Distinct interactions of GTP, UTP and CTP with G<sub>S</sub>-proteins

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Running title: $G_S$-protein activation by UTP and CTP
Abstract

Early studies showed that in addition to GTP, the pyrimidine nucleotides UTP and CTP support activation of the adenylyl cyclase (AC)-stimulating Gs-protein. The aim of this study was to elucidate the mechanism by which UTP and CTP support Gs activation. As models, we used S49 wild-type lymphoma cells representing a physiologically relevant system in which the β2-adrenoceptor (β2AR) couples to Gs, and Sf9 insect cell membranes expressing β2AR-Gsα fusion proteins. Fusion proteins provide a higher sensitivity for the analysis of β2AR/Gs-coupling than native systems. Nucleoside 5-triphosphates (NTPs) supported agonist-stimulated AC activity in the two systems and basal AC activity in membranes from cholera toxin-treated S49 cells in the order of efficacy GTP > UTP > CTP > ATP (ineffective). NTPs disrupted high-affinity agonist binding in β2AR-Gsα in the order of efficacy GTP > UTP > CTP > ATP (ineffective). In contrast, the order of efficacy of NTPs as substrates for nucleoside diphosphokinase, catalyzing the formation of GTP from GDP and NTP was ATP > UTP > CTP > GTP. NTPs inhibited β2AR-Gsα-catalyzed [γ-32P]GTP hydrolysis in the order of potency GTP > UTP > CTP. Molecular dynamics simulations revealed that UTP is accommodated more easily within the binding pocket of Gsα than CTP. Collectively, our data indicate that GTP, UTP and CTP differentially interact with Gs-proteins and that transphosphorylation of GDP to GTP is not involved in this G-protein activation. In certain cell systems, intracellular UTP- and CTP concentrations reach ~10 nmol/mg of protein and are higher than intracellular GTP concentrations, indicating that G-protein activation by UTP and CTP can occur physiologically.
G-protein activation by UTP and CTP could be of particular importance in pathological conditions such as cholera and Lesch-Nyhan syndrome.

**Introduction**

G-proteins consist of an $\alpha$-subunit and a $\beta\gamma$-complex and serve as signal transducers between agonist-activated GPCRs\(^1\) and effector systems (1-4). Upon binding of an agonist, GPCRs undergo a conformational change causing GDP dissociation from $G\alpha$. GDP dissociation is the rate-limiting step of the G-protein cycle. Agonist-occupied GPCRs then form a ternary complex with the nucleotide-free G-protein. The ternary complex possesses high agonist-affinity. Subsequently, GPCRs promotes binding of GTP to $G\alpha$. The binding of GTP to $G\alpha$ induces the active conformation of the G-protein, leading to the dissociation of the heterotrimer into $G\alpha$-GTP and the $\beta\gamma$-complex. Both $G\alpha$-GTP and $\beta\gamma$ can regulate the activity of effector systems. $G\alpha$ possesses GTPase activity. The GTPase cleaves GTP into GDP and $P_i$ and, thereby, deactivates the G-protein. $G\alpha$-GDP and $\beta\gamma$ reassociate, completing the G-protein cycle.

Intriguingly, not only the purine nucleotide GTP but also pyrimidine nucleotides exhibit effects on G-proteins. Particularly, various natural and synthetic uracil nucleotides disrupt the complex between the photoexcited light receptor rhodopsin and the retinal G-protein transducin, but the uracil nucleotides are less effective in this regard than the corresponding guanine nucleotides (5). \([\gamma^{32}\text{P}]\text{GTP}\) hydrolysis- and \([35\text{S}]\text{GTP}\gamma\text{S}\) binding competition studies showed that pyrimidine nucleotides bind to G-proteins with low affinity (5-8). Moreover, early studies
revealed that UTP and CTP support GPCR-mediated AC activation in membranes (9-11).

However, it remained unclear whether the effects of UTP and CTP on AC were mediated via NDPK, catalyzing the formation of GTP from GDP and NTP (12, 13), or via direct interaction of UTP and CTP with Gsα (6).

The aim of the present study was to elucidate the mechanism by which UTP and CTP support Gs activation. To achieve our aim we have studied AC regulation in S49 membranes. S49 cells are a widely used and physiologically relevant model system for the analysis of β2AR/Gs/AC interactions (14-17). Additionally, we have studied fusion proteins of the β2AR with individual Gsα isoforms, i.e. β2AR-GsαS, β2AR-GsαL and β2AR-Gsαolf, expressed in Sf9 insect cells. Fusion proteins provide close proximity of the coupling partners and ensure efficient GPCR/G-protein/effecter coupling (18, 19). In addition, fusion proteins allow for the analysis of the coupling of a given GPCR to various Gα isoforms under defined experimental conditions (20-23). Here, we report on distinct interactions of GTP, UTP and CTP with Gs-proteins.

**Experimental Procedures**

**Materials.** The generation of baculoviruses encoding for β2AR-GsαS, β2AR-GsαL and β2AR-Gsαolf was described elsewhere (20, 23, 24). [γ-32P]GTP (6000 Ci/mmol), [α-32P]ATP (3000 Ci/mmol), [32P]P₁ (8500-9000 Ci/mmol), [3H]DHA (85-90 Ci/mmol) and [3H]GDP (30-39 Ci/mmol) were from Perkin Elmer Life Sciences (Boston, MA). Unlabeled ATP (special quality, < 0.01% GTP as assessed by HPLC, # 519 979), GTP, UTP and CTP were of the highest
quality available and were obtained from Roche (Indianapolis, IN). [γ-32P]UTP and [γ-32P]CTP (~6000 Ci/mmol each) were synthesized as described (5, 25). S49 cells were obtained from the Cell Culture Facility of The University of California at San Francisco (San Francisco, CA). ISO, SAL, (±)-alprenolol and CTX were obtained from Sigma (St. Louis, MO).

**Cell culture and membrane preparation.** Sf9 cells were cultured and infected with recombinant baculoviruses as described (20, 24, 26). S49 cells were grown at 37°C in suspension in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine, 1000 U/ml penicillin, 100 µg/ml streptomycin and 10% (vol./vol.) heat-inactivated horse serum in a humidified atmosphere containing 7% (vol./vol.) CO2. S49 cells were maintained at a density of 0.2-2.0 x 10^6 cells/ml. To inactivate the GTPase of G_sα, S49 cells were treated with CTX (1 µg/ml) for 24 h before membrane preparation (27). DMEM medium was from Cellgro Mediatech (Herndon, VA). All other constituents for the culture of S49 cells were obtained from Bio Whittaker (Walkersville, MD). S49- and Sf9 membranes were prepared according to the previously described protocol (24). Membranes were suspended in “binding buffer” (12.5 mM MgCl_2, 1 mM EDTA, and 75 mM Tris/HCl, pH 7.4) at a concentration of ~1-2 mg of protein/ml and stored at ~80°C until use. Immediately prior to [3H]DHA binding-, AC-, NTPase- and NDPK experiments, membrane aliquots were thawed, suspended in binding buffer and centrifuged for 15 min at 4°C and 15,000 x g to remove, as far as possible, any remaining endogenous nucleotides (23).

**[3H]DHA binding.** The expression levels of β2AR-G_sαS, β2AR-G_sαL and β2AR-
G$_{olf}$ were determined by saturation binding using the $\beta_2$AR antagonist $[^3H]$DHA (20, 24). Tubes (500 µl) contained Sf9 membranes (10-20 µg of protein per tube) expressing fusion proteins, 10 nM $[^3H]$DHA and binding buffer. Non-specific binding was determined in the presence of 10 µM (±)-alprenolol. Incubations were performed for 90 min at 25°C and shaking at 250 rpm. Bound $[^3H]$DHA was separated from free $[^3H]$DHA by filtration through GF/C filters (Schleicher & Schuell, Dassel, Germany). For generation of concentration/response curves for the inhibitory effects of NTPs on high-affinity agonist binding, reaction mixtures contained Sf9 membranes (20 µg of protein per tube) expressing $\beta_2$AR-Gs$\alpha$ fusion proteins, 1 µM SAL, 1 nM $[^3H]$DHA as radioligand and NTPs at increasing concentrations (26). For determination of the extent of ternary complex formation and its sensitivity to disruption by NTPs, reaction mixtures contained Sf9 membranes (20 µg of protein per tube) expressing $\beta_2$AR-Gs$\alpha$L, 1 nM $[^3H]$DHA as radioligand and ISO at increasing concentrations in the absence and presence of NTPs at a fixed concentration (1 mM each) (23, 24).

**AC activity.** The determination of AC activity in S49 membranes and Sf9 membranes expressing $\beta_2$AR-Gs$\alpha$ fusion proteins was performed under identical experimental conditions and followed the previously published protocol (23). Briefly, tubes (30 µl) contained membranes (20-50 µg of protein per tube), 5 mM MgCl$_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, and NTPs at various concentrations in the absence or presence of ISO. Tubes were incubated for 3 min at 37°C before the addition of 20 µl of reaction mixture containing (final) [$\alpha$-$^{32}$P]ATP (1.0-1.5 µCi/tube) plus 40 µM ATP, 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 at 37°C. Stopping of reactions and separation of [α-32P]ATP from [32P]cAMP was performed as described (23).

**NTPase activity.** High-affinity GTPase activity in Sf9 membranes expressing β2AR-Gsα fusion proteins was determined as described (23). Briefly, tubes (80 µl) contained membranes (10 µg of protein per tube), 1.0 mM MgCl2, 0.1 mM EDTA, 0.1 mM ATP, 1 mM adenylylimidodiphosphate, 5 mM creatine phosphate, 40 µg of creatine kinase, 30 nM unlabeled GTP, 10 µM ISO and 0.05% (mass/vol.) BSA in 50 mM Tris/HCl, pH 7.4. Reaction mixtures were incubated for 3 min at 25°C before the addition of 20 µl of [γ-32P]GTP (0.2 µCi/tube). Non-enzymatic [γ-32P]GTP hydrolysis was determined in the presence of a large excess of unlabeled GTP (1 mM). Reactions were conducted for 20 min at 25°C. Stopping of reactions and recovery of [32P]P1 were performed as described (23).

UTPase- and CTPase activities in Sf9 membranes were determined essentially as GTPase activity except that reaction mixtures contained [γ-32P]UTP or [γ-32P]CTP (up to 2.5 µCi/tube) instead of [γ-32P]GTP. In addition, reaction mixtures contained unlabeled UTP or CTP (0.1100 µM) instead of unlabeled GTP. Non-enzymatic UTP- and CTP hydrolysis was determined in the presence of 1 mM unlabeled UTP and CTP, respectively.

**NDPK activity.** NDPK activity in S49 wt lymphoma membranes and Sf9 membranes was determined as described for soluble transducin preparations with modifications (28). Briefly, reaction mixtures contained S49 membranes (5.0-10.0 µg of protein per tube) or Sf9 membranes...
(0.5-1.0 µg of protein per tube), NTPs at various concentrations, 0.1% (mass/vol.) BSA, 1 mM MgCl₂ and 0.1 mM EDTA in 50 mM Tris/HCl, pH 7.4. Reaction mixtures were pre-incubated for 3 min at 37°C before the addition of 0.5 µM carrier-free [³H]GDP. The total reaction volume was 50 µl. Reactions were conducted for 10 min. To obtain blank values, tubes containing all components described above except for membranes were processed in parallel. Stopping of reactions, separation of nucleotides on poly(ethyleneimine)-cellulose TLC plates (Schleicher & Schuell), elution of nucleotides and counting of radioactivity were performed exactly as described (28).

Molecular modeling. Potential energy minimization and molecular dynamics simulations were carried out using the AMBER 6.0 program package (29), using the force field of Cornell et al. (30). Initial models for Gₛα"Mg²⁺NTP complexes were based on the coordinates of Gₛα"Mg²⁺GTPγS in the complex with the catalytic domains of AC (PDB ID: 1AZS) (31). We replaced the γS substituent of GTPγS by an sp² hybridized oxygen atom. Models of complexes with UTP and CTP were generated by substitution of the guanine base by uracil and cytosine, respectively, maintaining the glycosyl torsion angle (O4’-C1’-N9-C8) of GTPγS in the starting model. Partial charges assigned to phosphate groups were obtained from Dr. N. Ducleter-Savatier (Institut Pasteur, Paris, France)². Protein-bound water molecules observed in the crystal structure were included in the model. The non-bonded cutoff distance was set to 10 Å. The stereochemistry of the Mg²⁺ ligand field was restrained as an octahedral complex with the six coordinating oxygen atoms of the β- and γ-phosphate oxygens, the hydroxyl groups of Ser 54
(GsαL and GsαS) and Thr 204 (GsαL) and two water molecules, with oxygen-Mg²⁺ distances of 2.1 Å. This geometry was maintained by pseudo-van der Waals potentials between all pairs of atoms within the octahedral complex. In constructing models of the Gsα"Mg²⁺"UTP- and Gsα"Mg²⁺"CTP complexes, we placed a water molecule at the site occupied by the guanine exocyclic C(2) amine in the GTP complex. The included water molecule bridges the uracil exocyclic C(2) keto with the O1γ carboxylate oxygen of Asp 295 (GsαL) and Asp 280 (GsαS). The water molecule placed at this site did not move after energy minimization of the model complexes.

Energy relaxations were carried out using the SANDER module of AMBER, using steepest decent minimization for the first 250 cycles, followed by 250 cycles of conjugate gradient minimization. In all cases the total computed potential energy typically declined smoothly from values of -14500 to -16750 kcal·mol⁻¹, attaining a constant value after 400-450 cycles of minimization. For molecular dynamics simulations, a box of TIP3P water molecules was used to solvate the protein, leaving a 10 Å border between the edge of the box and the closest atoms of the protein. The system was heated to 300 K using the temperature scaling scheme of Berendsen et al. (32) and periodic boundary conditions. Simulations were carried out for 10 ps in steps of 1 fs.

**Miscellaneous.** Protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Data shown in Figs. 1-5 and Table I were analyzed by non-linear regression, using the Prism III program (GraphPad, Prism, San Diego, CA).

**Results**
UTP, CTP and GTP differentially support AC activation in S49 membranes. S49 cells
express the β2AR, the Gsα splice variants GsαS and GsαL and AC (17, 33, 34). In the absence
of added UTP, CTP or GTP, the full βAR agonist ISO at a maximally stimulatory concentration
(10 µM) had no stimulatory effect on AC activity in S49 membranes (Fig. 1). However, these
experimental conditions do not imply the complete absence of NTP, since the AC assay
contained 40 µM ATP as AC substrate (see Experimental Procedures). UTP, CTP and GTP had
little effects on basal AC activity in the absence of ISO. In agreement with the literature (1-4),
GTP was potent (EC50, 230 nM; 95% c. i., 150-340 nM) and effective at supporting AC
activation by ISO (Fig. 1C). UTP was much less potent (EC50, 82 µM, 95% c. i., 43-160 µM) in
this regard than GTP, but only moderately less efficient (~80% efficacy) than GTP (Fig. 1A).
CTP only poorly supported AC activation by ISO, both in terms of potency and efficacy (Fig.
1B).

CTX-catalyzed ADP-ribosylation of Arg 201 (GsαL) and Arg 186 (GsαS) blocks the
GTPase of Gsα (1, 27, 35). As a result, GTP, like the GTPase-resistant GTP analog GTPγS,
becomes an efficient AC activator even in the absence of GPCR agonist (1, 27, 35). In fact, in
membranes from CTX-treated S49 cells, GTP was far more efficient at activating AC than were
GTP plus ISO in control membranes (Fig. 1C). However, CTX did not increase the potency of
GTP (EC50, 560 nM; 95% c. i., 310-1000 nM). As was the case with GTP, CTX greatly
increased the efficacy of UTP at activating AC (Fig. 1A). The efficacy of UTP at activating AC
in membranes from CTX-treated S49 cells amounted to ~70% of the efficacy of GTP. In
contrast to the data obtained for GTP, CTX increased the potency of UTP ~4-fold (EC50, 21
µM; 95% c. i., 11-38 µM). Although CTX also substantially enhanced AC activation by CTP, this NTP was, nonetheless, much less efficient than UTP and GTP (Fig. 1B). Thus, CTX greatly amplifies the maximum effects of NTPs on basal AC activity without altering their relative efficacies.

**Rationale for conducting further studies with Sf9 membranes expressing β2AR-Gsα fusion proteins.** We wished to answer the question whether UTP and CTP, like GTP, disrupt the ternary complex consisting of agonist-occupied β2AR and nucleotide-free Gsα, whether UTP and CTP inhibit [γ-32P]GTP hydrolysis by Gsα and whether Gsα hydrolyzes [γ-32P]UTP and [γ-32P]CTP. However, the extent of ternary complex formation in S49 membranes is rather limited (17). Thus, we were concerned that this system would not be sensitive enough to dissect potentially small differences in efficacies of NTPs on high-affinity agonist binding. In addition, S49 membranes are not sensitive models for the analysis of the GTPase activity of Gs (36). We were also interested to answer the question whether the different Gsα isoforms, *i.e.* GsαS, GsαL and Gαolf, respond similarly to UTP and CTP. However, S49 cells express a mixture of GsαS and GsαL, and the sensitivity of the Gsα-deficient S49 *cyc*- cells as reconstitution system for the planned studies is limited, too (33, 34).

Sf9 membranes expressing β2AR-Gsα fusion proteins possess a sufficiently high sensitivity for dissecting differential effects of NTPs on ternary complex formation, surpassing the sensitivity of native and recombinant non-fused systems (17, 24, 26). Additionally, β2AR-
G_α fusion proteins are sensitive models for NTPase studies (24, 26). Moreover, β_2AR-G_α fusion proteins are suitable systems for dissecting biochemical differences between G_α isoforms (20, 22, 23). Based on these considerations, we decided to conduct all further studies with β_2AR-G_α fusion proteins.

**UTP, CTP and GTP differentially disrupt the ternary complex in Sf9 membranes expressing β_2AR-G_αS, β_2AR-G_αL and β_2AR-G_αolf.** We examined binding of a fixed concentration of the antagonist [³H]DHA (1 nM) to fusion proteins in the presence of an agonist (SAL) at a fixed sub-saturating concentration (1 µM) and NTPs at increasing concentrations.

NTPs decrease the affinity of the β_2AR for SAL and, as a result, increase [³H]DHA binding (20, 23, 26). GTP potently and efficiently disrupted the ternary complex in membranes expressing β_2AR-G_αS (EC₅₀, 180 nM; 95% c. i., 110-310 nM), β_2AR-G_αL (EC₅₀, 90 nM; 95% c. i., 50-150 nM) and β_2AR-G_αolf (EC₅₀, 590 nM; 95% c. i., 310-1100 nM) (Fig. 2). The efficacy of UTP at disrupting the ternary complex in β_2AR-G_α fusion proteins amounted to 53-61% of the efficacy of GTP. UTP was far less potent than GTP in membranes expressing β_2AR-G_αS (EC₅₀, 30 µM; 95% c. i., 9-101 µM), β_2AR-G_αL (EC₅₀, 12 µM; 95% c. i., 2-69 µM) and β_2AR-G_αolf (EC₅₀, 19 µM; 95% c. i., 7-62 µM). The efficacy of CTP at disrupting the ternary complex amounted to less than 25% of the efficacy of GTP, preventing us from calculating meaningful EC₅₀ values for CTP. Of particular importance was the finding that ATP did not disrupt the ternary complex in membranes expressing β_2AR-G_αS or β_2AR-G_αolf.
To corroborate the conclusion that the order of efficacy of NTPs at disrupting the ternary complex is GTP > UTP > CTP, we determined the extent of ternary complex formation in β2AR-GsαL by competing [3H]DHA binding by the full agonist ISO in the absence and presence of NTPs at a saturating concentration (1 mM). Fig. 3 shows the agonist-competition curves and Table I summarizes the non-linear regression analysis of these binding experiments. As reported before (20, 24), ISO inhibited [3H]DHA binding according to a biphasic function, with ~35% of the β2ARs being in a state of high agonist-affinity (Fig. 3A). GTP (1 mM) substantially shifted the ISO-competition curve to the right and converted the competition curve into a steep monophasic function, reflecting complete disruption of the ternary complex. In contrast, in the presence of 1 mM UTP, the agonist-competition curve was still biphasic (~10% high-affinity agonist binding remaining), and the low-affinity component of the competition curve was not shifted as far to the right as with GTP (Fig. 3B). These data confirm the notion that UTP disrupted the ternary complex only incompletely. CTP had only minimal inhibitory effects on ternary complex formation in membranes expressing, β2AR-GsαL i.e. the fraction of high-affinity binding sites was not decreased compared to control conditions (Fig. 3C and Table I). In addition, CTP increased the $K_i$ values for ISO only slightly relative to control. Taken together, the analysis of the effects of NTPs on ternary complex formation in membranes expressing β2AR-Gsα fusion proteins clearly showed that the order of efficacy is GTP > UTP > CTP > ATP (ineffective).

**Competition of β2AR-Gsα-catalyzed [γ-32P]GTP hydrolysis by GTP, UTP and CTP.**
In order to address the question whether UTP and CTP bind to the nucleotide-binding pocket of \( \alpha \), we stimulated \( \alpha \)-catalyzed \([\gamma-32P]GTP\) hydrolysis in \( \alpha \) fusion proteins by ISO and competed [\( \gamma-32P \)]GTP hydrolysis with unlabeled NTPs. For all three \( \alpha \) fusion proteins we obtained monophasic competition curves, indicating that [\( \gamma-32P \)]GTP and NTPs competed for binding to a single site (Fig. 4). The \( K_i \) values for GTP at the three fusion proteins ranged between 210-490 nM. The \( K_i \) values for UTP ranged between 170-910 \( \mu \)M, and those for CTP ranged between 3.4-4.4 mM. Thus, the order of affinity of \( \alpha \)S, \( \alpha \)L and \( \alpha \)olf for NTPs is GTP > UTP > CTP.

We also addressed the question whether \( \beta_2AR-\alpha \) fusion proteins hydrolyze [\( \gamma-32P \)]UTP and [\( \gamma-32P \)]CTP. However, despite using high amounts of [\( \gamma-32P \)]NTPs per tube (up to 2.5 \( \mu \)Ci/tube) and low basal UTPase- and CTPase activities in Sf9 membranes, we could not detect ISO-stimulated UTP- and CTP hydrolysis in Sf9 membranes expressing either of the three fusion proteins using UTP- and CTP concentrations between 0.1-100 \( \mu \)M (data not shown).

**UTP, CTP and GTP differentially support AC activation by \( \beta_2AR-\alpha S \), \( \beta_2AR-\alpha L \) and \( \beta_2AR-\alpha olf \) expressed in Sf9 membranes.** In the absence of GTP, UTP or CTP, *i.e.* in the presence of ATP alone, membranes expressing \( \beta_2AR-\alpha S \) exhibit a higher basal AC activity than membranes expressing \( \beta_2AR-\alpha L \) and \( \beta_2AR-\alpha olf \), and ISO efficiently reduced AC activity in membranes expressing \( \beta_2AR-\alpha S \) but not in membranes expressing \( \beta_2AR-\alpha L \) or \( \beta_2AR-\alpha olf \) (compare Figs. 5A, 5D and 5G). These differences are explained by the fact that
\( \beta_2 \text{AR-G}_s \alpha S \) possesses a higher GDP-affinity than \( \beta_2 \text{AR-G}_s \alpha L \) and \( \beta_2 \text{AR-G}_\text{olf} \) (20, 23). Accordingly, ISO efficiently promotes GDP-dissociation from \( \text{G}_s \alpha S \) and, thereby, reduces the concentration of \( \text{G}_s \alpha S \)-GDP. Since \( \text{G}_s \alpha \)-GDP is more efficient at activating AC than nucleotide-free \( \text{G}_s \alpha \), ISO reduces AC activity in the absence of GTP, UTP or CTP. In membranes expressing either of the three fusion proteins, NTPs supported ISO-stimulation of AC in the order of efficacy GTP ~ UTP > CTP, and UTP and CTP were considerably less potent than GTP (Figs. 5A-5I). Although there are certain differences in the regulation of AC by NTPs between S49- and Sf9 membranes with respect to the effect of ISO in the presence of ATP alone and the stimulatory effects of NTPs on basal AC activity, the overall pattern of AC regulation by NTPs is similar. Specifically, the order of efficacy of NTPs in all systems is GTP > UTP > CTP, and pyrimidine nucleotides are less potent than GTP. Fusion proteins facilitate detection of UTP- and CTP effects on \( \text{G}_s \) since the relative efficacies of CTP and particularly UTP with respect to ISO-stimulation of AC are larger with \( \beta_2 \text{AR-G}_s \alpha \) fusion proteins than with S49 membranes.

We observed certain differences in the effects of UTP and CTP at the various fusion proteins in the AC assay. Specifically, the maximum agonist-stimulated AC activities achieved with CTP in membranes expressing \( \beta_2 \text{AR-G}_s \alpha S \) and \( \beta_2 \text{AR-G}_s \alpha L \) amounted to ~65% of that obtained with UTP, whereas in membranes expressing \( \beta_2 \text{AR-G}_\text{olf} \), the maximum AC activity achieved with CTP amounted to only ~40% of the activity obtained with UTP (compare Figs. 5A with 5B, Figs. 5D with 5E, and Figs. 5G with 5H, respectively). There were also differences in
the relative stimulatory effects of ISO in the individual fusion proteins in the presence of different NTPs. In membranes expressing $\beta_2$AR-Gs$\alpha$S, the maximum stimulatory effects of ISO amounted to 74% (UTP), 55% (CTP) and 57% (GTP), respectively. The corresponding values for $\beta_2$AR-Gs$\alpha$L were 74% (UTP), 115% (CTP) and 60% (GTP), respectively. For $\beta_2$AR-Gsolf the stimulations were 195% (UTP), 77% (CTP) and 206% (GTP), respectively.

**NDPK activity in S49- and Sf9 membranes.** In S49 membranes, GTP was the most potent (EC$_{50}$ ~ 1 $\mu$M) but least efficient NDPK substrate (Fig. 6A). UTP and ATP were less potent (EC$_{50}$ ~ 5 $\mu$M) but more efficient as phosphoryl group donors than GTP. CTP was less potent (EC$_{50}$ ~ 10 $\mu$M) than UTP and ATP at serving as NDPK substrate, but similarly efficient. While GTP exhibited similar potency (EC$_{50}$ ~1 $\mu$M) and relative efficacy as NDPK substrate in Sf9- and S49 membranes (compare Figs. 6A and 6B), the properties of ATP, UTP and CTP were different in the two systems. The order of efficacy of NTPs in Sf9 membranes was ATP > UTP > CTP, and the order of potency was ATP (EC$_{50}$ ~ 5 $\mu$M) > UTP (EC$_{50}$ ~10 $\mu$M) > CTP (EC$_{50}$ ~ 20 $\mu$M).

**Molecular modeling of the interactions of GTP, UTP and CTP with Gs$\alpha$.** In order to provide an explanation for the different affinities and efficacies of NTPs at Gs$\alpha$-proteins we constructed models for the GTP-, CTP- and UTP complexes of Gs$\alpha$ based on the structure of the Gs$\alpha$Mg$^{2+}$GTP$\gamma$S complex (31) and subjected the complexes to potential energy minimization. However, none of the energy-minimized Gs$\alpha$Mg$^{2+}$NTP models differed substantially from the
corresponding $G_\alpha^\alpha''\text{Mg}^{2+}''\text{GTP}\gamma S$ complex (0.15Å - 0.18Å root mean square deviation between pairs of corresponding C$\alpha$ atoms) (data not shown). Furthermore, the position, orientation and conformation of NTP$''\text{Mg}^{2+}$ were unaltered after energy minimization. Hence, energy minimization experiments did not provide an explanation for the differences in affinity and efficacy of NTPs at $G_\alpha^\alpha$.

We then decided to investigate the molecular models of the GTP-, UTP- and CTP complexes of $G_\alpha^\alpha$ by molecular dynamics simulation. After 10 ps simulation at 300 K, the $G_\alpha^\alpha''\text{Mg}^{2+}''\text{GTP}$-, $G_\alpha^\alpha''\text{Mg}^{2+}''\text{UTP}$- and $G_\alpha^\alpha''\text{Mg}^{2+}''\text{CTP}$ models diverged from the corresponding $G_\alpha^\alpha''\text{Mg}^{2+}''\text{GTP}\gamma S$ complex by 0.94 Å, 0.95 Å, and 1.15 Å, root mean square, respectively, over all C$\alpha$ pairs. The deviations were smaller (0.6-0.8 Å) for the set limited to C$\alpha$ atoms within 6 Å of the NTPs. The models did not change further after 500 cycles of energy minimization. All of the guanine ring-$G_\alpha^\alpha$ hydrogen bonds and van der Waals interactions observed in the structure of the $G_\alpha^\alpha''\text{Mg}^{2+}''\text{GTP}\gamma S$ complex (31, 37) were retained in the model of the $G_\alpha^\alpha''\text{Mg}^{2+}''\text{GTP}$ complex after molecular dynamics simulation (Fig. 7A), although the ribosyl group shifted ~1 Å. Likewise, the molecular geometry of the triphosphate moiety and its interaction with Mg$^{2+}$ were unperturbed, indicating that the simulation with GTP preserves experimentally observed properties of the interaction of $G_\alpha^\alpha$ with GTP$\gamma S$.

In contrast, the molecular dynamics simulations of the $G_\alpha^\alpha''\text{Mg}^{2+}''\text{UTP}$- and
G$_{\alpha}'$Mg$^{2+}$CTP complexes had different outcomes. The position and orientation of the uracil ring of UTP remained near the starting position after 10 ps of dynamics simulation (Fig. 7B). The N3 imine stayed in position to donate a hydrogen bond to the O$\delta$2-carboxylate oxygen of Asp 295, and the hydrogen bond network involving the 2-keto oxygen, the imposed water molecule and O$\delta$1 of Asp 295 were intact, although with sub-optimal geometry. In contrast, the cytosine ring of CTP rotated ~10° from its initial orientation (Fig. 7C). The rotation was accommodated, without substantial change in ribose ring pucker or in the position of the triphosphate moiety, by a 30° rotation around the ribosyl C(5')-O(5') bond. By so rotating, the cytosine ring avoided steric conflict with the side chain of Asn 292 (G$_{\alpha}$L) and Asn 277 (G$_{\alpha}$S) (not shown). Val 367 (G$_{\alpha}$L) and Val 352 (G$_{\alpha}$S) also moved away from the cytosine ring (Fig. 7C). As another consequence of the rotation, the cytosine C(4) exocyclic amine was in position to donate a hydrogen bond to Asp 295; this interaction is not possible in the starting structure. However, neither the N(3) imine nor the C(2) exocyclic keto oxygen atom of the cytosine ring were within hydrogen bonding distance of the imposed water molecule, which remained bonded to Asp 295.

**Discussion**

Transphosphorylation of GDP to GTP cannot explain the effects of UTP and CTP on G$_{\alpha}$-proteins. UTP and CTP disrupt the ternary complex between the $\beta_2$AR and G$_{\alpha}$-proteins (Figs. 2 and 3 and Table I) and support AC activation by unmodified and ADP-ribosylated G$_{\alpha}$ (Figs. 1 and 5). An explanation for those observations could be transphosphorylation of endogenous GDP
to GTP by UTP or CTP via NDPK (12, 13, 38), but several findings argue against this notion. First, the concentration of GsαS-GDP in washed Sf9 membranes is higher than the concentration of GsαL-GDP and Gαolf-GDP (20, 23). Thus, for transphosphorylation, membranes expressing β2AR-GsαS should have been a considerably more efficient system than membranes expressing β2AR-GsαL and β2AR-Gαolf. However, UTP and CTP were not more efficient at disrupting the ternary complex and supporting AC activation in membranes expressing β2AR-GsαS than in membranes expressing β2AR-GsαL and β2AR-Gαolf (Figs. 2 and 5). Second, a given NTP should have had the same relative efficacies in the AC- and high-affinity agonist binding assays. However, UTP was less efficient than GTP at disrupting the ternary complex in β2AR-Gsα fusion proteins (Figs. 2 and 3 and Table I). In contrast, UTP was equally efficient as GTP at supporting ISO-stimulation of AC in Sf9 membranes expressing β2AR-Gsα fusion proteins (Fig. 5). Third, in S49- and Sf9 membranes, ATP was a potent (EC50 ~5 µM) and efficient phosphoryl group donor for GTP formation (Fig. 6), but we failed to detect a stimulatory effect of ISO on AC activity in S49- and Sf9 membranes in the presence of ATP (40 µM) (Figs. 1 and 5). Fourth, CTP and UTP were similarly efficient NDPK substrates in S49 membranes (Fig. 6A), but CTP was much less efficient than UTP at supporting AC activation in this system (Figs. 1A and 1B). Finally, in S49- and Sf9 membranes UTP was a more efficient phosphoryl group donor for [3H]GTP formation than GTP (Fig. 6), but UTP was not more efficient than GTP with respect to disruption of the ternary complex and AC activation.

Evidence that GTP, UTP and CTP stabilize distinct conformations of Gsα. We then
considered the hypothesis that UTP and CTP exert their effects on AC and ternary complex formation directly by binding to the nucleotide-binding pocket of Gₛα. Indeed, UTP and CTP have already been shown to bind with low affinity to various G-proteins including Gₛ (5-8). In agreement with the results of the earlier studies, our GTPase competition studies with β₂AR-Gₛα fusion proteins showed that NTPs bind to GₛαS, GₛαL and Gₛαlf in the order of affinity GTP > UTP > CTP (Fig. 4). We noted that the apparent affinities of UTP and CTP in the agonist binding- and AC studies with fusion proteins (Figs. 2 and 5) were considerably higher than in the GTPase competition studies (Fig. 4). An important difference between these experiments is that the GTPase studies were conducted in the presence of GTP, whereas the agonist binding- and AC studies were conducted in the absence of GTP (see Experimental Procedures). These data raise the intriguing hypothesis that in the presence of GTP, access of UTP and CTP to Gₛα is restricted. Evidence for restricted access of nucleotides to G-proteins was already obtained in a previous study (39).

If the NTPs studied had stabilized the same conformation in Gₛα we would have expected NTPs to exhibit the same maximum effects on ternary complex formation and AC activation at saturating concentrations. However, this was clearly not the case. The overall order of efficacy of NTPs with respect to these parameters was GTP > UTP > CTP (Figs. 1-3 and 5). The enhancing effects of CTX on maximum AC activation by NTPs (Fig. 1) are particularly intriguing. CTX unmask a strong stimulatory effect of GTP on AC by blocking the GTPase activity of Gₛα (1, 27, 35). Our present data are in agreement with this concept (Fig. 1C). CTX also greatly enhanced the stimulatory effects of UTP and CTP on AC activity (Figs. 1A and 1B),
suggesting that Gsα hydrolyzes UTP and CTP as well. However, we could not obtain evidence for the presence of UTPase- and CTPase activity in β2AR-Gsα-proteins although Sf9 membranes expressing these proteins are highly sensitive systems to detect NTPase activity (24, 26). These data imply that the mechanism of deactivation of Gsα-UTP and Gsα-CTP is NTP dissociation rather than NTP hydrolysis. Our data indicate that CTX-catalyzed ADP-ribosylation induces a conformational change in Gsα that is independent of the well-established GTPase inhibition and allows UTP and CTP to interact more productively with the G-protein. In support of this hypothesis is the fact that CTX-catalyzed ADP-ribosylation of Gsα increased the apparent affinity of the G-protein for UTP (Fig. 1A). In contrast to UTP, we did not observe an increase of affinity of Gsα for GTP following CTX-catalyzed ADP-ribosylation (Fig. 1C).

Taken together, our data indicate that GTP, UTP and CTP interact with Gs-proteins in non-identical fashions.

The molecular dynamics simulations are consistent with our experimental data. Specifically, the simulation experiments indicate that CTP binds with lower affinity to Gsα than UTP because optimal interactions require a conformational change in the protein with no net gain in stabilizing interactions relative to UTP (Fig. 7). In fact, CTP binds to Gs-proteins with lower affinity than UTP (Fig. 4). In addition, UTP and CTP are expected to bind to Gsα with lower affinity than GTP because they form fewer stabilizing hydrogen bonds to the G-protein. In particular, hydrogen bond analogous to that between the guanine N(7) and Asn 292 (31, 37, 40) cannot be formed with uracil and cytosine. The experimental data are in agreement with the modeling
Compared to CTP, UTP is more easily accommodated within the binding site of Gsα and forms more hydrogen bonds with the protein, assuming the participation of a water molecule captured from the solvent. The differences in interactions of GTP, UTP and CTP with Gsα could be interpreted in such a way that each of the NTPs stabilizes a distinct conformation of Gsα or that certain NTPs stabilize less productive states of the G-protein than GTP. Specifically, Gsα-GTP possesses a conformation that is highly efficient at disrupting the high-affinity interaction with the agonist-occupied β2AR and at activating AC, whereas Gsα-CTP possesses a conformation that is inefficient in these regards. Of particular interest, Gsα-UTP possesses a conformation that is similarly efficient as Gsα-GTP at activating AC (Fig. 5) but less efficient than Gsα-GTP at disrupting the high-affinity interaction with the agonist-occupied β2AR (Figs. 2 and 3 and Table I). In agreement with our present data on Gs, guanine nucleotides are more efficient at disrupting the complex between photoexcited rhodopsin and transducin than the corresponding uracil nucleotides (5). These data indicate that G-proteins do not simply act as on/off switches but rather exist in states with different functional capacities that are differentially stabilized by NTPs.

Although the overall pattern of effects of GTP, UTP and CTP at the various β2AR-Gsα fusion proteins was similar (Figs. 2 and 5), we noted that CTP was particularly effective at supporting ISO-stimulation of AC in Sf9 membranes expressing β2AR-GsαL (Fig. 5E). These data indicate that there are subtle differences in the conformations of GsαS-CTP, GsαL-CTP
and G\(_{\alpha}\)olf-CTP, exhibiting different efficacies at activating AC relative to the corresponding conformations of G\(_{3}\)\(\alpha\)-GTP and G\(_{3}\)\(\alpha\)-UTP. Studies with hydrolysis-resistant phosphorothioate analogs of UTP and CTP will be important to substantiate the concept of distinct active G\(_{3}\)\(\alpha\) conformations.

**Physiological and pathological relevance of G-protein activation by pyrimidine nucleotides.** Differential G\(_{3}\)-activation by NTPs was observed in Sf9 membranes expressing \(\beta_2\)AR-G\(_{3}\)\(\alpha\) fusion proteins (Figs. 2, 3 and 5) and in a physiological system, the S49 membranes. The effects of UTP and CTP on fused and non-fused G\(_{3}\)-proteins were observed at concentrations e 10 \(\mu\)M (Figs. 1-5). The bulk intracellular UTP- and CTP concentrations in various neuronal and astroglial cells may be as high as \(~10\) nmol/mg of protein and may exceed the intracellular GTP concentrations by up to \(~\)two-fold (41-44). Thus, it is likely that at least in some cellular systems the intracellular UTP- and CTP concentrations are sufficiently high for allowing these NTPs to activate G-proteins under physiological conditions, provided that the access of UTP and CTP to G\(_{3}\) is not severely restricted.

CTX-catalyzed ADP-ribosylation substantially increases both the potency and efficacy of UTP at activating AC (Fig. 1A). This effect of CTX appears to be due to a conformational change in G\(_{3}\)\(\alpha\) rather than due to GTPase/NTPase inhibition. In other words, CTX-catalyzed ADP-ribosylation may optimize the conformation of G\(_{3}\)\(\alpha\) to bind UTP. Thus, enhanced G\(_{3}\) activation by UTP may contribute to the clinical symptoms of cholera.

Lesch-Nyhan syndrome is caused by a defect of hypoxanthine-guanine
phosphoribosyltransferase, a key enzyme in the purine salvage pathway (45). This enzyme defect results in a decrease in intracellular GTP concentration and increases in intracellular UTP- and CTP concentrations (43, 44, 46-48). Intriguingly, patients with Lesch-Nyhan syndrome have alterations in the regulation of multiple neurotransmitter systems including the adrenergic system (49-54) and severe neuropsychiatric abnormalities (45). Given the fact that Lesch-Nyhan syndrome is associated with changes in intracellular GTP-, UTP- and CTP concentrations and that these NTPs differ in their effects on G-protein-mediated signaling, one could envisage that GPCR/G-protein/effecter coupling is altered in Lesch-Nyhan syndrome, resulting in impaired development and function of the central nervous system.

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Footnotes

1The abbreviations used are: AC, adenylyl cyclase; β2AR, β2-adrenoceptor; β2AR-Golf, fusion protein containing the β2AR and Golf; β2AR-GsL, fusion protein containing the β2AR and the long splice variant of Gsα; β2AR-GsS, fusion protein containing the β2AR and the short splice variant of Gsα; c. i., confidence interval; CTX, cholera toxin; [3H]DHA, [3H]dihydroalprenolol; EC50, concentration causing half-maximal stimulation; Gα, unspecified G-protein α-subunit; GPCR, G-protein-coupled receptor; GTPγS, guanosine 5-O-(3-thiotriphosphate); ISO, (-)-isoproterenol; NDPK, nucleoside diphosphokinase; NTP, nucleoside 5'-triphosphate; SAL, salbutamol; S49 cells, S49 wild-type lymphoma cells.

2personal communication.
Figure legends

Figure 1. Effects of UTP, CTP and GTP on AC activity in S49 wild-type lymphoma membranes.

AC activity in S49 wild-type (wt) lymphoma membranes was determined as described in Experimental Procedures. Reaction mixtures contained S49 membranes (20-50 µg of protein per tube) and NTPs at the concentrations indicated on the abscissa with solvent (basal) (i) or with 10 µM ISO ( ). 10^-9 designates the absence of added UTP, CTP or GTP. In additional experiments, reaction mixtures contained membranes from S49 cells that had been treated with CTX (1 µg/ml) for 24 h before membrane preparation. The experiments with membranes from CTX-treated cells were only conducted with solvent (basal) (²). The basal AC activity in membranes from untreated S49 cells in the absence of NTP and ISO amounted to 0.48 ± 0.51 pmol/mg/min and was set 0%. The AC activity in untreated S49 membranes in the presence of 10 µM GTP and 10 µM ISO was set 100% (control) and amounted to 2.36 ± 0.75 pmol/mg/min. All other AC activities were referred to those calibration points. Data were analyzed by non-linear regression analysis and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 6-8 experiments performed in duplicates.
Figure 2. Effects of UTP, CTP, GTP and ATP on ternary complex formation in Sf9 membranes expressing \( \beta_2 \text{AR-G}_S \alpha_S \), \( \beta_2 \text{AR-G}_S \alpha_L \) or \( \beta_2 \text{AR-G}_\alpha \text{olf} \): Concentration/response curves for NTPs. \[^3\text{H}]\text{DHA} \) binding in Sf9 membranes was performed as described in *Experimental Procedures*. Reaction mixtures contained Sf9 membranes (20 µg of protein per tube) expressing \( \beta_2 \text{AR-G}_S \alpha_S \), \( \beta_2 \text{AR-G}_S \alpha_L \) or \( \beta_2 \text{AR-G}_\alpha \text{olf} \), 1 nM \[^3\text{H}]\text{DHA}, 1 \mu\text{M SAL} \) and NTPs (\( \bar{I}, \text{GTP} \); \( \bar{I}, \text{UTP} \); \( \bar{I}, \text{CTP} \); \( \bar{I}, \text{ATP} \)) at the concentration indicated on the abscissa. 10\(^{-10}\) designates the absence of added NTP. \( A \), membranes expressing \( \beta_2 \text{AR-G}_S \alpha_S \) at 2.6-4.4 pmol/mg; \( B \), membranes expressing \( \beta_2 \text{AR-G}_S \alpha_L \) at 3.0-4.8 pmol/mg; \( C \), membranes expressing \( \beta_2 \text{AR-G}_\alpha \text{olf} \) at 3.3-4.2 pmol/mg. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 3-5 independent experiments performed in triplicate.

Figure 3. Effects of UTP, CTP and GTP on ternary complex formation in Sf9 membranes expressing \( \beta_2 \text{AR-G}_S \alpha_L \): Concentration/response curves for ISO. \[^3\text{H}]\text{DHA} \) binding in Sf9 membranes was performed as described in *Experimental Procedures*. Reaction mixtures contained Sf9 membranes (20 µg of protein per tube) expressing \( \beta_2 \text{AR-G}_S \alpha_L \) at 4.5-7.4 pmol/mg. Reaction mixtures additionally contained ISO at the concentrations indicated on the abscissa in the presence of solvent (control) (\( \bar{E} \)) or various NTPs (\( \bar{I} \)) at a concentration of 1 mM each. 10\(^{-10}\) designates the absence of ISO. Data were analyzed for best fit to monophasic or biphasic competition isotherms (\( F \)-test). Data shown are the means ± SD of 5 independent
experiments performed in triplicates. The dashed lines without data points shown in panels B and C represent the competition isotherms in the presence of solvent (control) and 1 mM GTP depicted in panel A to illustrate the relative position of the isotherms in the presence of UTP and CTP.

**Figure 4. Competition of [γ-32P]GTP hydrolysis in Sf9 membranes expressing β2AR-Gₛαₛ, β2AR-Gₛα₉ or β2AR-Gₒلد by GTP, UTP and CTP.** GTPase activity in Sf9 membranes was determined as described in *Experimental Procedures*. Reaction mixtures contained Sf9 membranes (10 μg of protein per tube) expressing various fusion proteins, 30 nM [γ-32P]GTP, 10 μM ISO and unlabeled GTP (Í), UTP ( ) or CTP (²) at increasing concentrations. 10⁻⁸ designates the absence of unlabeled UTP, CTP or GTP. GTPase activities in the absence of competitor (control) were as follows. β2AR-Gₒلد (expressed at 13.7 pmol/mg), 8.5 ± 0.4 pmol/mg/min; β2AR-Gₛαₛ (expressed at 4.0 pmol/mg/min), 3.3 ± 0.3 pmol/mg/min; β2AR-Gₛα₉ (expressed at 6.0 pmol/mg), 5.2 ± 0.6 pmol/mg/min. These GTPase activities were defined as 100% (control). Competition curves were extended until complete inhibition of GTP hydrolysis in Sf9 membranes. This point was defined as 0%. All other data points were referred to those calibration points. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 3 independent experiments performed in duplicate.
Figure 5. Effects of UTP, CTP and GTP on AC activity in Sf9 membranes expressing β2AR-GsαS, β2AR-GsαL or β2AR-Golf. AC activity in Sf9 membranes was determined as described in Experimental Procedures. Reaction mixtures contained Sf9 membranes (20 µg protein per tube) expressing β2AR-GsαS, β2AR-GsαL or β2AR-Golf, and NTPs at the concentrations indicated on the abscissa with solvent (basal) (¡) or with 10 μM ISO ( ). 10^-9 designates the absence of added UTP, CTP or GTP. A-C, membranes expressing β2AR-GsαS at 2.3-2.6 pmol/mg; D-F, membranes expressing β2AR-GsαL at 4.8-5.4 pmol/mg; G-I, membranes expressing β2AR-Golf at 3.6-4.1 pmol/mg. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± SD of 3-5 independent experiments performed in duplicate. Note that the scale of the ordinate in panels A-F is different from the scale in panels G-I. The different scales were chosen to facilitate comparison of the relative effects of NTPs at the various fusion proteins. Differences in the absolute efficacies of β2AR-Gsα fusion proteins at activating AC were reported before (20, 23).
Figure 6. \[^3\text{H}\]GTP formation from \[^3\text{H}\]GDP and NTP in S49 wild-type lymphoma membranes and Sf9 membranes. \[^3\text{H}\]GTP formation from \[^3\text{H}\]GDP and NTP was determined as described in Experimental Procedures. Reaction mixtures contained S49 wild-type (wt) lymphoma membranes (5.0-10.0 µg of protein per tube) (A) or membranes from Sf9 cells infected with β2AR-\(\text{G}_{\alpha}\)-olf baculovirus (0.5-1.0 µg of protein per tube) (Sf9 inf., B), 0.5 µM carrier-free \[^3\text{H}\]GDP and NTPs at the concentrations indicated on the abscissa. Data shown are the means ± SD of 3 experiments performed in duplicates. Note that the scale of the ordinate in panels A and B is different. The different scales were chosen to facilitate comparison of the relative effects of NTPs in the two membrane systems.

Figure 7. Three-dimensional model of the interaction of GTP, UTP and CTP with the nucleotide-binding pocket of \(G_s\). Molecular dynamics simulations were performed as described in Experimental Procedures. The environments of the NTP bases of \(G_s\)\(\text{Mg}^2+\)NTP complexes after molecular dynamics simulation are shown. A, \(G_s\)\(\text{Mg}^2+\)GTP complex. For clarity, Asn 292 (\(G_s\)\(\alpha\)L), which forms a hydrogen bond with the N(7) imine (31), is not shown. B, \(G_s\)\(\text{Mg}^2+\)UTP complex. The side chain of Val 367 (\(G_s\)\(\alpha\)L) is not shown for clarity; C, \(G_s\)\(\text{Mg}^2+\)CTP complex. Carbon atoms of \(G_s\) are colored orange, carbon atoms of the NTPs are colored green. Nitrogen and oxygen atoms in the protein and NTPs are colored blue and red, respectively. Selected hydrogen bonds are depicted as green dotted lines. The isolated red sphere
represents the included water molecule. In panels B and C, the $G_\alpha^{\text{Mg}^{2+}\text{-UTP}}$ and $G_\alpha^{\text{Mg}^{2+}\text{-CTP}}$ complexes, respectively, are superimposed on the $G_\alpha^{\text{Mg}^{2+}\text{-GTP}}$ complex that is shown as charcoal stick model.
Table I. Non-linear regression analysis of the effects of GTP, UTP and CTP on ternary complex formation in Sf9 membranes expressing β2AR-GsαL

| Addition      | $K_h$ (nM) | $K_l$ (nM) | $R_h$ (%) |
|---------------|------------|------------|-----------|
| Solvent (control) | 0.7 (0.3-1.3) | 91 (70-120) | 34.3 (29.4-39.2) |
| GTP (1 mM)    | -          | 180 (150-200) | -         |
| UTP (1 mM)    | 0.01 (0.005-1.8) | 100 (80-120) | 10.7 (3.4-18.0) |
| CTP (1 mM)    | 2.8 (1.0-8.1) | 130 (70-250) | 39.5 (24.6-54.4) |

[$^3$H]DHA binding in Sf9 membranes was performed as described in Experimental Procedures. Reaction mixtures contained Sf9 membranes (20 µg of protein per tube) expressing β2AR-GsαL at 4.5-7.4 pmol/mg. Reaction mixtures additionally contained ISO at the concentrations indicated on the abscissa in the presence of solvent (control) or various NTPs at a concentration of 1 mM each. The data shown in Fig. 3 were analyzed for best fit to monophasic or biphasic competition isotherms ($F$-test). Data shown are the means of 5 independent experiments performed in triplicates. Numbers in parentheses represent the 95% confidence intervals. $K_h$ and $K_l$ designate the dissociation constants for the high- and low-affinity state of β2AR-GsαL, respectively. $R_h$ (%) indicates the percentage of high-affinity binding sites.
Distinct interactions of GTP, UTP and CTP with Gs-proteins
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