlations between the efficacies of agonists at β_2AR-$G_{s\alpha}$ fusion proteins in the presence of GTP, ITP, and XTP. Multiple G-protein states versus different kinetics of NTP interactions with G-proteins

In a recent study, we showed that the patterns of efficacy of a series of β_2AR ligands for AC activation by $G_{s\alpha L}$ in the presence of GTP, ITP, and XTP are different from each other [17]. This is reflected by the non-linear relations between the efficacies of ligands at activating AC in the presence of GTP versus ITP, GTP versus XTP, and ITP versus XTP (Fig. 3D–F). Additionally, the potencies of individual agonists at activating AC by $G_{s\alpha L}$ in the presence of GTP, ITP, and XTP differed considerably from each other (Table 2). Our present data show that β_2AR agonists induce distinct responses in all three G_s-proteins. In particular, at β_2AR-$G_{s\alpha S}$ the efficacies of agonists at activating AC were similar with GTP, ITP, and XTP (Fig. 3A–C and Table 2), moderately similar for β_2AR-$G_{\alpha olf}$ (Fig. 3G–I and Table 2), and dissimilar for β_2AR-$G_{s\alpha L}$ (Fig. 3D–F and Table 2). When comparing AC regulation by β_2AR agonists at β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ with a given NTP, we observed similar efficacies with GTP (Fig. 4A–C) and with ITP for the comparison of β_2AR-$G_{s\alpha S}$ versus β_2AR-$G_{\alpha olf}$ (Fig. 4E), whereas as for the other comparisons with ITP (Fig. 4D and F) and for all comparisons with XTP (Fig. 4G–I) non-linear relations were observed. Furthermore, the relative potencies of β_2AR ligands at activating AC in the presence of GTP, ITP, and XTP were distinct for each fusion protein (see ratio EC_{50} NTP_1/EC_{50} NTP_2 in Table 2). We also observed differences in the efficacies of the agonist-free β_2AR at promoting NTP binding to G_s-proteins, as is reflected by the stimulatory effects of NTPs on basal AC activity (Fig. 2) [14,16,17]. Specifically, the agonist-free β_2AR promotes NTP binding to $G_{s\alpha L}$ in the order of efficacy GTP > ITP > XTP (Fig. 2D–F). High efficacy of the agonist-free β_2AR at promoting GTP binding to $G_{s\alpha L}$ also was observed in a co-expression system [22]. In contrast, the agonist-free β_2AR is inefficient at promoting NTP binding to $G_{s\alpha S}$ (Fig. 2A–C). Finally, the agonist-free β_2AR is similarly efficient at promoting the binding of GTP, ITP, and XTP to $G_{\alpha olf}$ (Fig. 2G–I).

With respect to the correlations including data with β_2AR-$G_{s\alpha L}$ in the presence of GTP, it may be argued that those correlations are not valid since the high basal AC activation by GTP (Fig. 2D) diminishes the signal-to-noise ratio for the analysis of agonists, specifically partial agonists. The signal-to-noise ratio of the GTPase assay is much greater than that of the AC assay [13,22]. Nonetheless, there is an excellent correlation between the efficacies of agonists at activating AC in the presence of GTP and GTPase in membranes expressing β_2AR-$G_{s\alpha L}$ [17]. In addition, the potencies of most agonists in the AC assay in the presence of GTP and the GTPase assay in membranes expressing β_2AR-$G_{s\alpha L}$ are in very good agreement [17]. Moreover, the correlations of the efficacies of agonists in the presence of GTP at the various fusion proteins were linear (Fig. 4A–C), although the signal-to noise-ratios for the analysis of AC activity in the presence of GTP in membranes expressing β_2AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$, and β_2AR-$G_{\alpha olf}$ were quite different (Fig. 2A, D and G). These data indicate that the non-linear correlations for agonist efficacies observed for GTP versus ITP and GTP versus XTP at β_2AR-$G_{s\alpha L}$ (Fig. 3D and E) were not the result of low assay sensitivity in the presence of GTP. Another concern could be that our correlations were distorted by the fact that the efficacies of agonists were normalized relative to ISO for each NTP (Table 2). This normalization procedure was chosen to facilitate comparison with our previous data [17]. It is unlikely that our data analysis systematically influenced correlations because some correlations were linear (Fig. 3A–C, G and H; Fig. 4A–C and E), whereas the other correlations were not linear.

The differential effects of GTP, ITP, and XTP on G_s-protein activation could be interpreted in two ways. (i) Multiple active conformations of the β_2AR may stabilize specific conformations in any given G_s-protein. These G-protein conformations could differ from each other in their ability to bind various NTPs. Once bound to $G_{s\alpha}$ proteins, GTP, ITP, and XTP each could stabilize unique conformations in $G_{s\alpha S}$, $G_{s\alpha L}$, and $G_{\alpha olf}$. The $G_{s\alpha}$-conformations stabilized by GTP, ITP, and XTP could differ from each other with regard to TC disruption and AC activation. Rigorous proof of multiple active G-protein states would require crystallographic studies unmasking structural differences of G-proteins bound to hydrolysis-resistant analogs of NTPs [27,31]. (ii) Alternatively, the differential effects of NTPs on G_s-protein activation may reflect differences in the kinetics of interaction of GTP, ITP, and XTP with the various G_s-proteins. Rigorous proof of this hypothesis would require detailed binding studies with phosphorothioate analogs of GTP, ITP, and XTP. However, such analogs are only readily available for GTP, and it is also doubtful whether the affinities of the ITP and XTP analogs would be high enough for binding studies [17,32]. Regardless of which of the two explanations is correct, our present data demonstrate that NTPs are highly efficient

regulators of signal transduction mediated through a specific G-protein.

4.5. Role of transducisomes

It is generally assumed that G-proteins sequentially shuttle between GPCRs and effectors [3–5]. This model may have to be modified to reconcile the agonist-specific effects on G_s-protein activation in the presence of GTP, ITP, and XTP (Figs. 3 and 4 and Table 2). Specifically, one could assume that G-proteins and GPCR stay in at least some contact with each other during the entire G-protein cycle. Thus, shuttling of G-proteins between GPCR and effector may be viewed more appropriately as a reorientation of molecules to each other than as an absolute coupling and uncoupling of signaling partners. It was also shown for other systems that reduction of the agonist-affinity of GPCR following GTP-binding to G_α does not automatically imply physical dissociation of the two proteins [33]. In addition, in β_2AR-$G_{s\alpha}$ fusion proteins with large truncations of the GPCR C-terminus, the mobility of G_α relative to GPCR is severely impaired, but the efficiency of agonist-mediated AC activation is enhanced [34]. Moreover, there is pre-coupling of GPCRs and G-proteins in the absence of agonist [35], and GPCRs and G-proteins often co-purify [36,37]. Taken together, all these data support the hypothesis that GPCRs, G-protein, and effectors are tightly packed together into supramolecular signaling complexes, i.e. transducisomes [38–40].

4.6. (Patho)physiological implications

While GPCR-$G_{s\alpha}$ fusion proteins ensure defined stoichiometry of the signaling partners and their efficient coupling [40], these proteins are nonetheless artificial. Possible limitations of the fusion protein techniques were discussed in two recent papers [14,41]. Therefore, to evaluate the possible (patho)physiological relevance of our data, it will be important to confirm the differential regulation of G_s-protein function by GTP, ITP, and XTP in co-expression studies with non-fused G-proteins.

The differential effects of GTP, ITP, and XTP on G_s-protein activation may help us understand the pathophysiology of Lesch–Nyhan syndrome. In this disease, hypoxanthine-guanine phosphoribosyltransferase, a key enzyme in the purine salvage pathway, is defective [42]. The enzyme defect results in decreased intracellular GTP levels and increases in intracellular inosine and xanthine levels [43–45]. Intriguingly, patients with Lesch–Nyhan syndrome have alterations in the regulation of multiple neurotransmitter receptors including adrenoceptors and severe neurological abnormalities among which choreoathetosis and self-injurious behavior are the most prominent [42,46]. Thus, one could envisage that in Lesch–Nyhan syndrome, altered intracellular GTP, ITP, and XTP levels change GPCR/G-protein coupling and impair development and function of the central nervous system.

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