Effects of Guanine, Inosine, and Xanthine Nucleotides on $\beta_2$-Adrenergic Receptor/Gs Interactions: Evidence for Multiple Receptor Conformations

ROLAND SEIFERT,1 ULRIK GETHER,2 KATHARINA WENZEL-SEIFERT,3 and BRIAN K. KOBILKA

Howard Hughes Medical Institute (R.S., U.K., K.W.-S., B.K.K.) and Division of Cardiovascular Medicine (B.K.K.), Stanford University Medical School, Stanford, California

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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 $\beta_2$-adrenergic receptor and the $\alpha$ subunit of the G protein $G_s$. GTP was more potent and efficient than ITP and XTP at inhibiting ternary complex formation and supporting adenyl cyclase (AC) activation. We also studied the effects of several $\beta_2$-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 $\beta_2$-adrenergic receptor ($\beta_2$-AR) is a prototypical G protein-coupled receptor (GPCR) that interacts with the G protein $G_s$ to activate adenyl cyclase (AC; Gilman, 1987; Kobilka, 1992). GPCRs activate G proteins by promoting GDP release from and GTP binding to G protein $\alpha$ subunits (Iiri et al., 1998). GTP-liganded $G_{\alpha s}$ 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., 1998; 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 $\alpha$ subunits (De Lean et al., 1980; Seifert et al., 1998a,b). The agonist-occupied receptor and nucleotide-free G protein $\alpha$ 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 $\beta_2$-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-
pecific 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 D₂ 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 regard 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 (Wollf and Cook, 1973; Bilezian 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 β₂-AR-modulated interactions between the G protein G₁, and the purine nucleotides GDP, GTP, IDP, ITP, and XTP. As an experimental system, we used a fusion protein of the β₂-AR and the long-splice variant of G₁ (Gₛₜ.reverse), expressed in Sf9 insect cells. Fusion of the two proteins to each other does not change the fundamental properties of either the β₂-AR or Gₛₜ.reverse and allows for sensitive analysis of GPCR/G protein coupling in terms of ternary complex formation, GDP hydrolysis, and AC regulation (Seifert et al., 1998a,b). The β₂-AR coupled to Gₛₜ.reverse, but not the β₂-AR coupled to Gₛₜ, 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 β₂-AR coupled to Gₛₜ.reverse can be explained by the relatively low GDP affinity of Gₛₜ.reverse compared with the short-splice variant of Gₛₜ (Gₛₜ₈). Specifically, Gₛₜ.reverse is more often guanine nucleotide-free than Gₛₜ₁₀ and therefore is more often available to stabilize the R* state. Here, we report that the potency and efficacy of a series of β₂-AR ligands at the β₂-AR Gₛₜ.reverse fusion protein is dependent on the purine nucleotide that binds to Gₛₜ.reverse. Our results provide further evidence for ligand-specific receptor conformational states.

**Experimental Procedures**

**Materials.** [γ-³²P]GTP (6000 Ci/mmol), [γ-³²P]ITP (4000 Ci/mmolel), and [γ-³²P]XTP (4000 Ci/mmolel) were custom synthesized by DuPont-NEC (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) (GTPS₅), 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 β₂-AR and Gₛₜ.reverse is described in Seifert et al. (1998a,b).

**Cell Culture and Membrane Preparation.** The β₂-AR or β₂-ARₘₙ fusion protein was expressed in Sf9 cells via recombinant baculovirus as described (Seifert et al., 1998a,b). Sf9 membranes expressing β₂-ARₘₙ were prepared according to Seifert et al. (1998a,b). The experiments described in this study were performed in the absence of mammalian βγ complex. The effect of mammalian βγ complex on the function of β₂-ARₘₙ was described previously (Seifert et al., 1998b).

[³²P]Dihydroalprenolol (DHA) Binding. [³²P]DHA binding studies were carried out as described (Seifert et al., 1998a,b). Tubes contained Sf9 membranes expressing β₂-AR at 6.1 pmol/mg of protein or β₂-ARₘₙ at 7.0 to 7.5 pmol/mg of protein (15–30 µg of protein/tube), 1 nM [³²P]DHA, 1 µM salbutamol (SAL) and various nucleotides at increasing concentrations. As reported before, the Kᵥ value for [³²P]DHA at β₂-ARₘₙ is 0.38 ± 0.03 nM (Seifert et al., 1998b). Nonspecific binding with 1 nM [³²P]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 β₂-AR at 6.1 pmol/mg of protein or β₂-ARₘₙ at 7.0 to 7.5 pmol/mg of protein (10 µg of protein), 1.0 mM MgCl₂, 0.1 mM EDTA, 0.1 mM ATP, 1 mM adenylylimidodiphosphate, 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 β₂-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-³²P]GTP, [γ-³²P]ITP, or [γ-³²P]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 NaH₂PO₄, pH 2.0. Charcoal absorbs nucleotides but not Pi. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000g. Seven hundred microliters of the supernatant fluid of reaction mixtures was removed, and [³²P]Pi, was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of [γ-³²P]NTP. Spontaneous [³²P]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 [³²P]NTP, prevents [³²P]NTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [³²P]NTP degradation was <1% of the total amount of radioactivity added. Note that, for NTPase studies, β₂-ARₘₙ was expressed at considerably lower levels than wild type. For AC studies, β₂-ARₘₙ was expressed at considerably lower levels than wild type.

AC Activity. Cyclic AMP (cAMP) formation in Sf9 membranes was carried out as described (Seifert et al., 1998a,b). Tubes contained Sf9 membranes expressing β₂-AR at 6.1 pmol/mg of protein or β₂-ARₘₙ at 2.3 to 2.7 pmol/mg of protein (15–20 µg of protein/tube), 5 mM MgCl₂, 0.4 mM EDTA, and 30 mM Tris/HCl, pH 7.4, and purine nucleotides and β₂-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-³²P]ATP and 3% (w/v) charcoal. Reactions were conducted for 20 min. [³²P]cAMP was separated from [α-³²P]ATP by reverse-phase HPLC as described (Seifert et al., 1998a,b). Note that, for AC studies, β₂-ARₘₙ was expressed at considerably lower levels than wild type.
levels than for NTPase studies. This was done to avoid AC availability being 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-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 β2-AR 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 Gs (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 GDP, GTP, GDP, 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 [3H]DHA in the presence of a saturating concentration of the full β2-AR agonist (-)-isoproterenol (ISO; Fig. 1, A and B) and the strong partial agonist SAL in Sf9 membranes expressing β2-ARGm. Nucleotides at increasing concentrations were added to the binding assays. Nucleotide binding to Gm reduces the affinity of the β2-AR for agonist and thereby increases [3H]DHA binding.

NTPs inhibited high-affinity binding of both (-)-ISO and SAL at β2-ARGm in the order of potency GTP > ITP > XTP > ATP (ineffective). This rank order to potency is in agreement with the data obtained for nonfused Gmr (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 β2-AR alone is guanine nucleotide-insensitive, ruling out the possibility that the β2-AR coupling to endogenous insect Gm-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 β2-AR and β2-ARGm. Membranes expressing β2-ARGm at 2.3 to 2.7 pmol/mg of protein had ~8-fold higher basal AC activity than membranes expressing β2-AR alone at a higher level (6.1 pmol/mg of protein; Fig. 2). In membranes expressing β2-ARm, GTP increased AC activity with an EC50 of 0.7 ± 0.1 μM. Compared with GTP, ITP was considerably less potent (EC50: 30 ± 5 μM) and effective at increasing basal AC activity in membranes expressing β2-ARGm. XTP had virtually no stimulatory effect on basal AC activity in membranes expressing β2-ARGm.

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 β2-ARGm, fusion protein be mediated by cross-activation of endogenous Gm-like G proteins of Sf9 cells by the fused β2-AR? To address this question, we studied the effects of NTPs on AC activity in membranes expressing nonfused β2-AR. Note that, for these studies, we expressed the β2-AR at a level more than twice as high as β2-ARGm1, to increase the effects seen with the nonfused receptor. Despite the high expression level of β2-AR, the absolute stimulation of AC by GTP, ITP, and XTP was much less efficient in membranes expressing β2-AR than in membranes expressing β2-ARGm1, and the maximal AC stimulation in membranes expressing nonfused β2-AR did not even approach basal AC activity in membranes expressing β2-ARGm1, 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-ARG_{αsL} 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_{αsL} or G_{αsL}. Therefore, we compared the effects of GTP (1 μM), ITP (10 μM), and XTP (100 μM) on AC activity in membranes expressing β_2-ARG_{αsL} (2.3–2.7 pmol/mg of protein) with the corresponding NTP effects in Sf9 membranes expressing β_2-ARG_{αsS} at a similar level (2.6 pmol/mg of protein). In membranes expressing β_2-ARG_{αsL}, 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 · mg⁻¹ protein · min⁻¹; data not shown). In contrast, with β_2-ARG_{αsL}, AC activities varied by 3-fold (4–12 pmol · mg⁻¹ protein · min⁻¹; Fig. 2). In agreement with our previous study (Seifert et al., 1998a), the AC activities with β_2-ARG_{αsL} are considerably higher than the AC activities achieved with β_2-ARG_{αsS}. Collectively, these data indicate that the observed NTP effects on AC in membranes expressing β_2-ARG_{αsL} are attributable to the fused G_{αsL} and not to activation of endogenous G_{αs-like} G proteins.

**Regulation of AC Activity by (−)-ISO and ICI in Sf9 Membranes Expressing β_2-ARG_{αsL} 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-ARG_{αsL}.** To obtain insight into the mechanism by which G_{αsL} activation by ITP and XTP is terminated, we studied ITPase and XTPase activities in membranes expressing β_2-ARG_{αsL} or β_2-AR. The basal ITPase activity in membranes expressing β_2-ARG_{αsL} 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 XTP hydrolysis in membranes expressing β_2-ARG_{αsL}. These data were the first indication that G_{αsL} exhibits substantial ITPase activity.

Membranes expressing β_2-AR and β_2-ARG_{αsL} both exhibited significant basal XTPase activity. However, in contrast to the data obtained for ITPase, the XTPase activity in membranes expressing β_2-ARG_{αsL} 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-ARG_{αsL}. 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-32P]XTP and membranes expressing β_2-ARG_{αsL} 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 MgCl₂ 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 G₁-protein-coupled αs proteins.

**Fig. 2.** Effects of GTP, ITP, and XTP on basal AC activity in Sf9 membranes expressing β_2-AR or β_2-ARG_{αsL}. AC activity in membranes expressing β_2-AR (6.1 pmol/mg of protein) or β_2-ARG_{αsS} (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, but is not hydrolyzed. To substantiate this hypothesis, we performed competition studies with [γ-32P]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 β2-ARG_s. GTPγS and GppNHp bind to G_s 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 (IC50, 402 ± 44 and 250 ± 33 μM, respectively). These findings show that GTPγS and GppNHp potently compete with ITP for binding to G_s. The ITPase that is inhibited by GppNHp 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 IC50 values of GTPγS and GppNHp for XTP hydrolysis were 294 ± 23 and 351 ± 44 μM, respectively, and were similar to the IC50 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 (IC50, 17 ± 4 nM). If XTP binds to but is not hydrolyzed by G_s, XTP is expected to block GTP hydrolysis, as does GTPγS. Indeed, XTP abolished (−)ISO-stimulated GTP hydrolysis, although with a much higher IC50 value than GTPγS (IC50, 139 ± 22 μM). Taken together, the nucleotide competition data and the similar basal XTPase activities in membranes expressing β2-ARG_s and β2-ARG_m indicate that basal XTP hydrolysis in Sf9 membranes is caused by endogenous nucleotidases and that G_s does not hydrolyze XTP to a measurable extent.

Kinetics of Agonist-Stimulated GTP and ITP Hydrolysis in Membranes Expressing β2-ARG_m. 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 β2-ARG_m expression level. By doing so, a Vmax of (−)ISO-stimulated GTP turnover of 1.37 ± 0.11 μmol min⁻¹ was obtained by nonlinear regression analysis (Fig. 7C). The Km value of the (−)ISO-stimulated GTPase is 0.18 ± 0.04 μM. These kinetic properties of β2-ARG_m agree with data obtained for reconstituted purified β-AR and G_s (Brandt and Ross, 1986). Because the affinity of
Gₐₐ 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 Vₘₐₓ of (−)-ISO-stimulated ITP hydrolysis was 3.06 ± 0.07 min⁻¹, and the Kₘₐₐ was 6.3 ± 0.5 μM.

**Ligand Efficacies at β₂-AR₉₉ 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 β₂-AR₉₉ (7.5 pmol/mg of protein). (−)-ISO stimulated GTPase, with an EC₅₀ of 13 ± 3 nM, and ICI reduced GTP hydrolysis, with an IC₅₀ 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 (EC₅₀, 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 Gₐₐ (see Fig. 4, A and B).

Table 1 summarizes the efficacies of a series of β₂-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 β₂-AR agonists at activating GTPase and AC in the presence of GTP were plotted against each other (Fig. 8A). However, when the efficacies of β₂-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 (−)-alpenolol 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 EC₅₀ values of selected β₂-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 Kᵢ values for the agonists studied. Moreover, we calculated potency ratios for the various parameters studied.
For (-)-ISO, (+)-ISO, SAL, and DOB, the EC\textsubscript{50} values for activation of AC in the presence of GTP were substantially lower than the EC\textsubscript{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\textsubscript{50} values of agonists at activating AC in the presence of GTP were similar to the EC\textsubscript{50} values of agonists at activating GTPase. Similarly, the EC\textsubscript{50} values of agonists at activating AC in the presence of ITP were similar to the EC\textsubscript{50} values of agonists at activating ITPase. When AC activation in the presence of XTP was considered, the EC\textsubscript{50} values of (-)-ISO, (+)-ISO, and DOB were even higher than the EC\textsubscript{50} values for AC activation in the presence of ITP.

### Discussion

**Interaction of Guanine, Inosine, and Xanthine Nucleotides with G\textsubscript{sa}**

Guanine, inosine, and xanthine nucleotides differentially form hydrogen bonds with a highly conserved aspartate residue in G protein \textalpha subunits (Noel et al., 1993; Fig. 10). The reduced affinity of G\textsubscript{sa} for IDP and ITP compared with GDP and GTP may be because the inosine ring forms only one hydrogen bond with Asp295 in G\textsubscript{sa} (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\textsubscript{sa} 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\textsubscript{sa} that influence interactions with the receptor and/or effector. Studies with fluorescent guanine nucleotides already provided evidence for...
the existence of nucleotide-specific G protein activation states (Remmers and Neubig, 1996).

Differences in the kinetics of interaction of nucleotides with Gs subunits could also contribute to the different efficacies of nucleotides. NTP hydrolysis is the principal mechanism by which Gs subunits is deactivated (Cassel and Selinger, 1976; Gilman, 1987). The faster NTP hydrolysis proceeds, the shorter the time for which Gs subunits 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 Gs subunits is greater than the rate of GTP dissociation (Florio and Sternweis, 1989). Fast dissociation from Gs subunits could be another factor that contributes to the lower efficacy of IDP and ITP compared with GDP and GTP. For AC activation by Gs subunits in the presence of XTP, nucleotide dissociation and not rapid hydrolysis appears to be the major mechanism by which Gs subunits is deactivated (Figs. 4, C and D, and 5B). Because of its low affinity for Gs subunits, XTP could be thought to dissociate from Gs subunits even before it can be cleaved. As a result of the rapid dissociation of XTP, Gs subunits 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 Gs subunits.

Another mechanism that could contribute to the observed differences in efficacy between GTP, ITP, and XTP is differential βγ-AR regulation of NTP binding to Gs subunits. 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 βγ-AR can effectively increase GTP binding to Gs subunits (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 Gs subunits (see Fig. 10, A and B), binding of ITP to Gs subunits is less stable than binding of GTP so that substantial agonist occupancy of βγ-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 Gs subunits may be so high that the agonist-free βγ-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).

### Table 1

<table>
<thead>
<tr>
<th>βγ-AR Ligand</th>
<th>AC + GTP</th>
<th>GTPase</th>
<th>AC + ITP</th>
<th>ITPase</th>
<th>AC + XTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)-ISO</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>(+)-ISO</td>
<td>0.98 ± 0.06</td>
<td>0.91 ± 0.03*</td>
<td>0.64 ± 0.01</td>
<td>1.07 ± 0.07**</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>SAL</td>
<td>1.02 ± 0.14</td>
<td>0.95 ± 0.02*</td>
<td>0.53 ± 0.15</td>
<td>1.22 ± 0.02**</td>
<td>0.49 ± 0.20</td>
</tr>
<tr>
<td>DOB</td>
<td>0.89 ± 0.16</td>
<td>0.76 ± 0.04*</td>
<td>0.71 ± 0.03</td>
<td>0.85 ± 0.05*</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>EPH</td>
<td>0.68 ± 0.16</td>
<td>0.66 ± 0.05*</td>
<td>0.21 ± 0.13</td>
<td>0.74 ± 0.29**</td>
<td>0.11 ± 0.11</td>
</tr>
<tr>
<td>DCl</td>
<td>0.56 ± 0.19</td>
<td>0.49 ± 0.08*</td>
<td>0.09 ± 0.06</td>
<td>0.62 ± 0.07**</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>(−)-Labetalol</td>
<td>0.33 ± 0.16</td>
<td>0.30 ± 0.03*</td>
<td>0.11 ± 0.11</td>
<td>0.26 ± 0.10*</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>(−)-Alpenrolol</td>
<td>0.22 ± 0.06</td>
<td>0.21 ± 0.04*</td>
<td>0.03 ± 0.03</td>
<td>0.13 ± 0.05**</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>(−)-Propanrolol</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.02</td>
<td>0.00 ± 0.01</td>
<td>0.01 ± 0.01*</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>TIM</td>
<td>−0.69 ± 0.13</td>
<td>−0.10 ± 0.02**</td>
<td>−0.13 ± 0.04</td>
<td>0.01 ± 0.04**</td>
<td>0.03 ± 0.07</td>
</tr>
<tr>
<td>ICI</td>
<td>−0.88 ± 0.02</td>
<td>−0.12 ± 0.03**</td>
<td>−0.14 ± 0.03</td>
<td>−0.02 ± 0.03**</td>
<td>0.19 ± 0.10</td>
</tr>
</tbody>
</table>

* 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 EC50 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 EC50 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 ligands are listed as K curves. The EC50 values of ligands were calculated by nonlinear regression analysis. To facilitate the analysis of the different potencies of ligands for various parameters, the efficacies of ligands at activating AC were determined in membranes expressing 2-ARGs (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 (r² = 0.999, 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 β2-AR (7.0–7.5 pmol/mg of protein) and AC in the presence of GTP in Sf9 membranes expressing β2-AR (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 (r² = 0.644; p < .0092). The dotted line indicates the 95% confidence interval of the regression line.

FIG. 9. Correlation between the efficacies of agonists at activating steady-state GTPase or ITPase activity in Sf9 membranes expressing β2-AR and 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 β2-AR (7.0–7.5 pmol/mg of protein) and AC in the presence of GTP in Sf9 membranes expressing β2-AR (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 (r² = 0.999, 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 β2-AR (7.0–7.5 pmol/mg of protein) and AC in the presence of ITp in Sf9 membranes expressing β2-AR (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 (r² = 0.644; p < .0092). The dotted line indicates the 95% confidence interval of the regression line.

### TABLE 2
Potencies of selected β2-AR ligands at β2-AR as assessed by AC activity in presence of GTP, ITp, or XTP and by GTPase and ITPase activity and agonist affinities

<table>
<thead>
<tr>
<th>β2-AR Ligand</th>
<th>(+)-ISO</th>
<th>(+)-ISO</th>
<th>SAL</th>
<th>DOB</th>
<th>DOB</th>
<th>EPB</th>
<th>DCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC + GTP</td>
<td>18 ± 8</td>
<td>115 ± 7</td>
<td>63 ± 20</td>
<td>300 ± 110</td>
<td>8400 ± 1100</td>
<td>38 ± 12</td>
<td></td>
</tr>
<tr>
<td>GTPase</td>
<td>13 ± 3</td>
<td>150 ± 15</td>
<td>93 ± 10</td>
<td>90 ± 19</td>
<td>560 ± 180</td>
<td>29 ± 4</td>
<td></td>
</tr>
<tr>
<td>AC + ITp</td>
<td>233 ± 34</td>
<td>1600 ± 750</td>
<td>1700 ± 200</td>
<td>850 ± 200</td>
<td>10000 ± 2000</td>
<td>48 ± 10</td>
<td></td>
</tr>
<tr>
<td>ITpase</td>
<td>57 ± 10</td>
<td>1100 ± 460</td>
<td>1200 ± 420</td>
<td>310 ± 80</td>
<td>5400 ± 2100</td>
<td>43 ± 10</td>
<td></td>
</tr>
<tr>
<td>AC + XTP</td>
<td>416 ± 44</td>
<td>5700 ± 2100</td>
<td>900 ± 300</td>
<td>3500 ± 800</td>
<td>N.A.</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Kᵦ (nM)</td>
<td>2.0 ± 1.3</td>
<td>30 ± 11</td>
<td>44 ± 20</td>
<td>61 ± 12</td>
<td>N.A.</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Ratio EC50</td>
<td>120 ± 62</td>
<td>2200 ± 610</td>
<td>1700 ± 420</td>
<td>3200 ± 940</td>
<td>5100 ± 1300</td>
<td>160 ± 49</td>
<td></td>
</tr>
<tr>
<td>AC + GTP(EC50 GTPase)</td>
<td>1.39</td>
<td>0.77</td>
<td>0.68</td>
<td>3.33</td>
<td>15.0</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>AC + ITp(EC50 ITpase)</td>
<td>4.09</td>
<td>1.45</td>
<td>1.41</td>
<td>2.74</td>
<td>1.85</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>AC + XTP(EC50 AC + GTP)</td>
<td>12.9</td>
<td>13.9</td>
<td>27.0</td>
<td>2.83</td>
<td>1.19</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>ITpase(EC50 GTPase)</td>
<td>23.1</td>
<td>49.6</td>
<td>14.3</td>
<td>11.7</td>
<td>N.A.</td>
<td>N.A.</td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable because effects of EPB and DCI on AC in the presence of XTP were too small to reliably calculate EC50 values.
al., 1998a). Most important, (−)-ISO binds to the purified β2-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 β2-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 $K_i$ 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 β2-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.

**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 β2-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 (Széle 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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**References**


Send reprint requests to: Brian Koblika, M.D., Howard Hughes Medical Institute, B-157, Beckman Center, Stanford University Medical School, Stanford, CA 94305-5428. E-mail: koblika@cmgm.stanford.edu