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Effects of Guanine, Inosine, and Xanthine Nucleotides on β_2-Adrenergic Receptor/G_s Interactions: Evidence for Multiple Receptor Conformations

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ABSTRACT

The aim of our study was to examine the effects of different purine nucleotides [GTP, ITP, and xanthosine 5′-triphosphate (XTP)] on receptor/G protein coupling. As a model system, we used a fusion protein of the β_2-adrenergic receptor and the α subunit of the G protein G_s. GTP was more potent and efficient than ITP and XTP at inhibiting ternary complex formation and supporting adenylyl cyclase (AC) activation. We also studied the effects of several β_2-adrenergic receptor ligands on nucleotide hydrolysis and on AC activity in the presence of GTP, ITP, and XTP. The efficacy of agonists at promoting GTP hydrolysis correlated well with the efficacy of agonists for stimulating AC in the presence of GTP. This was, however, not the case for ITP hydrolysis and AC activity in the presence of ITP. The efficacy of ligands at stimulating AC in the presence of XTP differed considerably from the efficacies of ligands in the presence of GTP and ITP, and there was no evidence for receptor-regulated XTP hydrolysis. Our findings support the concept of multiple ligand-specific receptor conformations and demonstrate the usefulness of purine nucleotides as tools to study conformational states of receptors.

The β_2-adrenergic receptor (β_2-AR) is a prototypical G protein-coupled receptor (GPCR) that interacts with the G protein G_s to activate adenylyl cyclase (AC; Gilman, 1987; Kobilka, 1992). GPCRs activate G proteins by promoting GDP release from and GTP binding to G protein α subunits (Iiri et al., 1998). GTP-liganded $G_{s\alpha}$ activates AC, and G protein deactivation is accomplished by GTP hydrolysis (Cassel and Selinger, 1976; Gilman, 1987). The extended ternary complex model assumes that GPCRs exist in an equilibrium between an inactive state (R) and an active state (R*) (Lefkowitz et al., 1993; Gether and Kobilka, 1998). According to this model, GPCRs can undergo R to R* isomerization in the absence of agonist, which gives rise to a receptor-dependent basal G protein and effector activity. Agonists stabilize the R* state and increase G protein activity above basal levels, whereas inverse agonists stabilize the R state and suppress basal G protein activity (see, e.g., Chidiac et al., 1994; Samama et al., 1994; Gether et al., 1995; Wenzel-Seifert et al., 1998a). The R* state is also stabilized by guanine nucleotide-free G protein α subunits (De Lean et al., 1980; Seifert et al., 1998a,b). The agonist-occupied receptor and nucleotide-free G protein α subunit form a ternary complex that is characterized by high agonist affinity. The ternary complex is disrupted by guanine nucleotide binding to the G protein (De Lean et al., 1980; Seifert et al., 1998a,b). An increasing number of experimental observations indicate that the extended ternary complex model cannot sufficiently explain the molecular mechanisms underlying GPCR activation. First, Chidiac et al. (1994) have shown that certain β_2-AR agonists can either act as partial agonists or as inverse agonists depending on whether effector system activity is assessed in intact cells or in cell membranes. Second, the extended ternary complex model proposes that inverse agonists stabilize an inactive and G protein-uncoupled state of GPCRs (Lefkowitz et al., 1993; Gether and Kobilka, 1998). However, the results from various studies suggest that inverse agonists induce a spe-

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ABBREVIATIONS: β_2-AR, β_2-adrenergic receptor; GPCR, G protein-coupled receptor; AC, adenylyl cyclase; XTP, xanthosine 5′-triphosphate; XDP, xanthosine 5′-diphosphate; $G_{s\alpha L}$, long-splice variant of $G_{s\alpha}$; $G_{s\alpha S}$, short-splice variant of $G_{s\alpha}$; GTPγS, guanine 5′-*O*-(3-thiotriphosphate); GppNHp, guanylyl imidodiphosphate; DHA, dihydroalprenolol; SAL, salbutamol; NTP, nucleoside 5′-triphosphate; cAMP, cyclic AMP; ICI, [erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol]; ISO, isoproterenol; NDP, nucleoside 5′-diphosphate; EPH, (−)-ephedrine; DCI, dichloroisoproterenol; DOB, dobutamine.

cific conformation in the GPCR that actively inhibits G protein function (Bouaboula et al., 1997; Seifert et al., 1998b). Third, the extended ternary complex model cannot explain why defined mutations in the dopamine D_2 receptor result in agonist-dependent changes in signaling (Wiens et al., 1998). Fourth, the observation that not only agonists but even antagonists can promote GPCR internalization (Roettger et al., 1997) and that some receptor ligands behave as antagonists with respect to G protein activation but as agonists with regard to ternary complex formation (Brown and Pasternak, 1998) cannot be reconciled with the extended ternary complex model. Finally, several reports showed that various synthetic and natural opioids interact differently with the μ-opioid receptor (Keith et al., 1996; Blake et al., 1997; Yu et al., 1997). Based on these and several other observations, it has been proposed that there are multiple, ligand-specific GPCR conformations (Kenakin, 1996; Tucek, 1997).

The aim of our study was to explore the usefulness of guanine, inosine, and xanthine nucleotides as experimental tools to explore ligand-specific GPCR conformations. Previous studies had shown that inosine and xanthine nucleotides can bind to various G proteins, although with lower affinity than guanine nucleotides (Northup et al., 1982; Kelleher et al., 1986; Florio and Sternweis, 1989; Klinker and Seifert, 1997). The idea to use nucleotides as tools for analyzing receptor conformations originated from previous studies showing that GTP, ITP, and xanthosine 5′-triphosphate (XTP) behave differently with respect to signaling mediated by different GPCRs that are coupled to the same G proteins and effector systems (Wolff and Cook, 1973; Bilezikian and Aurbach, 1974; Klinker and Seifert, 1997). In our study, we use purine nucleotides to examine ligand-specific differences in signaling mediated by a single GPCR. We examined the effects of different classes of ligands on β_2-AR-modulated interactions between the G protein G_s and the purine nucleotides GDP, GTP, IDP, ITP, xanthosine 5′-diphosphate (XDP), and XTP. As an experimental system, we used a fusion protein of the β_2-AR and the long-splice variant of $G_{s\alpha}$ ($G_{s\alpha L}$) expressed in Sf9 insect cells. Fusion of the two proteins to each other does not change the fundamental properties of either the β_2-AR or $G_{s\alpha}$ and allows for sensitive analysis of GPCR/G protein coupling in terms of ternary complex formation, GTP hydrolysis, and AC regulation (Seifert et al., 1998a,b). The β_2-AR coupled to $G_{s\alpha L}$, but not the β_2-AR coupled to $G_{s\alpha S}$, possesses the hallmarks of constitutive activity (high basal GTPase activity and high efficacy of inverse agonists and partial agonists; Seifert et al., 1998a). The apparent constitutive activity of the β_2-AR coupled to $G_{s\alpha L}$ can be explained by the relatively low GDP affinity of $G_{s\alpha L}$ compared with the short-splice variant of $G_{s\alpha}$ ($G_{s\alpha S}$). Specifically, $G_{s\alpha L}$ is more often guanine nucleotide-free than $G_{s\alpha S}$ and therefore is more often available to stabilize the R* state. Here, we report that the potency and efficacy of a series of β_2-AR ligands at the β_2-$ARG_{s\alpha L}$ fusion protein is dependent on the purine nucleotide that binds to $G_{s\alpha L}$. Our results provide further evidence for ligand-specific receptor conformational states.

Experimental Procedures

Materials. [γ-^{32}P]GTP (6000 Ci/mmol), [γ-^{32}P]ITP (4000 Ci/mmol), and [γ-^{32}P]XTP (4000 Ci/mmol) were custom synthesized by DuPont-NEN (Boston, MA). ITP, IDP, XTP, and XDP were of the highest purity available and were purchased from Sigma Chemical Co. (St. Louis, MO). GTP, guanine 5′-*O*-(3-thiotriphosphate) (GTPγS), guanylyl imidodiphosphate (GppNHp), GDP, and ATP were of the highest purity available and were purchased from Boehringer Mannheim (Mannheim, Germany). Nucleotide stock solutions (10 mM) were stored at −20°C. Nucleotide dilutions were prepared fresh daily. Sources of other materials have been described elsewhere (Seifert et al., 1998a,b). The construction of the fusion protein of the β_2-AR and $G_{s\alpha L}$ is described in Seifert et al. (1998a,b).

Cell Culture and Membrane Preparation. The β_2-AR or β_2-$ARG_{s\alpha}$ fusion protein was expressed in Sf9 cells via recombinant baculovirus, as described (Seifert et al., 1998a,b). Sf9 membranes expressing β_2-$ARG_{s\alpha}$ were prepared according to Seifert et al. (1998a,b). The experiments described in this study were performed in the absence of mammalian $\beta\gamma$ complex. The effect of mammalian $\beta_1\gamma_2$ complex on the function of β_2-$ARG_{s\alpha}$ was described previously (Seifert et al., 1998b).

[^{3}H]Dihydroalprenolol (DHA) Binding. [^{3}H]DHA binding studies were carried out as described (Seifert et al., 1998a,b). Tubes contained Sf9 membranes expressing β_2-$ARG_{s\alpha}$ at 5.0 to 7.5 pmol/mg of protein (15–30 μg of protein/tube), 1 nM [^{3}H]DHA, 1 μM salbutamol (SAL) and various nucleotides at increasing concentrations. As reported before, the K_d value for [^{3}H]DHA at β_2-$ARG_{s\alpha}$ is 0.36 ± 0.03 nM (Seifert et al., 1998b). Nonspecific binding with 1 nM [^{3}H]DHA, as assessed by the binding not competed for by 10 μM (−)-alprenolol, was less than 5% of total binding.

Steady-State Nucleoside 5′-Triphosphatase (NTPase) Activity. Nucleoside 5′-triphosphate (NTP) hydrolysis was determined according to Seifert et al. (1998a,b). Unless stated otherwise, assay tubes contained Sf9 membranes expressing β_2-AR at 6.1 pmol/mg of protein or β_2-$ARG_{s\alpha}$ at 7.0 to 7.5 pmol/mg of protein (10 μg of protein), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, and 0.2% (w/v) BSA in 50 mM Tris/HCl, pH 7.4. Tubes additionally contained β_2-AR ligands and unlabeled GTP, ITP, or XTP at various concentrations. Assay tubes (80 μl) were incubated for 3 min at 25°C before the addition of 20 μl of [γ-^{32}P]GTP, [γ-^{32}P]ITP, or [γ-^{32}P]XTP (0.75–2.0 μCi/tube). Reactions were conducted for 20 min at 25°C. Reactions were terminated by the addition of 900 μl of a slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not P_i. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000*g*. Seven hundred microliters of the supernatant fluid of reaction mixtures was removed, and $^{32}P_i$ was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]NTP. Spontaneous [γ-^{32}P]NTP degradation was determined in tubes containing all of the above-described components plus a very high concentration of unlabeled NTP (1 mM) that, by competition with the trace concentrations of [γ-^{32}P]NTP, prevents [γ-^{32}P]NTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]NTP degradation was <1% of the total amount of radioactivity added. Note that, for NTPase studies, β_2-$ARG_{s\alpha}$ was expressed at high levels to increase the sensitivity of the system (Seifert et al., 1998b).

AC Activity. Cyclic AMP (cAMP) formation in Sf9 membranes was carried out as described (Seifert et al., 1998a,b). Tubes contained Sf9 membranes expressing β_2-AR at 6.1 pmol/mg of protein or β_2-$ARG_{s\alpha}$ at 2.3 to 2.7 pmol/mg of protein (15–20 μg of protein/tube), 5 mM $MgCl_2$, 0.4 mM EDTA, and 30 mM Tris/HCl, pH 7.4, and purine nucleotides and β_2-AR ligands at various concentrations. Assay tubes (30 μl) were incubated for 3 min at 37°C before the addition of 20 μl of reaction mixture containing (final) 40 μM [α-^{32}P]ATP (2.5–3.0 μCi/tube), 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, 1 IU of myokinase, and 0.1 mM cAMP. Reactions were conducted for 20 min. [^{32}P]cAMP was separated from [α-^{32}P]ATP as described (Seifert et al., 1998a,b). Note that, for AC studies, β_2-$ARG_{s\alpha}$ was expressed at considerably lower

levels than for NTPase studies. This was done to avoid AC availability becoming limiting (Seifert et al., 1998b).

Miscellaneous. Protein was determined with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Data were analyzed by nonlinear or linear regression with the Prism program (GraphPAD, San Diego, CA). In this article, we use the term *efficacy* to describe the phenomenon that different agonists and nucleotides may vary in their ability to produce a response, although they may occupy the same proportion of receptors and G proteins, respectively. The efficacies of ligands on AC in the presence of GTP versus GTPase and on AC in the presence of ITP versus ITPase and the effect of [erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol] (ICI) on AC in the presence of XTP were compared with the *t* test.

Results

Regulation of High-Affinity Agonist Binding at β_2-AR$G_{s\alpha}$ by Guanine, Inosine, and Xanthine Nucleotides and ATP. One of the earliest steps of the G protein activation/deactivation cycle is formation of a ternary complex consisting of agonist, GPCR, and guanine nucleotide-free G_α (De Lean et al., 1980; Seifert et al., 1998a,b). The ternary complex is characterized by high agonist affinity. On binding of a guanine nucleotide, be it GMP, GDP, GTP, or a GTPase-resistant GTP analog, the ternary complex is disrupted and agonist affinity decreases (De Lean et al., 1980; Seifert et al., 1998a,b). To determine whether inosine and xanthine nucleotides can also disrupt the ternary complex, we examined binding of a fixed concentration of the antagonist [^{3}H]DHA in the presence of a subsaturating concentration of the full β_2-AR agonist (−)- isoproterenol (ISO; Fig. 1, A and B) and the strong partial agonist SAL in Sf9 membranes expressing β_2-AR$G_{s\alpha}$. Nucleotides at increasing concentrations were added to the binding assays. Nucleotide binding to $G_{s\alpha}$ reduces the affinity of the β_2-AR for agonist and thereby increases [^{3}H]DHA binding.

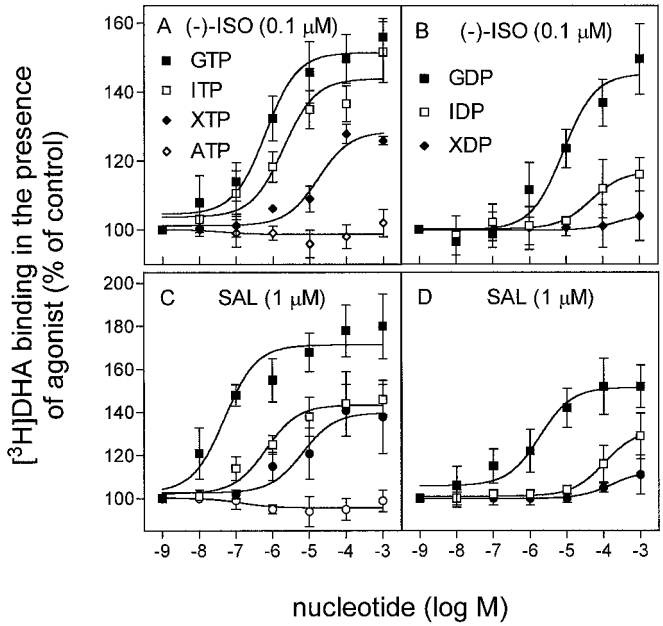

Fig. 1. Effects of GTP, ITP, XTP, and ATP on high-affinity agonist binding in Sf9 membranes expressing β_2-AR$G_{s\alpha}$. Binding experiments were carried out as described in *Experimental Procedures*, with membranes expressing β_2-AR$G_{s\alpha}$ at 5.0 to 7.5 pmol/mg of protein. Reaction mixtures additionally contained 1 nM [^{3}H]DHA, 1 μM SAL, and NTPs at the concentrations indicated on the abscissa. Data are means ± S.D. of three independent experiments performed in triplicate.

NTPs inhibited high-affinity binding of both (−)-ISO and SAL at β_2-AR$G_{s\alpha}$ in the order of potency GTP > ITP > XTP > ATP (ineffective). This rank order to potency is in agreement with the data obtained for nonfused $G_{s\alpha}$ (Northup et al., 1982). The lack of effect of ATP on high-affinity agonist binding indicates that nucleoside diphosphate kinase-catalyzed transphosphorylation of endogenous GDP to GTP by NTP cannot account for the effects of ITP and XTP. In a previous study (Seifert et al., 1998b), we showed that agonist binding in membranes expressing β_2-AR alone is guanine nucleotide-insensitive, ruling out the possibility that the β_2-AR coupling to endogenous insect $G_{s\alpha}$-like G proteins is responsible for the observed NTP effects. In agreement with the concept that the guanine nucleotide-free G protein α subunits support ternary complex formation (De Lean et al., 1980; Seifert et al., 1998a,b), we found that nucleoside 5′-diphosphates (NDPs) also inhibited ternary complex formation (order of potency, GDP > IDP > XDP). Whereas the observed order of potency of nucleotides to inhibit high-affinity agonist binding was expected (Northup et al., 1982; Klinker and Seifert, 1997), differences in potency and efficacy of nucleotides between (−)-ISO and SAL were somewhat unexpected. Specifically, NTPs and GDP were more potent at disrupting the ternary complex with SAL than with (−)-ISO (compare Fig. 1A with Fig. 1C and Fig. 1B with Fig. 1D). In addition, whereas ITP and GDP were less efficacious at inhibiting the high-affinity binding of SAL, these nucleotides were about similarly efficacious at inhibiting the high-affinity binding to (−)-ISO.

Regulation of Basal AC Activity by GTP, ITP, and XTP in Sf9 Membranes Expressing β_2-AR and β_2-AR$G_{s\alpha}$. Membranes expressing β_2-AR$G_{s\alpha}$ at 2.3 to 2.7 pmol/mg of protein had ~8-fold higher basal AC activity than membranes expressing β_2-AR alone at a higher level (6.1 pmol/mg of protein; Fig. 2). In membranes expressing β_2-AR$G_{s\alpha}$, GTP increased AC activity with an EC_{50} of 0.7 ± 0.1 μM. Compared with GTP, ITP was considerably less potent (EC_{50}, 30 ± 5 μM) and effective at increasing basal AC activity in membranes expressing β_2-AR$G_{s\alpha}$. XTP had virtually no stimulatory effect on basal AC activity in membranes expressing β_2-AR$G_{s\alpha}$.

It has been shown that, in certain systems expressing fusion proteins, there can be cross talk between the fused GPCR and endogenous G proteins of the host cell (Burt et al., 1998). Could the stimulatory effects of GTP and ITP on basal AC activity in membranes expressing the β_2-AR$G_{s\alpha L}$ fusion protein be mediated by cross-activation of endogenous $G_{s\alpha}$-like G proteins of Sf9 cells by the fused β_2-AR? To address this question, we studied the effects of NTPs on AC activity in membranes expressing nonfused β_2-AR. Note that, for these studies, we expressed the β_2-AR at a level more than twice as high as β_2-AR$G_{s\alpha L}$ to increase the effects seen with the nonfused receptor. Despite the high expression level of β_2-AR, the absolute stimulation of AC by GTP, ITP, and XTP was much less efficient in membranes expressing β_2-AR than in membranes expressing β_2-AR$G_{s\alpha L}$, and the maximal AC stimulation in membranes expressing nonfused β_2-AR did not even approach basal AC activity in membranes expressing β_2-AR$G_{s\alpha L}$ in the absence of added nucleotides. We also had to rule out the possibility that the stimulatory effects of

GTP and ITP in membranes expressing β_2-AR$G_{s\alpha L}$ had been caused by a nonspecific fusion-dependent perturbation of the β_2-AR, resulting in high constitutive activity of the GPCR. If this were the case, we would observe a similar level of constitutive activity when the β_2-AR is fused to either $G_{s\alpha S}$ or $G_{s\alpha L}$. Therefore, we compared the effects of GTP (1 μM), ITP (10 μM), and XTP (100 μM) on AC activity in membranes expressing β_2-AR$G_{s\alpha L}$ (2.3–2.7 pmol/mg of protein) with the corresponding NTP effects in Sf9 membranes expressing β_2-AR$G_{s\alpha S}$ at a similar level (2.6 pmol/mg of protein). In membranes expressing β_2-AR$G_{s\alpha S}$, NTPs did not have substantial effects on basal AC activity; i.e., the AC activities in the presence of the different nucleotides were in the same range (17–22 pmol $\cdot$ mg^{-1} protein $\cdot$ min^{-1}; data not shown). In contrast, with β_2-AR$G_{s\alpha L}$, AC activities varied by 3-fold (4–12 pmol $\cdot$ mg^{-1} protein $\cdot$ min^{-1}; Fig. 2). In agreement with our previous study (Seifert et al., 1998a), the AC activities with β_2-AR$G_{s\alpha S}$ are considerably higher than the AC activities achieved with β_2-AR$G_{s\alpha L}$. Collectively, these data indicate that the observed NTP effects on AC in membranes expressing β_2-AR$G_{s\alpha L}$ are attributable to the fused $G_{s\alpha L}$ and not to activation of endogenous $G_{s\alpha}$-like G proteins.

Regulation of AC Activity by (−)-ISO and ICI in Sf9 Membranes Expressing β_2-AR$G_{s\alpha}$ in Presence of GTP, ITP, and XTP. The full β_2-AR agonist (−)-ISO further increased AC activity in the presence of GTP, but the stimulatory effect of (−)-ISO did not exceed 50% (Fig. 3A). The inverse agonist ICI suppressed GTP-dependent AC activity by ~50%. In the presence of ITP, (−)-ISO increased AC activity by up to 100%, whereas ICI decreased basal AC activity by not more than 17% (Fig. 3B). The absolute agonist-stimulated AC activity with ITP was substantially lower than with GTP. In the presence of XTP (0.1–1 mM), (−)-ISO increased AC activity by up to 110%, but the absolute agonist-stimulated AC activity with XTP was lower than the corresponding AC activity with ITP (Fig. 3C). Whereas ICI behaved as an inverse agonist by suppressing AC activity in the presence of GTP and ITP, it behaved as a partial agonist in the presence of XTP. ICI increased AC activity by up to 20% in the presence of 100 μM XTP. In Fig. 3F, the stimulatory effect of ICI on AC in the presence of XTP is seen more clearly than in Fig. 3C, because Fig. 3F shows AC activities normalized to basal values.

We also studied the concentration dependence of the effects of (−)-ISO and ICI on AC activity in the presence of GTP, ITP, and XTP at fixed concentrations. NTPs were used at concentrations that gave the highest relative agonist stimulation of AC. In the presence of GTP, (−)-ISO increased AC activity, with an EC_{50} of 18 ± 8 nM (Fig. 3D). Compared with GTP, the concentration-response curves for (−)-ISO were shifted to the right with ITP (EC_{50}, 233 ± 34 nM; Fig. 4E) and XTP (EC_{50}, 416 ± 44 nM; Fig. 3F). The IC_{50} values of ICI to inhibit AC in the presence of GTP and ITP were similar (16 ± 8 and 22 ± 12 nM, respectively). The stimulatory effect of ICI on AC in the presence of XTP was half-maximal at 7 ± 4 nM and reached a maximum at 0.1 to 1.0 μM. At 0.1 and 1.0 μM, the stimulatory effect of ICI on AC in the presence of XTP was significant ($p < .05$).

Analysis of GTPase, ITPase, and XTPase Activity in Membranes Expressing β_2-AR$G_{s\alpha}$. To obtain insight into the mechanism by which $G_{s\alpha}$ activation by ITP and XTP is terminated, we studied ITPase and XTPase activities in membranes expressing β_2-AR$G_{s\alpha}$ or β_2-AR. The basal ITPase activity in membranes expressing β_2-AR$G_{s\alpha}$ was almost twice that of ITPase activity in membranes expressing β_2-AR alone (Fig. 4, A and B). Whereas (−)-ISO had no stimulatory effect on ITP hydrolysis in membranes expressing β_2-AR, (−)-ISO significantly increased ITP hydrolysis in membranes expressing β_2-AR$G_{s\alpha}$. These data were the first indication that $G_{s\alpha}$ exhibits substantial ITPase activity.

Membanes expressing β_2-AR and β_2-AR$G_{s\alpha}$ both exhibited significant basal XTPase activity. However, in contrast to the data obtained for ITPase, the XTPase activity in membranes expressing β_2-AR$G_{s\alpha}$ was not higher than the XTPase activity in membranes expressing β_2-AR alone (Fig. 4, C and D). In addition, we did not detect a significant stimulatory effect of (−)-ISO on XTP hydrolysis in membranes expressing β_2-AR or β_2-AR$G_{s\alpha}$. It is unlikely that our failure to detect a stimulatory effect of (−)-ISO on XTP hydrolysis was because of an insensitive method, because we used high amounts of [γ-^{32}P]XTP and membranes expressing β_2-AR$G_{s\alpha}$ at high levels (see *Experimental Procedures*). Varying the concentration of (−)-ISO from 1 nM to 1 mM, with another agonist (SAL at 10 nM to 1 mM) and changing the concentration of $MgCl_2$ between 0.1 and 10 mM did not unmask β_2-AR-ligand effects on XTP hydrolysis. The lack of ligand regulation of XTPase activity was also reported for G_i protein-coupled

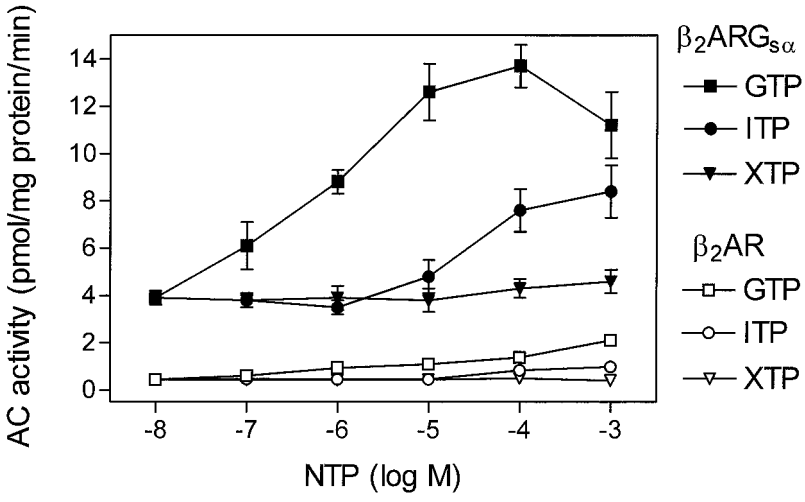

Fig. 2. Effects of GTP, ITP, and XTP on basal AC activity in Sf9 membranes expressing β_2-AR or β_2-AR$G_{s\alpha}$. AC activity in membranes expressing β_2-AR (6.1 pmol/mg of protein) or β_2-AR$G_{s\alpha}$ (2.3–2.7 pmol/mg of protein) was determined as described in *Experimental Procedures*. AC activity was determined in the presence of NTPs at the concentrations indicated on the abscissa. Data are means ± S.D. of three to six independent experiments performed in duplicate.

chemoattractant receptors in HL-60 membranes (Klinker and Seifert, 1997).

The XTPase experiments (Fig. 4, C and D) together with the agonist competition and AC studies (Figs. 1 and 3C) suggest that XTP binds to $G_{s\alpha}$ but is not hydrolyzed. To substantiate this hypothesis, we performed competition studies with [γ-^{32}P]NTPs and various unlabeled NTPs. In a previous study, it had already been demonstrated that the GTPase-resistant GTP analog GTPγS efficiently inhibits β-AR-mediated GTP hydrolysis in turkey erythrocyte membranes (Cassel and Selinger, 1977a).

In a first set of experiments, we studied the effects of the nucleotidase-resistant GTP analogs GTPγS and GppNHp on ITP and XTP hydrolysis in the presence of (−)-ISO in membranes expressing β_2-$ARG_{s\alpha}$. GTPγS and GppNHp bind to $G_{s\alpha}$ with high affinity, and GTPγS is ~7-fold more potent in this regard than GppNHp (Northup et al., 1982). GTPγS and GppNHp inhibited ITP hydrolysis according to a biphasic function (Fig. 5A). About 40% of the inhibition of ITP hydrolysis by stable GTP analogs was attributable to a high-affinity interaction, whereas the remaining 60% was attributable to a low-affinity interaction. GTPγS inhibited the high-affinity component of ITP hydrolysis approximately nine times more potently than GppNHp, whereas, for the low-affinity component, no such difference in potency between GTPγS and GppNHp was observed (IC_{50}, 402 ± 44 and 250 ± 33 μM, respectively). These findings show that GTPγS and GppNHp potently compete with ITP for binding to $G_{s\alpha}$. The ITPase that is inhibited by GppHNp and GTPγS with low affinity presumably represents the activity of nonspecific nucleotidases of Sf9 cells.

In marked contrast to the biphasic competition of ITP hydrolysis by GTPγS and GppNHp, no high-affinity inhibition of XTPase by GTPγS and GppNHp was detected (Fig. 5B). The IC_{50} values of GTPγS and GppNHp for XTP hydrolysis were 294 ± 23 and 351 ± 44 μM, respectively, and were similar to the IC_{50} values for the low-affinity inhibition of ITPase by GTPγS and GppNHp.

In a second set of experiments, we compared the effects of GTPγS and XTP on (−)-ISO-stimulated GTP hydrolysis (Fig. 6). As expected from previous experiments (Cassel and Selinger, 1977a), GTPγS inhibited GTP hydrolysis (IC_{50}, 17 ± 4 nM). If XTP binds to but is not hydrolyzed by $G_{s\alpha}$, XTP is expected to block GTP hydrolysis, as does GTPγS. Indeed, XTP abolished (−)-ISO-stimulated GTP hydrolysis, although with a much higher IC_{50} value than GTPγS (IC_{50}, 139 ± 22 μM). Taken together, the nucleotide competition data and the similar basal XTPase activities in membranes expressing β_2-AR and β_2-$ARG_{s\alpha}$ indicate that basal XTP hydrolysis in Sf9 membranes is caused by endogenous nucleotidases and that $G_{s\alpha}$ does not hydrolyze XTP to a measurable extent.

Kinetics of Agonist-Stimulated GTP and ITP Hydrolysis in Membranes Expressing β_2-$ARG_{s\alpha}$. Because of the fixed 1:1 stoichiometry of GPCR to G protein in fusion proteins, the G protein concentration can be determined by receptor-antagonist saturation binding (Wise et al., 1997; Seifert et al., 1998a,b). These properties of fusion proteins allow calculation of agonist-stimulated NTP turnover of the fused G protein (Wise et al., 1997; Seifert et al., 1998b). With GTP at concentrations between 0.01 and 1.00 μM, (−)-ISO stimulated GTP hydrolysis up to 250% (Fig. 7A). For each substrate concentration, the basal GTP hydrolysis rates were subtracted from the GTP hydrolysis rates observed in the presence of (−)-ISO and referred to the β_2-$ARG_{s\alpha}$ expression level. By doing so, a V_{max} of (−)-ISO-stimulated GTP turnover of 1.37 ± 0.11 min^{-1} was obtained by nonlinear regression analysis (Fig. 7C). The K_m value of the (−)-ISO-stimulated GTPase is 0.18 ± 0.04 μM. These kinetic properties of β_2-$ARG_{s\alpha}$ agree with data obtained for reconstituted purified β-AR and G_s (Brandt and Ross, 1986). Because the affinity of

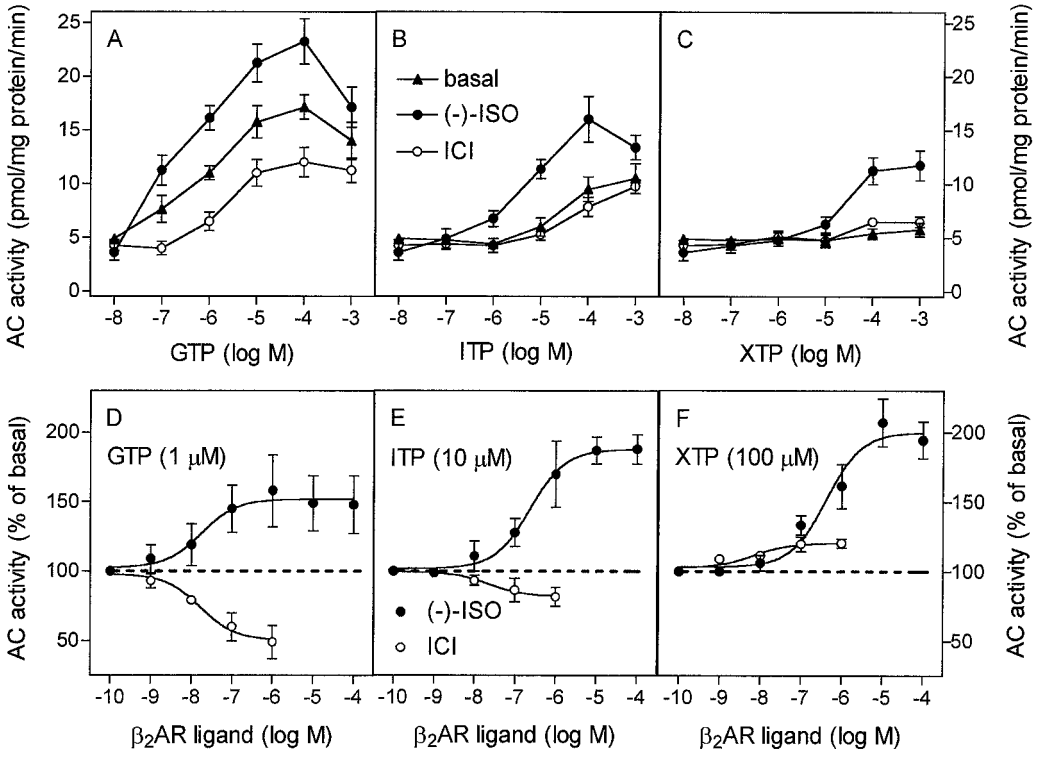

Fig. 3. Effects of (−)-ISO and ICI on AC activity in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ in the presence of GTP, ITP, or XTP. AC activity in membranes expressing β_2-$ARG_{s\alpha}$ (2.3–2.7 pmol/mg of protein) was determined as described in *Experimental Procedures*. A–C, AC activity was determined in the presence of NTPs at the concentrations indicated on the abscissa without or with (−)-ISO (10 μM) or ICI (1 μM). D–F, AC activity was determined in the presence of purine nucleotides at the indicated concentrations and (−)-ISO or ICI at increasing concentrations. Data are means ± S.D. of four to six independent experiments performed in duplicate. The dotted lines in A–C are extrapolations of basal AC activities to illustrate the relative contributions of (−)-ISO and ICI at the ligand-regulated enzyme activities.

$G_{s\alpha}$ for ITP is lower than for GTP (Figs. 1 and 2; Northup et al., 1982), ITP hydrolysis was studied with higher substrate concentrations than GTP hydrolysis. We readily detected stimulatory effects of (−)-ISO on ITP hydrolysis, with substrate concentrations from 0.1 to 100.0 μM (Fig. 7, B and D). The V_{max} of (−)-ISO-stimulated ITP hydrolysis was 3.06 ± 0.07 min^{-1}, and the K_m was 6.3 ± 0.5 μM.

Ligand Efficacies at β_2-ARG$_{s\alpha}$ in GTPase, ITPase, and AC Studies. Fig. 8 shows the concentration-response curves for the effects of (−)-ISO and ICI on GTP hydrolysis and ITP hydrolysis in membranes expressing β_2-ARG$_{s\alpha}$ (7.5 pmol/mg of protein). (−)-ISO stimulated GTPase, with an EC_{50} of 13 ± 3 nM, and ICI reduced GTP hydrolysis, with an IC_{50} of 3.0 ± 1.2 nM. (−)-ISO increased GTP hydrolysis by up to 230%, whereas ICI reduced GTP hydrolysis by up to 50%. In agreement with the reduced potency of (−)-ISO to stimulate AC in the presence of ITP (Fig. 3, D and E), the potency of (−)-ISO to activate ITPase was lower (EC_{50}, 57 ± 10 nM) than the potency to stimulate GTPase (Fig. 8, A and B). In the presence of ITP at 3.0 μM, (−)-ISO increased ITP hydrolysis by ~30% above basal (Fig. 8B). We could not detect an inhibitory effect of ICI on ITP hydrolysis, despite high assay sensitivity and presumably high basal ITPase activity of $G_{s\alpha}$ (see Fig. 4, A and B).

Table 1 summarizes the efficacies of a series of β_2-AR ligands on GTPase and ITPase activity and AC activities measured in the presence of GTP, ITP, or XTP. A highly significant correlation was obtained when the efficacies of β_2-AR agonists at activating GTPase and AC in the presence of GTP were plotted against each other (Fig. 8A). However, when the efficacies of β_2-AR agonists at activating ITPase and AC in the presence of ITP were plotted against each other, the correlation was much less stringent than in the corresponding experiments with GTP (compare Fig. 9, A and B). The efficacies of SAL, (−)-ephedrine (EPH), dichloroisoproterenol (DCI), and (−)-alprenolol at activating AC in the presence of ITP differed significantly from the respective efficacies of the ligands at activating ITP hydrolysis. The inverse agonists timolol and ICI were significantly more efficacious at reducing AC activity in the presence of GTP or ITP than at reducing hydrolysis of the respective NTP (see Table 1). Note that the efficacies of partial agonists on AC in the presence of XTP, most prominently the efficacies of dobutamine (DOB) and EPH, were very low.

Table 2 summarizes the EC_{50} values of selected β_2-AR agonists on GTPase and ITPase activity and AC activities in the presence of GTP, ITP, or XTP. For comparison with agonist potencies, Table 2 also contains the high- and low-affinity K_i values for the agonists studied. Moreover, we calculated potency ratios for the various parameters studied.

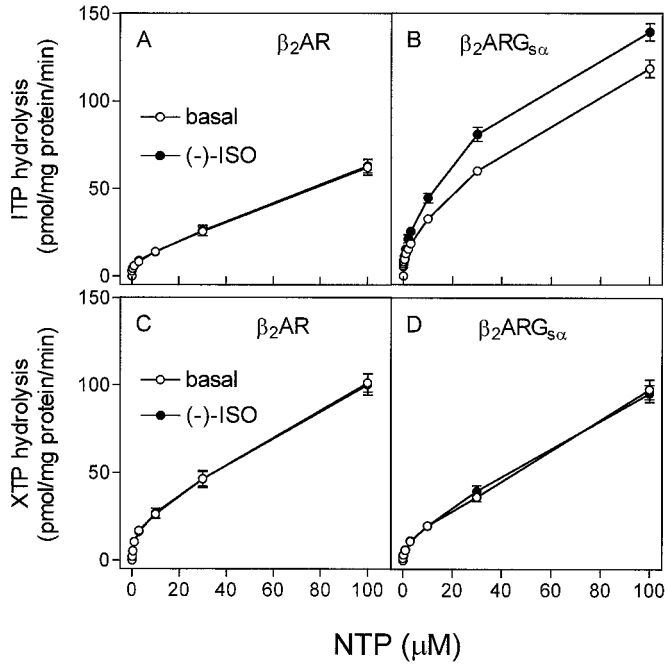

Fig. 4. Kinetics of steady-state ITP and XTP hydrolysis in Sf9 membranes expressing β_2-AR or β_2-ARG$_{s\alpha}$. ITPase and XTPase activity in membranes expressing β_2-ARG$_{s\alpha}$ was determined as described in *Experimental Procedures*. Reaction mixtures contained 0.1 to 2.0 μM [γ-^{32}P]ITP (1.0 μCi/tube; A and B) or 0.1 to 100 μM [γ-^{32}P]XTP (2.0 μCi/tube; C and D). NTPase activities were determined in membranes expressing β_2-AR (6.1 pmol/mg of protein; A and C) or β_2-ARG$_{s\alpha}$ (7.5 pmol/mg of protein). Reaction mixtures additionally contained solvent (basal) or (−)-ISO (10 μM). Data are means ± S.D. of two experiments performed in duplicate.

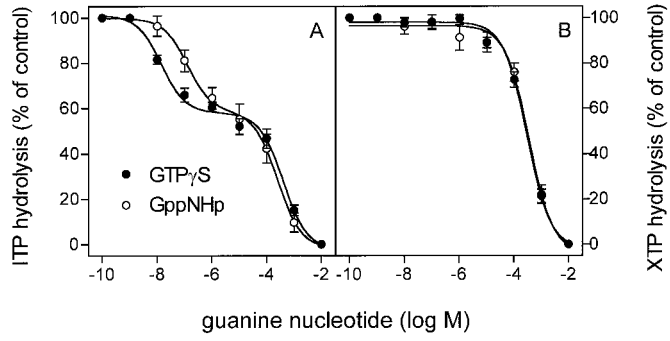

Fig. 5. Competition of ITP and XTP hydrolysis by GTPγS and GppNHp in Sf9 membranes expressing β_2-ARG$_{s\alpha}$. ITPase and XTPase activity in membranes expressing β_2-ARG$_{s\alpha}$ (7.5 pmol/mg of protein) was determined as described in *Experimental Procedures*. Reaction mixtures contained 1 μM [γ-^{32}P]ITP (1.0 μCi/tube; A) or 1 μM [γ-^{32}P]XTP (1.0 μCi/tube; B). Reaction mixtures additionally contained (−)-ISO (10 μM) and GTPγS or GppNHp at the concentrations indicated on the abscissa. The activities observed in the presence of (−)-ISO were set 100% (control). Data are means ± S.D. of two experiments performed in duplicate.

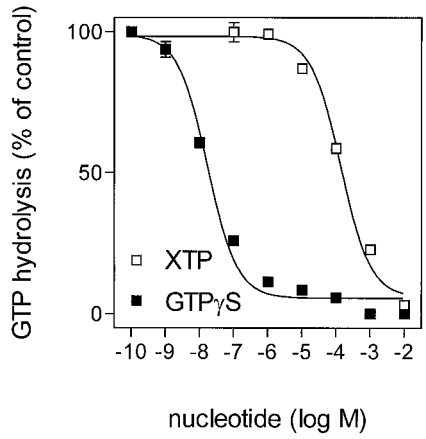

Fig. 6. Competition of GTP hydrolysis by GTPγS and XTP in Sf9 membranes expressing β_2-ARG$_{s\alpha}$. GTPase activity in membranes expressing β_2-ARG$_{s\alpha}$ (7.5 pmol/mg of protein) was determined as described in *Experimental Procedures*. Reaction mixtures contained 100 nM [γ-^{32}P]GTP (0.75 μCi/tube), 10 μM (−)-ISO, and GTPγS or XTP at the concentrations indicated on the abscissa. The activities observed in the presence of (−)-ISO were set 100% (control). Data are means ± S.D. of two experiments performed in duplicate.

For (−)-ISO, (+)-ISO, SAL, and DOB, the EC_{50} values for activation of AC in the presence of GTP were substantially lower than the EC_{50} values for activation of AC in the presence of ITP. The same was true for the comparison of agonist potencies for activation of GTPase versus ITPase activation. With the exception of EPH, the EC_{50} values of agonists at activating AC in the presence of GTP were similar to the EC_{50} values of agonists at activating GTPase. Similarly, the EC_{50} values of agonists at activating AC in the presence of ITP were similar to the EC_{50} values of agonists at activating ITPase. When AC activation in the presence of XTP was considered, the EC_{50} values of (−)-ISO, (+)-ISO, and DOB were even higher than the EC_{50} values for AC activation in the presence of ITP. When EPH was considered, we noted an unexpectedly low EC_{50} value of the agonist at activating GTPase. However, the EC_{50} values of (−)-ISO, (+)-ISO, and EPH in certain functional assays were even higher than the low-affinity K_i values. In contrast, the EC_{50} values for DCI in functional assays were lower than was expected from the K_i values in the [^{3}H]DHA competition-binding assay.

Discussion

Interaction of Guanine, Inosine, and Xanthine Nucleotides with $G_{s\alpha}$. Guanine, inosine, and xanthine nucleotides differentially form hydrogen bonds with a highly conserved aspartate residue in G protein α subunits (Noel et al., 1993; Fig. 10). The reduced affinity of $G_{s\alpha}$ for IDP and ITP compared with GDP and GTP may be because the inosine ring forms only one hydrogen bond with Asp295 in $G_{s\alpha}$ (see Figs. 1, 2, and 10; Northup et al., 1982). Repulsion of the electronegative groups in the xanthine ring and Asp295 may explain why XDP and XTP have an even lower affinity for $G_{S\alpha}$ than IDP and ITP (see Figs. 1, 2, and 10). Factors that influence nucleotide affinity may also affect the efficacy of nucleotides. GTP, ITP, and XTP do not have the same maximal effect with respect to disruption of the ternary complex and AC activation (Figs. 1–3). Thus, there may be nucleotide-specific conformational changes in $G_{s\alpha}$ that influence interactions with the receptor and/or effector. Studies with fluorescent guanine nucleotides already provided evidence for

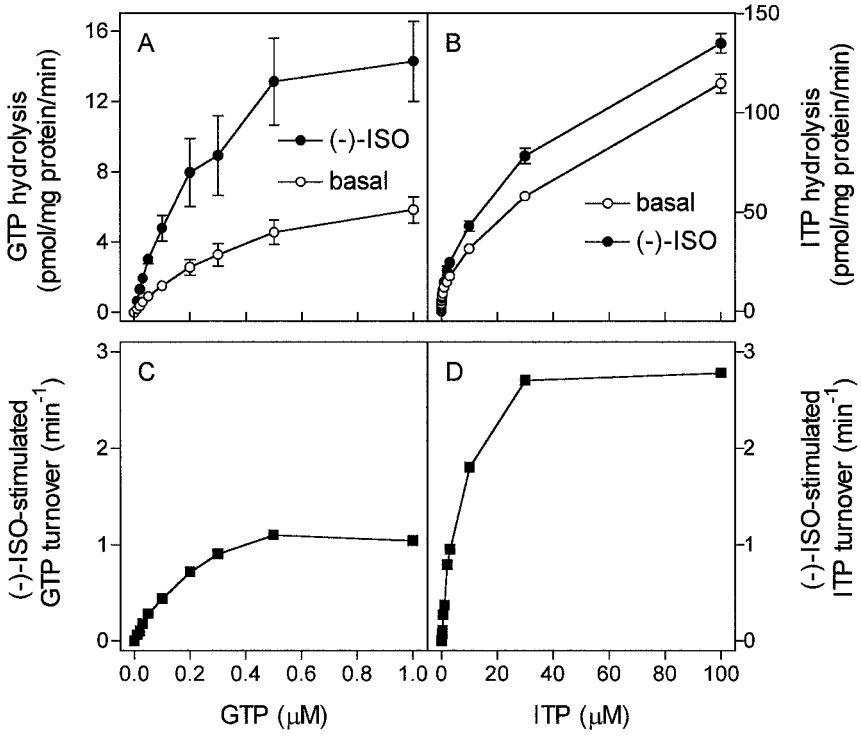

Fig. 7. Kinetics of steady-state GTP and ITP hydrolysis in Sf9 membranes expressing β_2-$ARG_{s\alpha}$. GTPase and ITPase activity in membranes expressing β_2-$ARG_{s\alpha}$ was determined as described in *Experimental Procedures*. Reaction mixtures contained 0.01 to 1.00 μM [γ-^{32}P]GTP (1.0 μCi/tube; A) or 0.1 to 100.0 μM [γ-^{32}P]ITP (2.0 μCi/tube; B). Reaction mixtures additionally contained solvent (basal) or (−)-ISO (10 μM). Data are means ± S.D. of two experiments performed in duplicate. C, basal GTPase activity for each substrate concentration was subtracted from GTPase activity in the presence of (−)-ISO and normalized for β_2-$ARG_{s\alpha}$ expression level (7.5 pmol/mg of protein) to calculate molar GTP turnover of fused $G_{s\alpha}$. D, basal ITPase activity for each substrate concentration was subtracted from ITPase activity in the presence of (−)-ISO and normalized for β_2-$ARG_{s\alpha}$ expression level (7.5 pmol/mg of protein) to calculate molar ITP turnover of fused $G_{s\alpha}$.

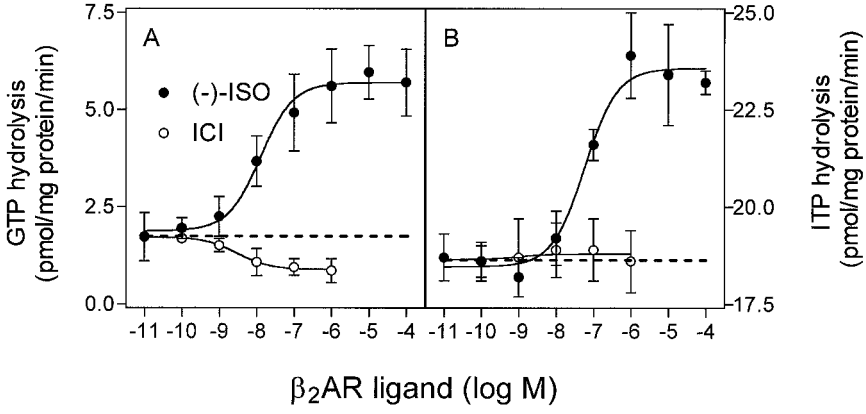

Fig. 8. Concentration-response curves for the effects of (−)-ISO and ICI on GTPase and ITPase activity in Sf9 membranes expressing β_2-$ARG_{s\alpha}$. GTPase and ITPase activity in membranes expressing β_2-$ARG_{s\alpha}$ (7.5 pmol/mg of protein) was determined as described in *Experimental Procedures*. Reaction mixtures contained 0.1 μM [γ-^{32}P]GTP (0.75 μCi/tube; A) or 3.0 μM [γ-^{32}P]ITP (1.5 μCi/tube; B) and (−)-ISO and ICI at the concentrations indicated on the abscissa. The dotted lines are extrapolations of basal GTPase and ITPase activities to illustrate the relative contributions of (−)-ISO and ICI at the ligand-regulated enzyme activities. Data are means ± S.D. of three or four independent experiments performed in duplicate. The data shown in A are identical with the data shown in Fig. 2B in Seifert et al. (1998a).

the existence of nucleotide-specific G protein activation states (Remmers and Neubig, 1996).

Differences in the kinetics of interaction of nucleotides with $G_{s\alpha}$ could also contribute to the different efficacies of nucleotides. NTP hydrolysis is the principal mechanism by which $G_{s\alpha}$ is deactivated (Cassel and Selinger, 1976; Gilman, 1987). The faster NTP hydrolysis proceeds, the shorter the time for which $G_{s\alpha}$ can stay in an active conformation (Wenzel-Seifert et al., 1998b). Thus, the lower efficacy of ITP compared with GTP at disrupting the ternary complex and activating AC could result from the higher ITP hydrolysis rate compared with GTP hydrolysis rate (see Figs. 1–3 and 7). Based on the dissociation rates of GDP and IDP from G proteins, it is also likely that the rate of ITP dissociation from $G_{s\alpha}$ is greater than the rate of GTP dissociation (Florio and Sternweis, 1989). Fast dissociation from $G_{s\alpha}$ could be another factor that contributes to the lower efficacy of IDP and ITP compared with GDP and GTP. For AC activation by $G_{S\alpha}$ in the presence of XTP, nucleotide dissociation and not rapid hydrolysis appears to be the major mechanism by which $G_{s\alpha}$ is deactivated (Figs. 4, C and D, and 5B). Because of its low affinity for $G_{s\alpha}$, XTP could be thought to dissociate from $G_{s\alpha}$ even before it can be cleaved. As a result of the rapid dissociation of XTP, $G_{s\alpha}$ stays in the active state only for short periods. NTP dissociation as a major mechanism of G protein deactivation is conceivable in view of the fact that even highly potent G protein ligands such as GTPγS or GppNHp can dissociate from G protein α subunits (Cassel and Selinger, 1977b; Hilf et al., 1992; Breivogel et al., 1998). We could not directly study dissociation of ITP and XTP because of the low affinity of these nucleotides for $G_{s\alpha}$.

Another mechanism that could contribute to the observed differences in efficacy between GTP, ITP, and XTP is differential β_2-AR regulation of NTP binding to $G_{s\alpha}$. GTP binding to G protein α subunits does not passively follow GDP release but is actively catalyzed by GPCR (Iiri et al., 1998). The dual hydrogen bonding of the guanine ring at Asp295 could be envisaged to stabilize GTP binding to such an extent that even the agonist-free β_2-AR can effectively increase GTP binding to $G_{s\alpha}$ (see Fig. 10A). This assumption is supported by the strong stimulatory effect of GTP on basal AC activity and the high inverse agonist efficacy of ICI and timolol (see Figs. 2 and 3; Table 1). Because of the weaker hydrogen bonding of the inosine ring to $G_{s\alpha}$ (see Fig. 10, A and B), binding of ITP to $G_{s\alpha}$ is less stable than binding of GTP so that substantial agonist occupancy of β_2-AR is required to stabilize ITP binding. In accordance with this model are our findings that ITP is less efficient at increasing basal AC activity than GTP and that the inverse agonist efficacy in the presence of ITP is lower than in the presence of GTP (see Figs. 2, 3, and 8; Table 1). Additionally, agonist potency is lower in the ITPase assay and the AC assay with ITP than in the GTPase assay and the AC assay with GTP (see Figs. 4, D and E, and 9). Because of the repulsion of the electronegative groups, the energy barrier for XTP to bind to $G_{s\alpha}$ may be so high that the agonist-free β_2-AR is virtually ineffective at promoting this XTP binding (see Fig. 10C). In support of this assumption, XTP only minimally increased basal AC activity, no inverse agonist effects were observed, and agonist potency was very low (see Figs. 2 and 3, C and E, and Table 1).

GTP, ITP, and XTP as Tools to Analyze Ligand-Specific GPCR Conformations. If receptor-stimulated NTP hydrolysis is assumed to be a function of receptor-promoted nucleotide binding and the intrinsic nucleotidase activity of the G protein, then the efficacies and potencies of ligands at activating AC and NTP hydrolysis should be identical. In agreement with previously published data, we found that a strong correlation exists when AC activation in the presence of GTP and GTPase activation is considered (see Fig. 9A and Table 1; Pike and Lefkowitz, 1980). However, we made several observations that are not compatible with this model. First, there was a much weaker correlation between the efficacies of agonists at activating ITPase and activating AC in the presence of ITP (see Fig. 9B and Table 1). Moreover, despite the lack of agonist regulation of XTP hydrolysis, we observed efficient agonist regulation of AC activity in the presence of XTP (see Figs. 3F and 4D; Table 1). Furthermore, certain ligands exhibited unexpected pharmacological properties when we examined AC regulation in the presence of XTP. Most notably, ICI, although an inverse agonist in the presence of GTP and ITP, displayed weak partial agonistic activity in the presence of XTP (Fig. 1). As another example,

TABLE 1

Efficacies of β_2-AR ligands at β_2-$ARG_{s\alpha}$ as assessed by AC Activity in presence of GTP, ITP, or XTP and by GTPase and ITPase activity

AC activity was determined as described in "Experimental Procedures" in membranes expressing β_2-$ARG_{s\alpha}$ (2.3–2.7 pmol/mg of protein) in the presence of GTP (1 μM), ITP (10 μM), or XTP (100 μM). GTPase activity was determined in membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg of protein) in the presence of 100 nM [γ-^{32}P]GTP. ITPase activity was determined in membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg of protein) in the presence of 3 μM [γ-^{32}P]ITP. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. The concentration-response curves were generated by nonlinear regression analysis, and the plateau values for (−)-ISO were set 100%. The efficacies of the other agonists are referred to the efficacy of (−)-ISO. The inhibitory effects of TIM and ICI are referred to the stimulatory effect of (−)-ISO as well. Data are means ± S.D. of three to seven independent experiments performed in duplicate or triplicate. Comparison of the efficacies of ligands on AC in the presence of GTP versus GTPase and on AC in the presence of ITP versus ITPase.

β_2-AR Ligand	β_2-AR Ligand Efficacy				
	AC + GTP	GTPase	AC + ITP	ITPase	AC + XTP
(−)-ISO	1.00	1.00	1.00	1.00	1.00
(+)-ISO	0.98 ± 0.06	0.91 ± 0.03*	0.64 ± 0.01	1.07 ± 0.07**	0.78 ± 0.09
SAL	1.02 ± 0.14	0.95 ± 0.02*	0.53 ± 0.15	1.22 ± 0.02**	0.49 ± 0.20
DOB	0.89 ± 0.16	0.76 ± 0.04*	0.71 ± 0.03	0.85 ± 0.05*	0.21 ± 0.10
EPH	0.68 ± 0.16	0.66 ± 0.05*	0.21 ± 0.13	0.74 ± 0.22**	0.11 ± 0.11
DCI	0.56 ± 0.19	0.49 ± 0.08*	0.09 ± 0.06	0.62 ± 0.07**	0.03 ± 0.04
(±)-Labetalol	0.33 ± 0.16	0.30 ± 0.03*	0.11 ± 0.11	0.26 ± 0.10*	0.06 ± 0.06
(−)-Alprenolol	0.22 ± 0.06	0.21 ± 0.04*	0.03 ± 0.03	0.13 ± 0.05**	0.01 ± 0.01
(−)-Propranolol	0.01 ± 0.01	0.01 ± 0.02*	0.00 ± 0.01	0.01 ± 0.01*	0.00 ± 0.01
TIM	−0.69 ± 0.13	−0.10 ± 0.02**	−0.13 ± 0.04	0.01 ± 0.04**	0.03 ± 0.07
ICI	−0.88 ± 0.02	−0.12 ± 0.03**	−0.14 ± 0.03	−0.02 ± 0.03**	0.19 ± 0.10

* Not significant. ** $p < .05$.

DOB is a strong partial agonist with respect to AC activation in the presence of GTP and ITP but only a weak partial agonist in the presence of XTP, having an efficacy comparable to the efficacy of ICI. We also observed differences in the EC_{50} values of some agonists when comparing their stimulation of AC with their stimulation of GTPase or ITPase activity (Table 2). It is unlikely that these differences are the result of differences in the experimental conditions in the AC versus NTPase assay, because such differences should have affected EC_{50} values and efficacies of agonists in a systematic manner.

The divergent effects of agonists and inverse agonists on NTP hydrolysis and AC activity in the presence of various NTPs could be reconciled by proposing multiple ligand-specific GPCR conformations. Each ligand stabilizes a unique β_2-AR conformation that allows the receptor to interact with $G_{s\alpha}$ in a ligand-specific manner, i.e., induces ligand-specific conformational changes in the G protein. As a result of this ligand-specific receptor/G protein coupling, all of the kinetic properties of NTP interaction with $G_{s\alpha}$, i.e., NTP binding, dissociation, and hydrolysis, are differentially altered, ultimately leading to ligand-specific effector activation. Thus, in the presence of ITP, the efficacy of (−)-ISO is greater than that of SAL in stimulating AC, whereas SAL has a higher efficacy at stimulating ITP hydrolysis (Table 1). This might be explained by proposing that SAL and (−)-ISO are equally effective in promoting ITP binding, but SAL is more efficient than (−)-ISO in promoting ITP hydrolysis. As a result, $G_{s\alpha}$ activated by SAL-liganded β_2-AR is more rapidly inactivated and thus less effective in stimulating AC. Perhaps most interesting is the behavior of ICI in the presence of XTP. Whereas the β_2-AR conformation stabilized by ICI may be efficient at preventing GTP binding to $G_{s\alpha}$, it promotes, at least to some extent, the binding of XTP (see Fig. 3, D and F). As another example, DOB may stabilize a specific β_2-AR conformation that is much more efficient at enhancing GTP and ITP binding to $G_{s\alpha}$ than XTP binding (see Table 1).

The apparent discrepancies between agonist affinity as determined in [^{3}H]DHA competition studies and agonist potencies in AC and NTPase studies also provide insight into the mechanisms underlying receptor/G protein coupling (Table 2). We expected that the EC_{50} values of agonists in AC assays would not be higher than the low-affinity K_i values for agonist binding (which is actually assumed to represent the G protein-uncoupled state; De Lean et al., 1980; Lefkowitz et al., 1993). However, this was not the case; i.e., the EC_{50} of (−)-ISO and (+)-ISO in the AC assays with XTP were higher than their low-affinity K_i values (Table 2). Similar discrepancies between agonist potency and agonist affinity have been observed before for several GPCRs, including the β_2-AR (Gether et al., 1995; Albrecht et al., 1998; Wenzel-Seifert et

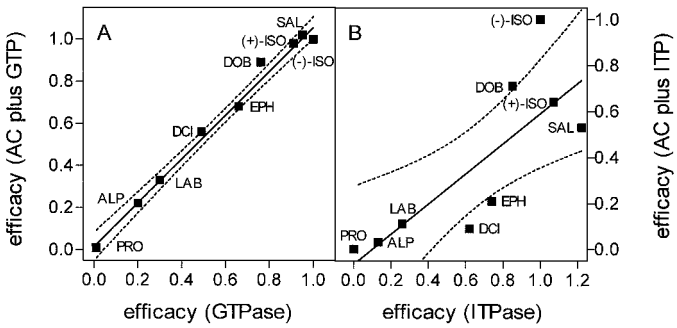

Fig. 9. Correlation between the efficacies of agonists at activating steady-state GTPase or ITPase activity in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ with the efficacies of ligands at activating AC in the presence of GTP and ITP, respectively. A, the efficacies of partial and full agonists at activating steady-state GTP hydrolysis in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg of protein) and AC in the presence of GTP in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ (2.0–2.7 pmol/mg of protein) as shown in Table 1 were plotted against each other. Data were analyzed by linear regression analysis ($r^2 = 0.990$, $p < .0001$). The dotted line indicates the 95% confidence interval of the regression line. B, the efficacies of partial and full agonists at activating steady-state ITP hydrolysis in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg of protein) and AC in the presence of ITP in Sf9 membranes expressing β_2-$ARG_{s\alpha}$ (2.0–2.7 pmol/mg of protein) as shown in Table 1 were plotted against each other. Data were analyzed by linear regression analysis ($r^2 = 0.644$; $p < .0092$). The dotted line indicates the 95% confidence interval of the regression line. LAB, (±)-labetalol; PRO, (−)-propranolol; ALP, (−)-alprenolol.

TABLE 2

Potencies of selected β_2-AR ligands at β_2-$ARG_{s\alpha}$ as assessed by AC activity in presence of GTP, ITP, or XTP and by GTPase and ITPase activity and agonist affinities

AC activity was determined in membranes expressing β_2-$ARG_{s\alpha}$ (2.3–2.7 pmol/mg) in the presence of GTP (1 μM), ITP (10 μM), or XTP (100 μM). GTPase activity was determined in membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg) in the presence of 100 nM [γ-^{32}P]GTP. ITPase activity was determined in membranes expressing β_2-$ARG_{s\alpha}$ (7.0–7.5 pmol/mg) in the presence of 3 μM [γ-^{32}P]ITP. Reaction mixtures contained ligands at 0.1 nM to 1 mM as appropriate to obtain saturated concentration-response curves. The EC_{50} values of ligands were calculated by nonlinear regression analysis. To facilitate the analysis of the different potencies of ligands for various parameters, we also calculated potency ratios by dividing EC_{50} values for two parameters (dimensionless). For comparison of agonist potencies with agonist affinities, the K_i values for high-affinity (K_h) and low-affinity (K_l) agonist binding are shown as well (expressed in nM). When distinct high- and low-affinity sites could not be discriminated, K_i values are listed as K_i values. Data for ligand affinities were taken from Seifert et al. (1998a).

β_2-AR Ligand	β_2-AR Ligand Potency/Affinity					
	(−)-ISO	(+)-ISO	SAL	DOB	EPH	DCI
EC_{50} (nM)						
AC + GTP	18 ± 8	115 ± 7	63 ± 20	300 ± 110	8400 ± 1100	38 ± 12
GTPase	13 ± 3	150 ± 15	93 ± 10	90 ± 19	560 ± 180	29 ± 4
AC + ITP	233 ± 34	1600 ± 750	1700 ± 200	850 ± 200	10000 ± 2000	48 ± 10
ITPase	57 ± 10	1100 ± 460	1200 ± 420	310 ± 80	5400 ± 2100	43 ± 10
AC + XTP	416 ± 44	5700 ± 2100	900 ± 300	3500 ± 800	N.A.	N.A.
K_h (nM)	2.0 ± 1.3	30 ± 11	44 ± 20	61 ± 12		
K_l (nM)	130 ± 62	2200 ± 610	1700 ± 420	3200 ± 940	5100 ± 1300	160 ± 49
Ratio EC_{50}						
AC + GTP/EC_{50} GTPase	1.39	0.77	0.68	3.33	15.0	1.31
AC + ITP/EC_{50} ITPase	4.09	1.45	1.41	2.74	1.85	1.12
AC + ITP/EC_{50} AC + GTP	12.9	13.9	27.0	2.83	1.19	1.26
AC + XTP/EC_{50} AC + GTP	23.1	49.6	14.3	11.7	N.A.	N.A.
ITPase/EC_{50} GTPase	4.38	7.33	12.9	3.44	9.64	1.48

N.A., not applicable because effects of EPH and DCI on AC in the presence of XTP were too small to reliably calculate EC_{50} values.

al., 1998a). Most important, (−)-ISO binds to the purified β_2-AR with a K_i value of 1 μM, but (−)-ISO induces a conformational change in the receptor only with an EC_{50} of ~30 μM (Gether et al., 1995). These findings indicate that the β_2-AR (and other GPCRs) may exist in a state of ultralow agonist affinity that is difficult to detect in ligand-binding studies, either because this ultralow agonist-affinity state is in a rapid equilibrium with K_l or the proportion of receptors in this state is small. Of interest in this context is the fact that, for the partial agonist DCI, EC_{50} values were always lower than K_i values in binding assays (Table 2). In this case, a fraction of the β_2-AR that is too small to be detected in the binding assay may exhibit high affinity for DCI and mediate G protein coupling, regardless of whether GTP or ITP is the nucleotide present. Collectively, the dissociation of agonist affinities and agonist potencies provide further support for the existence of multiple GPCR conformations (Kenakin, 1996; Tucek, 1997) and indicate that ligand-binding studies cannot detect all existing and functionally relevant receptor states.

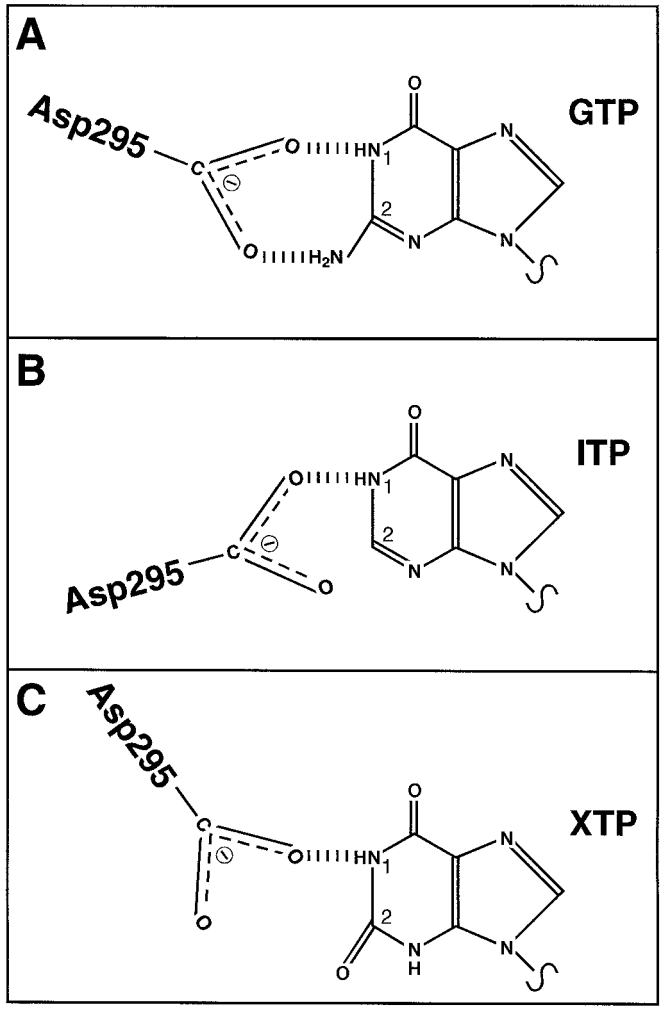

Fig. 10. Model for the interaction of guanine, inosine, and xanthine nucleotides with Asp295 in $G_{s\alpha}$. A, a highly conserved aspartate residue in G protein α subunits (Asp295 in $G_{s\alpha L}$) forms hydrogen bonds with the substituted nitrogen at position 1 of the guanine ring and the amino group in position 2. B, in inosine nucleotides, the amino group at position 2 is missing. Therefore, hydrogen bonding at this position cannot take place, and the orientation of IDP/ITP relative to Asp295 is presumably different from the orientation of GDP/GTP relative to Asp295. C, in xanthine nucleotides, position 2 of the purine ring is substituted with a keto group. Therefore, hydrogen bonding with Asp295 at position 2 of the purine ring cannot take place. The orientation of XDP/XTP relative to Asp295 may be different from the orientations of IDP/ITP relative to Asp295, because there is repulsion between the electronegative keto group of the xanthine ring and the carboxyl group of the aspartate residue. For the sake of simplicity, the sugar moiety and phosphate chain of NDPs/NTPs are not shown.

Conclusions. Guanine, inosine, and xanthine nucleotides can be used as probes to detect ligand-specific G protein-coupling states of receptors. We observed that the efficacy and potency of a panel of β_2-AR ligands are influenced by the nucleotide bound to $G_{s\alpha}$ and that purine nucleotides differentially disrupt the ternary complex stabilized by different ligands, supporting the concept of multiple active receptor conformations. Our results suggest that unique ligand-induced receptor states not only promote NDP release and NTP binding but may also influence NTP dissociation and hydrolysis. Moreover, the efficacy and potency of a ligand at regulating nucleotide binding may differ from its efficacy at promoting nucleotide hydrolysis or dissociation. This conclusion implies that G proteins retain a "memory" of the ligand-specific receptor conformation. The molecular basis for such G protein memory could be continuous physical interaction of a GPCR with the G protein during the entire G protein activation/deactivation cycle. The fact that ternary complex formation is at least partially preserved when $G_{s\alpha}$ binds ITP, XTP, GDP, IDP, or XDP clearly points to persistent receptor/G protein contact even after nucleotide binding. Although it is conceivable that such interaction of receptor and G protein during the entire cycle can happen in the conformationally constrained fusion protein system, such interaction may not be restricted to such systems. Specifically, guanine nucleotide-insensitive high-affinity-agonist binding has also been observed in nonfused systems (Szele and Pritchett, 1993; Wild et al., 1993; Seifert et al., 1998b), and guanine nucleotides do not prevent copurification of receptors and G proteins (Matesic et al., 1989). Moreover, cytoskeletal elements may restrict the mobility of receptors and G proteins in vivo, thereby forcing their close association(Neubig, 1994).

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