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Functionally Nonequivalent Interactions of Guanosine 5′-Triphosphate, Inosine 5′-Triphosphate, and Xanthosine 5′-Triphosphate with the Retinal G-Protein, Transducin, and with G_i-Proteins in HL-60 Leukemia Cell Membranes

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ABSTRACT. G-proteins mediate signal transfer from receptors to effector systems. In their guanosine 5′-triphosphate (GTP)-bound form, G-protein α-subunits activate effector systems. Termination of G-protein activation is achieved by the high-affinity GTPase [E.C. 3.6.1.−] of their α-subunits. Like GTP, inosine 5′-triphosphate (ITP) and xanthosine 5′-triphosphate (XTP) can support effector system activation. We studied the interactions of GTP, ITP, and XTP with the retinal G-protein, transducin (TD), and with G-proteins in HL-60 leukemia cell membranes. TD hydrolyzed nucleoside 5′-triphosphates (NTPs) in the order of efficacy GTP > ITP > XTP. NTPs eluted TD from rod outer segment disk membranes in the same order of efficacy. ITP and XTP competitively inhibited TD-catalyzed GTP hydrolysis. In HL-60 membranes, the chemoattractants *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and leukotriene B_4 (LTB_4) effectively activated GTP and ITP hydrolysis by G_i-proteins. fMLP and LTB_4 were at least 10-fold more potent activators of ITPase than of GTPase. Complement C5a effectively activated the GTPase of G_i-proteins but was only a weak stimulator of ITPase. The potency of C5a to activate GTP and ITP hydrolysis was similar. The fMLP-stimulated GTPase had a lower K_m value than the fMLP-stimulated ITPase, whereas the opposite was true for the V_{max} values. fMLP, C5a, and LTB_4 did not stimulate XTP hydrolysis. Collectively, our data show that GTP, ITP, and XTP bind to G-proteins with different affinities, that G-proteins hydrolyze NTPs with different efficacies, and that chemoattractants stimulate GTP and ITP hydrolysis by G_i-proteins in a receptor-specific manner. On the basis of our results and the data in the literature, we put forward the hypothesis that GTP, ITP, and XTP act as differential signal amplifiers and signal sorters at the G-protein level. BIOCHEM PHARMACOL **54**;5:551–562, 1997.

KEY WORDS. GTPase; ITPase; XTPase; transducin; G_i-proteins; chemoattractants

G-proteins are membrane-attached proteins consisting of an α-subunit and a βγ-complex, and mediate coupling of agonist-occupied heptahelical receptors to cellular effector systems. In their resting state, α-subunits are GDP liganded. Interaction of an activated receptor with G-proteins leads to the exchange of GDP for guanosine 5′-triphosphate (GTP).[§] GDP release is the rate-limiting step of G-protein activation. Once they have bound GTP, G-protein α-subunits activate or inhibit effector systems (for review, see [1, 2]). Examples of G-protein–regulated effectors are adenylyl cyclase [E.C. 4.6.1.1.] (activated by G_s via numerous receptors), the retinal cGMP-degrading phosphodiesterase [E.C. 3.1.4.35.] (activated by transducin (TD) via rhodopsin) and phospholipase C-β [E.C. 3.1.4.10.] (activated by G_i-proteins via chemoattractant receptors in neutrophilic cells) (for review, see [1–4]). Termination of G-protein activation is achieved by the high-affinity GTPase [E.C. 3.6.1.−] of α-subunits that cleaves GTP to GDP and P_i (for review, see [1–4]).

Even in the early days of G-protein research, it was recognized that inosine 5′-triphosphate (ITP) can substitute for GTP in G-protein activation [5–10]. The chemical difference between the bases of GTP and ITP concerns substitution of C_2 of the purine ring. In guanine, C_2 is substituted with an amino group, whereas hypoxanthine,

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§ *Abbreviations:* fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GppNHp, guanosine 5′-[β,γ-imido]triphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-O-(3-thiotriphosphate); ITP, inosine 5′-triphosphate; LTB_4, leukotriene B_4; NDPK, nucleoside diphosphate kinase; NTP, nucleoside 5′-triphosphate; PTX, pertussis toxin; ROS, retinal rod outer segment; TD, transducin; TD_α, α-subunit of transducin; TD_β, β-subunit of transducin; XppNHp, xanthosine 5′-[β,γ-imido]triphosphate; XTP, xanthosine 5′-triphosphate; XTPγS, xanthosine 5′-O-(3-thiotriphosphate).

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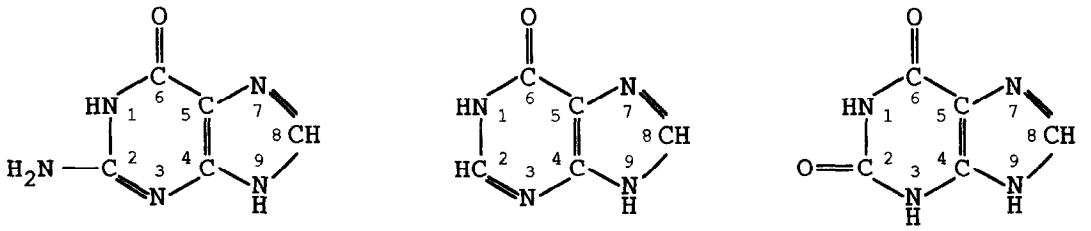

FIG. 1. Chemical structures of guanine, hypoxanthine, and xanthine. Guanine is the base present in GTP, hypoxanthine the base present in ITP, and xanthine is present in XTP. The amino group at C_2 of the purine ring in GTP is important for hydrogen bonding of the nucleotide with a highly conserved aspartic acid residue in G-protein α-subunits [11]. Omission of the amino group (in ITP) lowers the affinity of the nucleotide for the G-protein. Substitution of C_2 with a keto group (in XTP) further lowers the affinity of the nucleotide for α-subunits.

the base of ITP, bears a hydrogen at this position (Fig. 1). The crystal structure analysis of G-protein α-subunits revealed that the amino group of the guanine ring is crucial for hydrogen bonding with a highly conserved aspartic acid residue of the α-subunits [11]. If the amino group of GTP is missing, nucleotide binding to G-protein α-subunits is expected to be of lower affinity. It has been shown that ITP supports activation (via G_s) and inhibition (via G_i-proteins) of adenylyl cyclase [5–9], activation of retinal phosphodiesterase [10], and secretion in permeabilized mast cells, HL-60 leukemia cells, and chromaffin cells [12–14]. Moreover, ITP can disrupt high-affinity agonist binding to various G-protein–coupled receptors [15, 16], and inhibits receptor-stimulated high-affinity GTPase [7]. ITP competes with [^{35}S]guanosine 5′-O-(3-thiotriphosphate) ([^{35}S]GTPγS) for binding to purified G-proteins, including TD and G_s [17–20]. As predicted from the crystal structure of TDα [11], the affinity of ITP for G-protein α-subunits is substantially lower than that of GTP [17–20]. However, the reduced affinity of ITP to G-proteins as compared to GTP does not necessarily imply that the efficacy of ITP at maximally stimulatory concentrations to activate G-proteins is lower than that of GTP. For example, ITP supports thyrotropin-mediated AC activation in thyroid membranes more effectively than GTP [5], and ITP stimulates secretion in permeabilized cells more efficiently than GTP [13–15]. Ross *et al.* [8] suggested that G-proteins hydrolyze ITP with a lower V_{max} than GTP, but to the best of our knowledge, neither basal nor receptor agonist-stimulated ITP hydrolysis of purified G-proteins or G-proteins in native membranes has been reported so far.

Xanthosine 5′-triphosphate (XTP) is another GTP analogue. The base of XTP, xanthine, bears a keto group instead of an amino group at C_2 of the purine ring (see Fig. 1). By analogy to low-molecular mass GTP-binding proteins [21, 22], XTP is expected to possess an even lower affinity for G-protein α-subunits than GTP. There are few published data on the role of XTP in G-protein activation. As predicted, the affinity of XTP for G_s is lower than for ITP, as assessed by competition of [^{35}S]GTPγS binding [17]. Despite its low affinity to G-proteins, XTP can support adenylyl cyclase activation by receptor agonists [5, 6] and stimulate secretion in permeabilized cells [12–14, 23] even more effectively than GTP. The question of whether or not G-proteins hydrolyze XTP remains unanswered.

The aim of the present study was to analyze the interactions of GTP, ITP, and XTP with TD and G-proteins in differentiated HL-60 cells. In the latter cells, receptors for the chemoattractants *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), complement C5a, and leukotriene B_4 (LTB_4) couple to pertussis toxin (PTX)-sensitive G-proteins of the G_i family and mediate phospholipase C-β activation (for review, see [4]). We were specifically interested in treating the question as to whether ITP and XTP, like GTP, are hydrolyzed by G-proteins. Therefore, we measured GTP, ITP, and XTP hydrolysis catalyzed by soluble bovine TD and by G-proteins in HL-60 membranes.

MATERIALS AND METHODS

Materials

GTP, GDP, ITP, ADP, ATP, adenylyl 5′-[β,γ-imido]-triphosphate, and guanylyl 5′-[β,γ-imido]triphosphate (GppNHp), were from Boehringer–Mannheim, Mannheim, Germany. All other nucleotides were from Sigma Chemie, Deisenhofen, Germany. Nucleotides were of the highest purity available and were stored as stock solutions of 10 mM each in distilled water at −20°C. Nucleotide dilutions were prepared fresh daily in distilled water. [^{32}P]P_i (8500–9100 Ci/mmol) was obtained from Dupont/New England Nuclear, Bad Homburg, Germany.

[γ-^{32}P]Nucleoside 5′-Triphosphate ([γ-^{32}P]NTP) Synthesis

[γ-^{32}P]NTP synthesis was performed according to Walseth and Johnson [24]. The final concentration of ADP in reaction mixtures (50–80 μL) was 100 μM, and the final concentration of GDP, IDP, and XDP in reaction mixtures was 250 μM. Reactions were conducted for 15 min at 37°C and were terminated by the addition of ethanol (final concentration 50%, v/v). Conversion of NDPs to the corresponding [γ-^{32}P]NTPs was routinely >99.5%.

TD Preparation and TD Elution from Rod Outer Segment (ROS) Disk Membranes

Bovine ROS disk membranes were prepared according to Papermaster and Dreyer [25]. The membranes were washed six times with isotonic buffer (5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl, and 10 mM Tris/HCl, pH 7.5) and six times with hypotonic buffer (0.1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris/HCl, pH 7.5) [26]. TD was eluted from ROS disk membranes in hypotonic buffer substituted with 100 μM GTP. Membranes were centrifuged for 40 min at 4°C and 226,000 × g. The TD-containing supernatant fluid was carefully removed and concentrated in an Amicon concentration chamber (PM 10 membrane) (Amicon, Witten, Germany). Thereafter, TD was diluted with GTP-free hypotonic buffer [26]. This concentration/dilution procedure was repeated three times to remove free GTP. TD (50–60 μM) was stored in 50–100 μL aliquots at −80°C. Analysis of TD by SDS polyacrylamide gel electrophoresis and subsequent silver staining revealed a purity of >98% (data not shown).

To study TD elution with various NTPs, ROS disk membranes were subjected to six isotonic and six hypotonic washes as described above. ROS disk membranes were suspended at 3.0–3.7 mg of protein/mL in hypotonic buffer. Two hundred microliters of this suspension were placed into ultracentrifuge tubes containing stock solutions of NTPs to give the desired final concentrations. The contents of tubes were mixed, immediately placed into a Beckman TLA 100.1 rotor (Beckman, Munich, Germany) and centrifuged for 15 min at 365,000 × g in a Beckman TL-100 ultracentrifuge. All procedures were performed at 4°C. Thereafter, 100 μL of the supernatant fluid were removed, diluted with 200 μL of hypotonic buffer, and stored at −20°C until further analysis by SDS polyacrylamide electrophoresis and silver staining.

ATPase, GTPase, ITPase, and XTPase Assays

To determine the ATPase, GTPase, ITPase, and XTPase activity of TD, reaction mixtures (100 μL) contained TD (200–300 nM), unlabeled NTPs at various concentrations, the corresponding [γ-^{32}P]NTPs (0.5–1.5 μCi/tube), and a buffer consisting of 5 mM EDTA, 1 mM dithiothreitol, 0.2% (w/v) bovine serum albumin, and 50 mM triethanolamine/HCl, pH 7.4. To obtain blank values, tubes containing all components described above and hypotonic buffer instead of TD were processed in parallel with the TD-containing tubes. Reactions were conducted for 30–45 min at 25°C and were terminated by the addition of 900 μL of a stirred suspension consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Under these conditions, [^{32}P]P_i release was linear. Reaction mixtures were centrifuged for 40 min at 4°C at 2,500 × g. Seven hundred microliters of the supernatant fluid of reaction mixtures were removed, and Čerenkov radiation of [^{32}P]P_i was determined.

To determine the GTPase, ITPase, and XTPase activity in HL-60 membranes, membranes (800–1200 μg of protein) were suspended in 1.5 mL of 10 mM triethanolamine/HCl, pH 7.4, and centrifuged for 10 min at 30,000 × g at 4°C to remove endogenous nucleotides as far as possible. Thereafter, membranes were suspended at 500–1000 μg of protein/mL in 10 mM triethanolamine/HCl, pH 7.4, and immediately used for experiments. Reaction mixtures (100 μL) contained washed HL-60 membranes (5–10 μg of protein/tube), 0.5 μM [γ-^{32}P]NTPs (0.2–0.3 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenylyl 5′-[β,γ-imido]triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM dithiothreitol, and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. In some experiments, EDTA (5 mM) was included into reaction mixtures instead of $MgCl_2$/EGTA. Reactions were conducted for 15–20 min at 25°C. Under these conditions, [^{32}P]P_i release was linear. Stopping of reactions and extraction of [^{32}P]P_i was performed as described above. To study the substrate concentration dependency of NTP hydrolysis, reaction mixtures contained unlabeled NTPs at various concentrations and the corresponding [γ-^{32}P]NTPs (0.5 μCi/tube).

Miscellaneous

Protein was determined according to Peterson [27]. SDS polyacrylamide gel electrophoresis and silver staining of gels were performed as described [28, 29]. Immunoblotting with G-protein α_{common}- and β_{common}-peptide antisera was performed as described [30]. HL-60 cells were grown in suspension culture and differentiated with dibutyryl cAMP (0.2 mM) for 48 hr [31]. Treatment of cells with PTX was carried out as described [31]. HL-60 membranes were prepared as described [32]. Concentration–response curves were analyzed by nonlinear regression, using the Prism program (GraphPad, Prism, San Diego, CA).

RESULTS

To study GTP, ITP, and XTP hydrolysis by TD, we designed experimental conditions that could exclude a contribution of nucleoside diphosphate kinase [E.C. 2.6.4.6.] (NDPK) on NTP hydrolysis. Certain types of NDPKs can be closely associated with G-proteins and catalyze the formation of GDP to GTP by NTP in a strictly Mg^{2+}-dependent manner [33, 34]. Therefore, in the presence of Mg^{2+}, coupled NDPK/GTPase reactions may result in apparent NTPase activity [35]. To avoid this potential complication in the interpretation of the NTP hydrolysis studies, we assessed NTP hydrolysis by soluble TD in the presence of EDTA (see Materials and Methods). EDTA inhibits NDPK-catalyzed transphosphorylation [33, 34], but binding of guanine nucleotides to the α-subunit of TD (TD_α) is not influenced by Mg^{2+} [36]. In the presence of EDTA, TD catalyzed the hydrolysis of GTP, ITP, and XTP in a substrate concentration-dependent and saturable man-

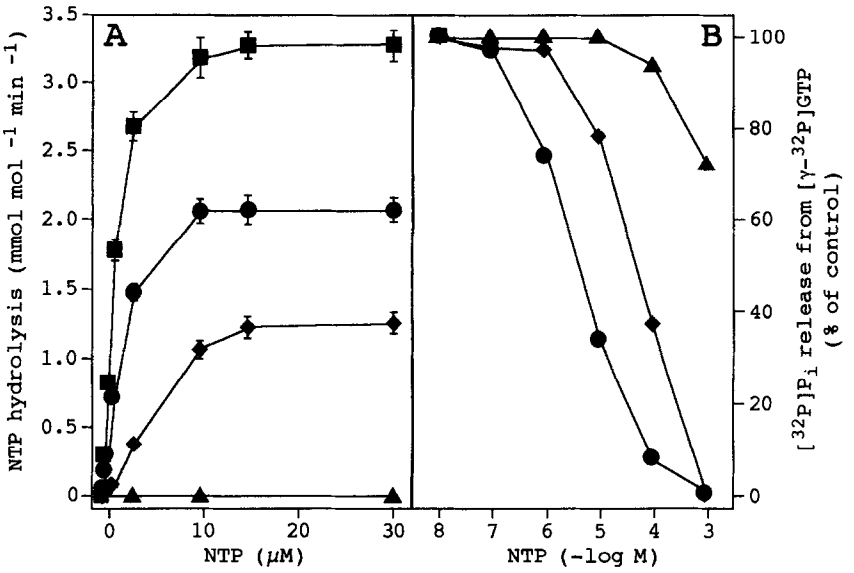

FIG. 2. GTP, ITP, XTP, and ATP hydrolysis by TD and competition of GTP hydrolysis by ITP, XTP, and ATP. The NTPase activities in soluble bovine TD preparations were assessed as described in Materials and Methods. (A) Concentration-dependence of GTP hydrolysis (■), ITP hydrolysis (●), XTP hydrolysis (◆), and ATP hydrolysis (▲). Data shown are the means ± SD of three independent experiments. (B) The effects of unlabeled ITP (●), XTP (◆), and ATP (▲) on [^{32}P]P_i release from [γ-^{32}P]GTP (100 nM) were studied. Data shown are the means of three independent experiments. The SD values were less than 5% of the means.

ner (Fig. 2A). The NTPase activities were analyzed by nonlinear regression (Table 1). The K_m value for the GTPase was lower than that for the ITPase and XTPase. The maximum efficiency (V_{max} values) of TD in hydrolyzing NTPs was GTP > ITP > XTP (see Table 1). TD did not show ATPase activity in the presence of EDTA (see Fig. 2A).

The GTP analogue guanylyl 5′-[β,γ-imido]triphosphate (GppNHp) competitively inhibits the binding of GTP to the α-subunit of TD (TD_α) [37]. We studied the effects of GppNHp at different fixed concentrations on the hydrolysis of GTP at various concentrations and analyzed the data according to Lineweaver-Burk (Fig. 3A). GppNHp caused shifts in the plot intersection with the abscissa. This result confirms that GppHNp competes with GTP for the binding to TD_α in a competitive manner [37]. By analogy to GppNHp, ITP and XTP also inhibited TD-catalyzed GTP hydrolysis in a concentration-dependent and competitive manner (see Fig. 2B and Fig. 3B and C). The K_i values for ITP and XTP were 4.2 μM and 54.9 μM, respectively. ATP only poorly inhibited TD-catalyzed GTP hydrolysis (see Fig. 2B).

We also addressed the question as to whether binding of ITP and XTP to TD induces an active conformation of the G-protein. The measurement of TD-stimulated phosphodiesterase activity requires the presence of Mg^{2+} [38]. There-

TABLE 1. Kinetic properties of the GTPase, ITPase, and XTPase of TD

Parameter	GTPase	ITPase	XTPase
K_m (μM)	0.92 ± 0.03	4.49 ± 1.65	4.50 ± 1.50
V_{max} (mmol mol^{-1} min^{-1})	3.48 ± 0.02	2.79 ± 0.30	1.46 ± 0.15
Goodness of fit (R^2)	0.999	0.951	0.967

GTP, ITP, and XTP hydrolysis by soluble bovine TD was assessed as described in Materials and Methods. The data shown in Fig. 2A were subjected to nonlinear regression analysis and are the means ± SD of three independent experiments.

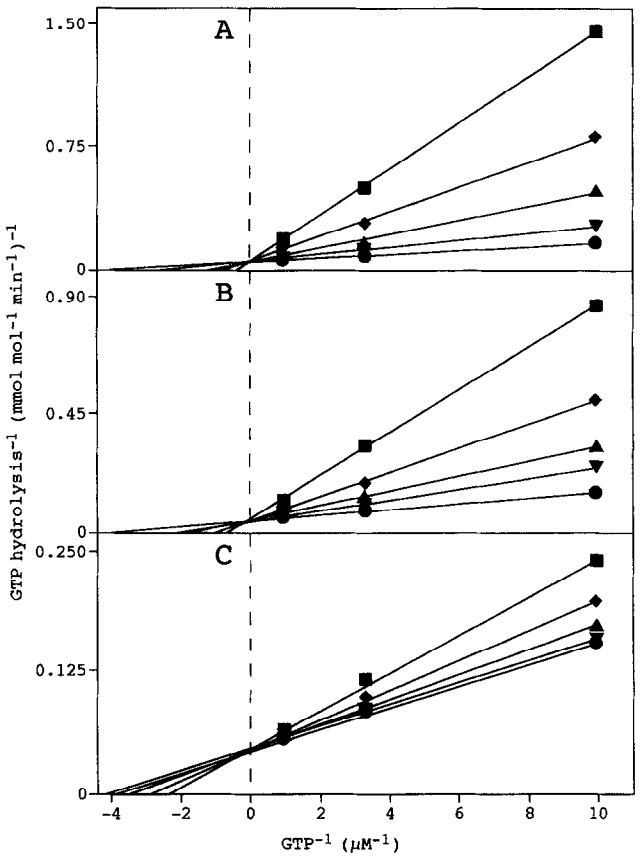

FIG. 3. Kinetic analysis of the effects of GppNHp, ITP, and XTP on TD-catalyzed GTP hydrolysis. The GTPase activity of soluble bovine TD was assessed as described in Materials and Methods, using GTP at concentrations between 0.1–1.0 μM. Reaction mixtures additionally contained H_2O (control) or nucleotides at various fixed concentrations. Lineweaver-Burk plots of typical experiments (repeated at least twice) are shown. (A) Effect of GppNHp. (B) Effect of ITP. (C) Effect of XTP. Note that the concentrations of competing nucleotides employed in the experiments shown in A–C were the same. Control (no nucleotide added) (●); nucleotide at 1 μM (▼); nucleotide at 3 μM (▲); nucleotide at 10 μM (◆); nucleotide at 30 μM (■).

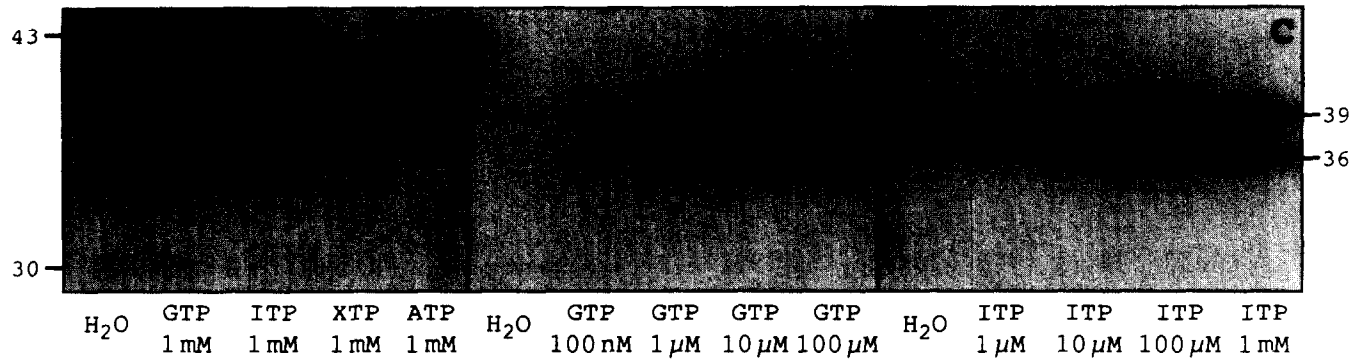

FIG. 4. Effects of NTPs on elution of TD from ROS disk membranes. Elution experiments were performed as described in Materials and Methods. Identical volumes of supernatant fluids obtained from ROS disk membranes after treatment with H_2O (control) or NTPs at the indicated concentrations were loaded onto SDS gels containing 12.5% (w/v) acrylamide and stained with silver. (A) Comparison of the effects of GTP, ITP, XTP, and ATP on TD elution. (B) Concentration-dependent stimulatory effect of GTP. (C) Concentration-dependent stimulatory effect of ITP. Numbers at the left margin designate apparent molecular masses (kDa) of marker proteins. Numbers at the right margin designate apparent molecular masses of TD_α (39 kDa) and TD_β (36 kDa). The data shown are representative for four independent experiments.

fore, a contribution of NDPK-catalyzed transphosphorylation reactions in any NTP effects cannot definitively be excluded in this assay. However, elution of TD from ROS disk membranes can be achieved in the absence of Mg^{2+} [26]. Moreover, the effects of guanine nucleotides on phosphodiesterase activity and TD elution parallel each other [37]. Thus, the TD elution assay can be used as a method to assess TD activation while avoiding NDPK involvement. GTP is known to elute TD from ROS disk membranes [26, 39]. We found that NTPs (1 mM each) eluted TD (TD_α, 39 kDa; β-subunit of TD (TD_β), 36 kDa) from ROS disk membranes in the order of efficacy GTP > ITP ≫ XTP (Fig. 4A). The 39 kDa and 36 kDa proteins comigrated with bands recognized by G-protein α_{common}-peptide antiserum (AS 8) and G-protein β_{common}-peptide antiserum (AS 11), respectively (data not shown). The stimulatory effect of GTP on TD elution was half-maximal at ca. 1 μM and reached a maximum at 10 μM (Fig. 4B). The corresponding values for ITP were ca. 10 μM and 100 μM, respectively (Fig. 4C). XTP (10 μM and 100 μM) (data not shown) and ATP (1 mM) (see Fig. 4A) did not elute TD from ROS disk membranes.

In dibutyryl cAMP-differentiated HL-60 cells, receptors for the chemoattractants fMLP, complement C5a and LTB_4 couple to G_i-proteins and mediate PTX-sensitive stimulations of high-affinity GTP hydrolysis [4]. Figure 5A–C shows the absolute rates of GTP, ITP, and XTP hydrolysis at a substrate concentration of 500 nM each in membranes of dibutyryl cAMP-differentiated HL-60 cells in the absence and presence of fMLP, C5a, and LTB_4 at increasing concentrations. The experiments were performed in the presence of Mg^{2+} (see Materials and Methods). Under these experimental conditions, the basal GTP hydrolysis rate was higher than that of ITP, which in turn, was higher than the hydrolysis rate of XTP. Figure 5D–F depict the relative stimulatory effects of chemoattractants on NTP hydrolysis, i.e. the percentage stimulations above basal induced by fMLP, C5a, and LTB_4. In agreement with our previously published data [40], fMLP, C5a, and LTB_4 were almost equally effective activators of GTPase. However, fMLP and LTB_4 were more effective activators of ITPase than C5a. This difference in the efficacy of chemoattractants to increase ITP hydrolysis was consistently observed in six independent experiments (Table 2). It was also noteworthy that fMLP activated ITPase with a ca. 10-fold higher potency than GTPase (Table 3). LTB_4 activated ITP hydrolysis approximately 20-fold more potently than GTP hydrolysis. The EC_{50} for ITPase activation by C5a was comparable to that of GTPase activation. EDTA strongly reduced basal GTP and ITP hydrolysis in HL-60 membranes and virtually abolished the stimulatory effects of chemoattractants on GTPase and ITPase (data not shown). fMLP, C5a, and LTB_4 had no stimulatory effect on XTP hydrolysis in the presence of Mg^{2+} (see Fig. 5A–F).

TABLE 2. Stimulatory effects of chemoattractants on ITP hydrolysis in membranes of dibutyryl cAMP-differentiated HL-60 cells: comparison of the results obtained in various experiments

Experiment No.	Relative stimulatory effect of chemoattractants on ITP hydrolysis (% stimulation)		
	fMLP (10 μM)	C5a (100 nM)	LTB_4 (1 μM)
1	33.3	2.4	34.7
2	33.2	4.9	21.0
3	39.4	10.3	19.8
4	28.2	5.1	33.1
5	30.4	4.8	22.2
6	32.9	7.8	25.8
mean ± SD	32.9 ± 3.8	5.9 ± 2.8	26.1 ± 6.4

The stimulatory effects of chemoattractants at the indicated concentrations on the hydrolysis of ITP (500 nM) in membranes of dibutyryl cAMP-differentiated HL-60 cells were determined as described in Materials and Methods. The increases in ITP hydrolysis mediated by fMLP, C5a, and LTB_4 are referred to basal hydrolysis, which was set at 100%. Absolute values of basal ITP hydrolysis are given in Table 4, in Figs. 5A–C, and in Fig. 7A.

TABLE 3. Potencies of chemoattractants to activate GTP and ITP hydrolysis in membranes of dibutyryl cAMP-differentiated HL-60 cells

Parameter	Chemoattractant		
	fMLP	C5a	LTB_4
EC_{50} GTPase (nM)			
mean	1220	0.40	42.4
95% confidence interval	793–1876	0.20–0.79	26.2–68.6
Goodness of fit (R^2)	0.990	0.986	0.989
EC_{50} ITPase (nM)			
mean	115	0.48	2.18
95% confidence interval	71–185	0.24–0.94	1.22–3.90
Goodness of fit (R^2)	0.989	0.987	0.985

The stimulatory effects of chemoattractants at the concentrations shown in Fig. 5 on the hydrolysis of GTP and ITP (500 nM each) in membranes of dibutyryl cAMP-differentiated HL-60 cells were determined as described in Materials and Methods. Concentration–response curves to chemoattractants obtained in six independent experiments were subjected to analysis by nonlinear regression to calculate EC_{50} values.

TABLE 4. Chemoattractant-stimulated ITP hydrolysis in membranes of dibutyryl cAMP-differentiated HL-60 cells: effect of PTX

Stimulus	ITP hydrolysis (pmol mg^{-1} min^{-1})	
	control	PTX
H_2O (basal)	6.9 ± 0.5	4.2 ± 0.3
fMLP (10 μM)	9.2 ± 0.3	4.3 ± 0.4
C5a (100 nM)	7.5 ± 0.4	4.1 ± 0.4
LTB_4 (1 μM)	9.3 ± 0.5	4.2 ± 0.5

Treatments with carrier (control) and PTX were performed as described in Materials and Methods. The effects of chemoattractants at the indicated concentrations on the hydrolysis of ITP (500 nM) in membranes of control and PTX-treated cells were determined as described in Materials and Methods. Data shown are the means ± SD of a representative experiment. Similar results were obtained in three independent experiments.

In membranes obtained from PTX-treated HL-60 cells, the stimulatory effects of fMLP, C5a, and LTB_4 on ITP hydrolysis were abolished (Table 4). These findings indicate that chemoattractant-stimulated ITP hydrolysis was exclusively mediated via G_i-proteins. By analogy to GTP hydrolysis [40], PTX treatment led to a reduction of basal ITP hydrolysis (see Table 4). This inhibition presumably reflects uncoupling of agonist-free chemoattractant receptors from G_i-proteins [41].

Figure 6A shows the substrate concentration-dependency of basal and fMLP-stimulated GTP hydrolysis in HL-60 membranes. It should be noted that the GTP hydrolysis rates depicted represent total rates, i.e. the hydrolysis uncorrected for low-affinity GTPase. At any of the substrate concentrations studied (0.1–3 μM), fMLP clearly increased GTP hydrolysis. With increasing concentrations of GTP, the relative stimulatory effect of fMLP, i.e. the stimulation in percent above basal, declined (Fig. 6B, inset). FMLP-

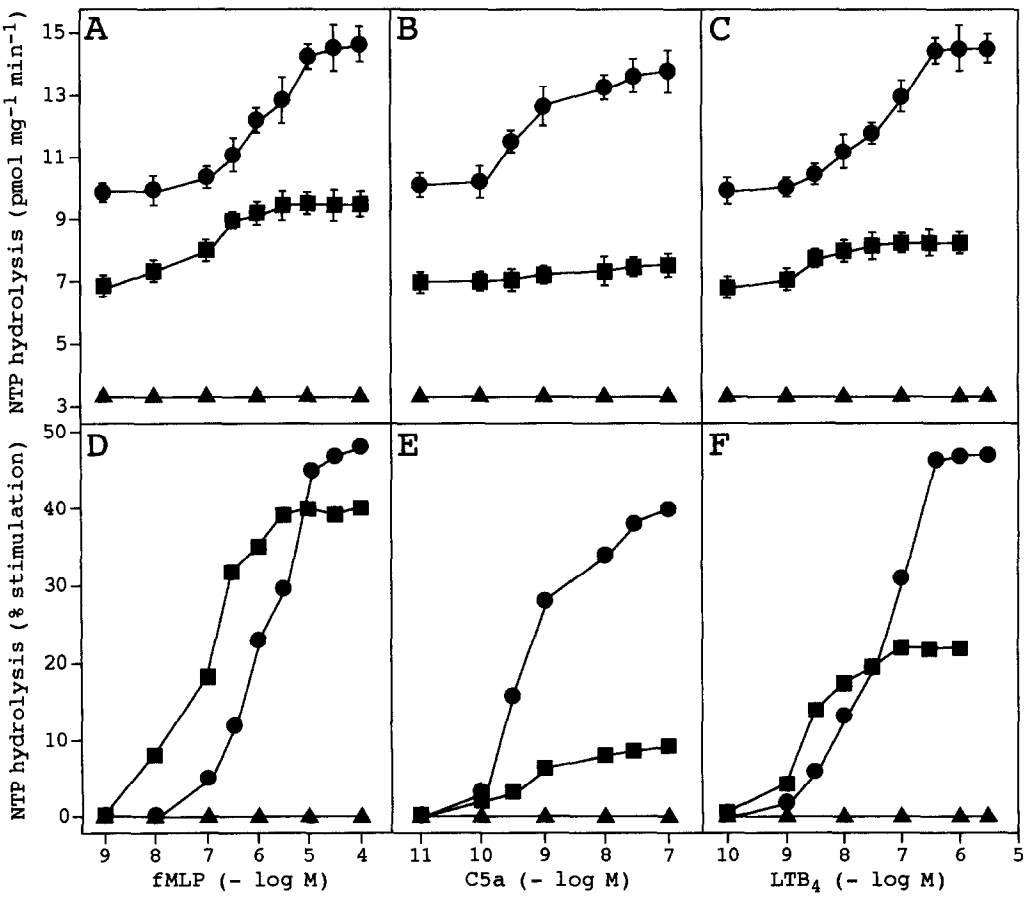

FIG. 5. Concentration–response curves for the effects of chemoattractants on GTP, ITP, and XTP hydrolysis in membranes of dibutyryl cAMP-differentiated HL-60 cells. GTP hydrolysis (●), ITP hydrolysis (■), and XTP hydrolysis (▲) were determined in the presence of fMLP, C5a and LTB_4 at the indicated concentrations as described in Materials and Methods. (A–C) Absolute rates of GTP, ITP, and XTP hydrolysis. (D–F) Replot of the data shown in panels A–C. The relative stimulatory effects of fMLP and C5a on GTP, ITP, and XTP hydrolysis are shown. To obtain these values, the basal NTP hydrolysis rates were set at 100%, and the increase mediated by chemoattractants at the different concentrations are referred to these values. Data shown are the means ± SD of a representative experiment. Similar results were obtained in six independent experiments.

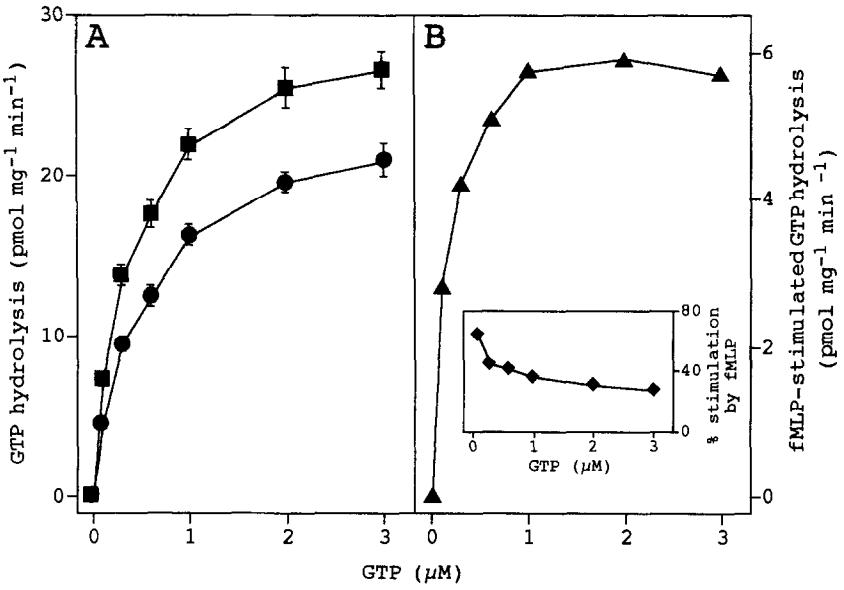

FIG. 6. Effects of fMLP on GTPase activity in membranes of dibutyryl cAMP-differentiated HL-60 cells at different GTP concentrations. The GTPase activity in HL-60 membranes was determined as described in Materials and Methods, using GTP at concentrations ranging from 0.1–3 μM. (A) Total GTP hydrolysis rates under basal conditions (●) or in the presence of 10 μM fMLP (■). Data shown are the means ± SD of a representative experiment. Similar results were obtained in three independent experiments. (B) Replot of the data shown in A. The mean values of the absolute basal GTP hydrolysis rates were subtracted from the mean values of the GTP hydrolysis rates observed in the presence of fMLP for each substrate concentration. The fMLP-stimulated GTP hydrolysis is unequivocally attributable to G_i-proteins because fMLP-stimulated GTP hydrolysis is fully PTX sensitive [40]. The inset shows the relative stimulatory effects of fMLP on GTP hydrolysis. To obtain these values, the basal GTP hydrolysis rates were set at 100%, and the increases mediated by fMLP at the different GTP concentrations are referred to these values.

stimulated GTP hydrolysis in HL-60 membranes is fully PTX sensitive [40]. Therefore, this proportion of GTP hydrolysis is unequivocally attributable to G_i-proteins, whereas PTX-sensitive and -insensitive G-proteins, low-molecular mass GTP-binding proteins, and other nucleotidases may contribute to basal GTPase activity to various and not precisely measurable extents. For each substrate concentration, we subtracted the basal GTP hydrolysis from the GTP hydrolysis observed in the presence of fMLP and replotted these data (Fig. 6B). The fMLP-stimulated GTP hydrolysis gives a hyperbolic function and shows saturation with GTP at 1–3 μM. Analysis of the hydrolysis rates by nonlinear regression revealed that the fMLP-stimulated GTP hydrolysis has a K_m value of ca. 0.15 μM and a V_{max} of ca. 6.5 pmol/mg/min (Table 5).

By analogy to the procedure adopted for GTP hydrolysis in HL-60 membranes (see Fig. 6), ITP hydrolysis was analyzed. With increasing concentrations of ITP, the total hydrolysis rates of ITP increased (Fig. 7A). A stimulatory effect of fMLP on ITP hydrolysis was observed with ITP at all concentrations studied (0.1–30 μM). The relative stimulatory effect of fMLP on ITP hydrolysis was highest at a substrate concentration of 3 μM (Fig. 7B, inset). The fMLP-stimulated ITP hydrolysis, i.e. the ITP hydrolysis unequivocally attributable to G_i-proteins, is replotted in Fig. 7B. fMLP stimulated ITP hydrolysis according to a hyperbolic function that showed saturation at a substrate concentration of 10–30 μM. Analysis by nonlinear regression revealed that the K_m value of the fMLP-stimulated ITPase was ca. 10-fold higher than that of the GTPase, whereas the V_{max} of the ITPase was ca. twofold higher than that of the GTPase (see Table 5).

TABLE 5. Kinetic properties of the fMLP-stimulated GTPase and ITPase in membranes of dibutyryl cAMP-differentiated HL-60 cells

Parameter	GTPase	ITPase
K_m (μM)	0.14 ± 0.03	1.79 ± 0.28
V_{max} (mmol mol^{-1} min^{-1})	6.41 ± 0.25	12.63 ± 0.55
Goodness of fit (R^2)	0.983	0.987

The fMLP-stimulated GTP and ITP hydrolysis in membranes was studied with various substrate concentrations as described in Materials and Methods and the legends to Figs. 6B and 7B. The data obtained in three independent experiments were subjected to nonlinear regression analysis.

DISCUSSION

For more than 20 years, it has been known that ITP can substitute for GTP in the receptor-mediated regulation of effector systems [5–9]. Additionally, ITP competes with [^{35}S]GTPγS for binding to purified G-proteins [17–19]. The affinity of G-proteins for ITP is ca. 10- to 170-fold lower than for GTP, depending on the G-protein studied and the type of assay employed [17–19]. However, the question of whether G-proteins undergo IDP/ITP exchange with subsequent ITP hydrolysis has thus far gone unanswered. Here, we show that TD indeed possesses ITPase activity (see Fig. 2A and Table 1). A contribution of coupled NDPK/GTPase reactions to the ITPase activity can be ruled out because reactions were conducted in the presence of EDTA (see Materials and Methods). The K_m value of the ITPase is higher than that of the GTPase, a finding that fits to the data obtained with ITP in the competition experiments concerning [^{35}S]GTPγS binding [17–19]. Additionally, TD eluted TD from ROS disk membranes less potentially than GTP (see Fig. 4). The finding that ITP binds to TD_α with lower affinity than GTP is predicted by the crystal structure of the α-subunit [11].

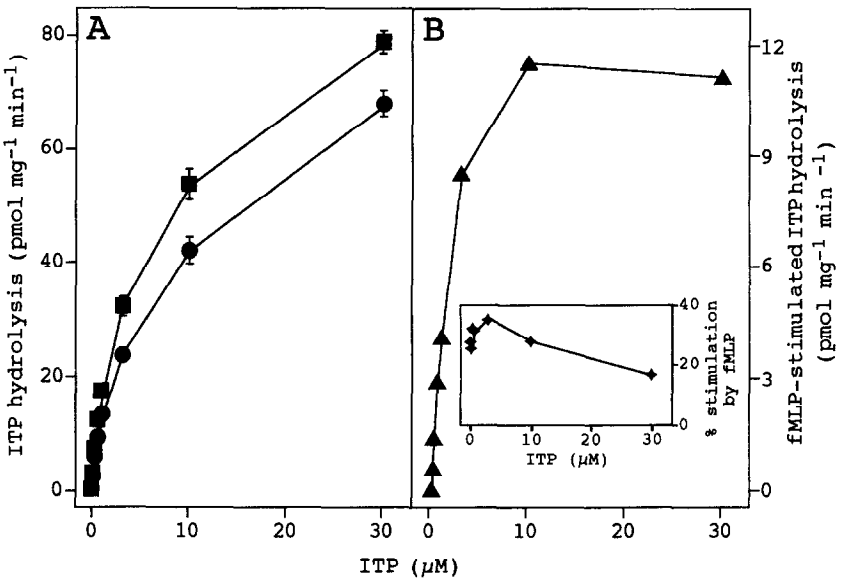

FIG. 7. Effects of fMLP on ITPase activity in membranes of dibutyryl cAMP-differentiated HL-60 cells at different ITP concentrations. The ITPase activity in HL-60 membranes was determined as described in Materials and Methods, using ITP at concentrations ranging from 0.1–30 μM. (A) Total ITP hydrolysis rates under basal conditions (●) or in the presence of 10 μM fMLP (■). Data shown are the means ± SD of a representative experiment performed in triplicate. Similar results were obtained in three independent experiments. (B) Replot of the data shown in A. The mean values of the basal ITP hydrolysis rates were subtracted from the mean values of the ITP hydrolysis rates observed in the presence of fMLP for each ITP concentration. The fMLP-stimulated ITPase activity is unequivocally attributable to G_i-proteins because fMLP-stimulated ITP hydrolysis is fully PTX sensitive (see Table 4). The inset shows the relative stimulatory effects of fMLP on ITP hydrolysis. To obtain these values, the basal ITP hydrolysis rates were set at 100%, and the increases mediated by fMLP at the different ITP concentrations are referred to these values.

The V_{max} of TD-catalyzed ITP hydrolysis was lower than the V_{max} of GTP hydrolysis (see Fig. 2A and Table 1), and ITP was less effective than GTP in eluting TD from ROS disk membranes (see Fig. 4). These findings raise the question as to whether ITP, compared to GTP, could be a partial activator of TD. To further address this question, conformational changes of TD must be monitored, either by studying the effects of ITP and GTP on endogenous tryptophane fluorescence of TD_α [42] or by studying the fluorescense of TD in the presence of the fluorescent NTP analogues of GTP and ITP, *N*-methyl-3′-O-anthranoyl-GTP and *N*-methyl-3′-O-anthranoyl-ITP, respectively [43]. Moreover, it will be necessary to compare the effects of the transphosphorylation-resistant ITP analogue, inosine 5′-[β,γ-imido]triphosphate, with the effect of GppNHp on phosphodiesterase activation because even in the presence of Mg^{2+}, there will be no interference with NDPK. It is of interest that various guanine nucleotides have already been shown to induce different extents of G-protein activation [43].

Not only does TD possess ITPase activity, but G_i-proteins do as well (see Figs. 5–7 and Table 4). Because the agonist-stimulated GTP and ITP hydrolysis by G_i-proteins in HL-60 membranes is virtually completely Mg^{2+} dependent, we were not able to study ITPase regulation in the presence of EDTA to exclude the involvement of coupled NDPK/GTPase reactions to the ITPase activity. However, not only is ITP a NDPK substrate, but any NTP, including XTP, is as well [33, 34]. Thus, if NDPK had contributed to coupled NDPK/GTPase reactions in HL-60 membranes, one would have expected the presence of an fMLP-stimulated apparent XTPase activity as well. Evidently, this was not the case. On the basis of these findings, we conclude that the fMLP-stimulated ITP hydrolysis is independent of coupled NDPK/GTPase reactions and represents the intrinsic enzymatic activity of G_i-protein α-subunits.

In accordance with the kinetic data of the GTPase and ITPase of TD, the fMLP-stimulated ITPase of G_i-proteins had a lower K_m than the GTPase (see Figs. 2, 6, and 7 and Tables 1 and 5). Surprisingly, the V_{max} of the ITPase activity of G_i-proteins was greater than that of the GTPase (see Table 4). How can this difference be explained? It is generally accepted that the release of GDP from G-protein α-subunits is the rate-limiting step of G-protein activation [1, 2]. Data obtained with G_o-proteins, which are structurally closely related to G_i-proteins [1, 2], indicate that IDP is released from G_o-protein α-subunits more rapidly than GDP [44]. Thus, agonist-occupied formyl peptide receptors may stimulate IDP release more rapidly than GDP release. A consequence of rapid IDP release would be rapid ITP uptake with a subsequent high ITP hydrolysis rate. Accordingly, the G-protein α-subunit would spend a longer period of time in its ITP-bound form than in its IDP-bound or nucleotide-free form. As has been shown for accelerated GDP/GTP exchange [45], the result of a rapid IDP/ITP exchange could be an enhanced activation of effector systems. Such a model could explain the findings that ITP may support receptor-stimulated adenylyl cyclase activation more effectively than GTP [5, 6] and that ITP may be a more efficient inducer of exocytosis in permeabilized cells than GTP [12–14].

Currently, we do not know whether agonist-occupied formyl peptide receptors promote only IDP release from α-subunits or whether they also stimulate ITP binding. From studies with guanine nucleotides, it is known that receptor agonists can stimulate GDP release and GTP binding [44]. Unfortunately, we are currently limited in terms of the approaches available to study IDP/ITP ex-

change on G_i-proteins and effector system activation in HL-60 membranes. First, in view of the already rapid spontaneous GDP release from G_i-proteins in HL-60 membranes [46], it is highly unlikely that receptor-stimulated IDP release can be detected in this system. Second, radiolabeled ITP analogues such as [^{35}S]inosine 5′-O-(3-thiotriphosphate) or [^{3}H]inosine 5′-[β,γ-imido]triphosphate are not generally available, and it is doubtful whether the affinity of these radioligands for G-protein α-subunits is high enough for binding experiments. Third, the effects of ITP on fMLP-stimulated phospholipase C activation in HL-60 membranes cannot be interpreted unequivocally because ITP is also an effective purinoceptor agonist and because purinoceptors activate phospholipase C via G_i-proteins as do formyl peptide receptors [47, 48]. These limitations also apply for XTP (see Discussion below).

We should like to emphasize that the observed relative differences in ITP hydrolysis rates between TD and G_i-proteins in HL-60 membranes (see Tables 1 and 5) do not necessarily reflect G-protein subtype-specific differences in NTP hydrolysis. The differences could equally well be attributed to differences in the experimental conditions. Specifically, NTP hydrolysis by TD was studied under basal conditions, i.e. in the absence of exogenously added rhodopsin and Mg^{2+}, whereas NTP hydrolysis by G_i-proteins in HL-60 membranes was assessed under maximal receptor agonist stimulation and in the presence of Mg^{2+}.

fMLP and LTB_4 stimulated ITP hydrolysis more potently than GTP hydrolysis (see Fig. 5D and 5F and Table 3). This could indicate that formyl peptide and LTB_4 receptors couple to G_i-proteins with higher affinity in the presence of IDP/ITP than in the presence of GDP/GTP. In other words, GTP, at the substrate concentration studied (500 nM), could uncouple formyl peptide- and LTB_4-receptors from G_i-proteins more efficiently than ITP (500 nM) because GTP has a higher affinity for G-proteins than ITP (see Figs. 2, 6, and 7 and Tables 1 and 5) [17–20]. However, such a model cannot explain why there is no such difference in potency concerning GTPase- and ITPase activation by C5a receptors and why C5a is such a weak activator of ITP hydrolysis relative to GTP hydrolysis (see Fig. 5 and Table 2).

Intriguingly, differential effects of GTP vs ITP on receptor/G-protein interaction have previously been reported. Specifically, ITP supports thyrotropin-stimulated adenylyl cyclase activation in bovine thyroid membranes more efficiently than GTP [5]. In contrast, no such difference between GTP and ITP was found for prostaglandin E_1 in this system [5]. In turkey erythrocyte membranes, ITP gives rise to a more effective adenylyl cyclase stimulation by β-adrenoceptor agonists than GTP [6]. Collectively, our present data and previously published results clearly show that GDP/GTP and IDP/ITP differentially affect the interaction of various receptors that are linked to the same class of G-proteins. A possible explanation for these observations could be that GDP- and IDP-liganded G-protein α-subunits adopt different conformations that fit differentially to various receptors. As has already been discussed above, biophysical studies would be required to substantiate the hypothesis that qualitative and/or quantitative conformational differences between guanine- and hypoxanthine nucleotide-liganded G-protein α-subunits exist.

Compared to ITP, considerably less is known about the function and role of XTP in G-protein activation. Previous studies had shown that XTP can substitute for GTP in supporting receptor-mediated adenylyl cyclase activation [6]. Here, we show that XTP, like GppNHp and ITP, competitively inhibits the binding of GTP to the guanine nucleotide-binding site of TD_α (see Figs. 2A and 3C). Moreover, TD shows measurable XTPase activity (see Fig. 2A and Table 1), and XTP elutes TD from ROS disk membranes (see Fig. 4A). Compared to ITP, XTP was much less potent than ITP in inhibiting GTP hydrolysis and in eluting TD from ROS disk membranes. This finding is not surprising, because hydrogen bonding of the keto group-substituted C_2 of the purine ring (see Fig. 1) with the highly conserved aspartic acid residue-268 in TD_α cannot occur [11]. Analogous observations as with TD have been made for the binding of GTP, ITP, and XTP to bacterial GTPases [49, 50], elongation factor-Tu [51] and low-molecular mass GTP-binding proteins [21, 22].

TD hydrolyzes XTP at a lower maximal rate than GTP and ITP (see Fig. 2A and Table 1), and XTP elutes TD from ROS disk membranes much less efficiently than GTP and ITP (see Fig. 4A). On the basis of these findings, XTP could be considered as a weak partial activator of TD. If this were true, then XTP would be predicted to induce smaller maximal changes in endogenous tryptophane fluorescence of TD than GTP and ITP, and N-methyl-3′-O-anthranoyl-XTP would be expected to induce smaller fluorescence signals upon binding to TD than the corresponding GTP and ITP analogues [43].

However, with regard to other G-proteins than TD, XTP may be a more effective activator than GTP. Specifically, XTP is more effective than GTP in supporting β-adrenoceptor–mediated adenylyl cyclase activation in turkey erythrocyte membranes [6], and XTP stimulates exocytosis in permeabilized cells more effectively than GTP or even GTPase-resistant GTP analogues [12–14, 23]. It is possible that XTP, once it has bound to certain G-protein α-subunits, stabilizes a conformation of the G-protein that shows high biological activity. By analogy to observations made with the GTPase-resistant GTP analogues GTPγS and GppNHp [1, 2], the activity of an XTP-liganded G-protein α-subunit would be expected to be increased if the XTP hydrolysis rate were low. In fact, in HL-60 membranes, we could not detect receptor agonist-stimulated XTPase activity although chemoattractant-stimulated GTP- and ITPase activity was readily observed (see Fig. 5). These findings indicate that G_i-proteins in HL-60 membranes do not possess XTPase activity or that their XTPase activity is below the detection limit of our assay.

To further analyze the mechanism of interaction of XTP with G-proteins, studies with XTP analogues such as

xanthosine 5′-O-(3-thiotriphosphate) (XTPγS) and xanthosine 5′-[β,γ-imido]triphosphate (XppNHp) must be performed. However, we anticipate that the affinity of these nucleotides to G-protein α-subunits will still be low. Thus, it will be necessary to mutate the highly conserved aspartic acid residue involved in hydrogen bonding with the amino group of C_2 of guanine (see Fig. 1) [11] into an asparagine residue. The consequence of analogous mutations in bacterial GTPases, elongation factor Tu, and low molecular mass GTP-binding proteins is a dramatic increase in affinity of the mutated proteins for xanthine nucleotides with a parallel decrease in affinity for guanine nucleotides [21, 22, 49–51]. Only with xanthine nucleotide-preferring G-protein α-subunits can release experiments with [^{3}H]XDP or [α-^{32}P]XDP, and binding studies with [^{35}S]XTPγS and [^{3}H]XppNHp be performed.

Could the effects of ITP and XTP on G-proteins be of physiological relevance? The bulk intracellular GTP concentration is estimated to be ca. 50 μM (for review, see [52]). Thus, G-proteins would be expected to always be saturated with GTP. However, GTP cannot be assumed to have free access to the G-proteins. Rather, NDPK-generated GTP may be preferred over exogenous GTP for G-protein activation [52–54]. Little is known about the physiological intracellular concentrations of IDP/ITP and XDP/XTP, but we assume that the bulk intracellular concentrations of these nucleotides are far lower than those of GDP/GTP. This does, however, not exclude the possibility that under certain physiological and/or pathological conditions, sufficient XTP and ITP is available to bind to G-protein α-subunits. ITP and XTP could reach the α-subunits either via diffusion from the cytosol or could locally be generated by NDPK from IDP and XDP, respectively, because NDPK is not base specific. The observed chemoattractant-specific differences in GTPase and ITPase activation of G_i-proteins in HL-60 membranes could provide, at least in part, the molecular basis for the quite different effects of fMLP, C5a, and LTB_4 in intact HL-60 cells [4].

XTP and ITP may also be of pharmacological importance for G-protein activation. Specifically, depletion of intracellular guanine nucleotide pools in HL-60 cells by inosine monophosphate dehydrogenase [E.C. 1.1.1.205.] inhibitors such as tiazofurin results in differentiation and changes in G-protein function [55–57]. It will be interesting to treat the question as to whether tiazofurin leads to increases in the cellular concentrations of ITP and XTP with consequent differential G-protein activation by these NTPs compared to GTP. Intriguingly, GTP depletion in SH-SY5Y neuroblastoma cells differentially affects the potency of agonists for various receptors to inhibit adenylyl cyclase [58].

In conclusion, GTP, ITP, and XTP bind to G-protein α-subunits with different affinities. G-proteins hydrolyze GTP, ITP, and XTP to different maximal extents and may lead to the formation of differently activated α-subunits. Chemoattractants stimulate the ITPase and GTPase of G_i-proteins in a receptor-specific manner. These findings suggest that GTP, ITP, and XTP are differential signal sorters and signal amplifiers at the G-protein level.

Future research on the roles of GTP, ITP, and XTP in G-protein activation will follow various directions. First, biophysical studies with purified G-proteins using nonfluorescent and fluorescent nucleotides must be performed. Second, the effects of transphosphorylation-resistant ITP and XTP analogues on activation of effector systems must be studied. Third, the molecular basis of the receptor-specific effects of GTP and ITP on G-protein–mediated signalling must be explored, because this may be a novel target for receptor-specific pharmacological manipulation of signalling processes at a postreceptor level. Fourth, the intracellular GTP, ITP, and XTP concentrations under basal and receptor-stimulated conditions, in pathological settings such as trauma and hypoxia, and under GTP-depleting conditions have to be carefully compared. Finally, studies with xanthosine nucleotide-preferring mutants of G-protein α-subunits are expected to provide substantial insight into the mechanisms underlying the interactions of XDP/XTP and their analogues with G-proteins.

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