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Guanosine 5′-[γ-thio]triphosphate-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate in HL-60 granulocytes

Evidence that the guanine nucleotide acts by relieving phospholipase C from an inhibitory constraint

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Myeloid differentiated human leukaemia (HL-60) cells contain a soluble phospholipase C that hydrolysed phosphatidylinositol 4,5-bisphosphate and was markedly stimulated by the metabolically stable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[S]). Half-maximal and maximal (up to 5-fold) stimulation of inositol phosphate formation by GTP[S] occurred at 1.5 μM and 30 μM respectively. Other nucleotides (GTP, GDP, GMP, guanosine 5′-[β-thio]diphosphate, ATP, adenosine 5′-[γ-thio]triphosphate, UTP) did not affect phospholipase C activity. GTP[S] stimulation of inositol phosphate accumulation was inhibited by excess GDP, but not by ADP. The effect of GTP[S] on inositol phosphate formation was absolutely dependent on and markedly stimulated by free Ca^{2+} (median effective concn. ≈ 100 nM). Analysis of inositol phosphates by anion-exchange chromatography revealed $InsP_3$ as the major product of GTP[S]-stimulated phospholipase C activity. In the absence of GTP[S], specific phospholipase C activity was markedly decreased when tested at high protein concentrations, whereas GTP[S] stimulation of the enzyme was markedly enhanced under these conditions. As both basal and GTP[S]-stimulated inositol phosphate formation were linear with time whether studied at low or high protein concentration, these results suggest that (*a*) phospholipase C is under an inhibitory constraint and (*b*) GTP[S] relieves this inhibition, most likely by activating a soluble GTP-binding protein.

INTRODUCTION

Many hormones, neurotransmitters and growth factors regulate the functions of their target cells by stimulating the hydrolysis of $PtdIns(4,5)P_2$, thus leading to generation of the two intracellular second messengers $Ins(1,4,5)P_3$ and diacylglycerol. $Ins(1,4,5)P_3$ regulates the level of intracellular Ca^{2+} by mobilizing Ca^{2+} from internal stores [1], whereas diacylglycerol acts by stimulating members of the protein kinase C family [2]. Similarly to other transmembrane signalling systems, hormonal activation of phospholipase C in most cases requires the interaction of at least three protein components: a receptor moiety, a guanine-nucleotide-binding protein (G-protein) and the effector enzyme phospholipase C [3–5]. On the basis of a differential sensitivity to pertussis toxin, at least two distinct G-proteins have been postulated to be involved in stimulation of phospholipase C [5]. Despite the tremendous recent progress in identifying various phospholipase C isoenzymes [6,7] and new members of the family of signal-transducing G-proteins [8], the identities of the G-protein-regulated phospholipase C enzyme(s) and of the relevant regulatory G-protein(s), as well as the mechanisms of interaction of these components, are largely unknown.

Neutrophils and related granulocytes, e.g. myeloid differentiated human leukaemia (HL-60) cells, express high numbers of several distinct cell-surface receptors for stimuli such as the chemotactic peptide fMet-Leu-Phe, leukotriene B_4 and complement component C5a, which are specifically coupled to stimulation of phospholipase C [5,9,10]. The phospholipase C-stimulating G-protein of neutrophils is sensitive to both cholera and pertussis toxins [11,12]. Neutrophils have therefore been widely used as a cellular model system to study the mechanisms of hormone- and/or guanine-nucleotide-stimulated inositol phospholipid hydrolysis. Towards this aim, the stimulation of phospholipase C has been examined in intact or permeabilized neutrophils, or in membrane preparations after the radiolabelling of the inositol phospholipids with either [^{3}H]inositol or [^{32}P]ATP (see refs. [5], [9], and [10] for recent reviews).

Several reports have appeared suggesting the existence of guanine-nucleotide-regulated phospholipase C enzymes in cytosolic preparations of a variety of tissues or cell types, e.g. human platelets [13,14], calf or bovine brain [6,14], human epidermis [15] and calf thymocytes [16]. Moreover, evidence has been provided that many of the purified cytosolic phospholipase C enzymes may not significantly differ in structure from their membrane counterparts [6]. These findings, together with our previous observation that pertussis-toxin substrates exist in both particulate and cytosolic preparations of neutrophils [17], prompted us to determine whether cytosolic preparations of granulocytes contain phospholipase C activity, and to what extent this activity is regulated by guanine nucleotides. Using exogeneous [^{3}H]$PtdIns(4,5)P_2$-containing phospholipid vesicles as substrate, we demonstrate here that GTP[S] stimulates a phospholipase C present in cytosolic preparations of myeloid differentiated HL-60 cells. The results suggest that GTP[S] stimulates phospholipase C by relieving the enzyme from an inhibitory constraint.

MATERIALS AND METHODS

Materials

[^{3}H]$PtdIns(4,5)P_2$ was from Amersham Buchler (Braunschweig, Germany). Nucleotides and unlabelled inositol phospholipids were obtained from Boehringer (Mannheim, Germany). Phosphatidylethanolamine was from Serva (Heidelberg,

Abbreviations used: G-protein, signal-transducing heterotrimeric guanine-nucleotide-binding protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine; EC_{50}, median effective concentration.

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Germany) or from Sigma (Deisenhofen, Germany). Sodium deoxycholate was purchased from Aldrich (Steinheim, Germany). $Na_4P_2O_7$ (anhydrous) and imidodiphosphate (sodium salt) were from Sigma. All other materials were from sources previously described [17,18].

Cell culture and preparation of cytosol

HL-60 cells were grown in suspension and induced to differentiate into mature myeloid forms by addition of 1.25% (v/v) dimethyl sulphoxide to the culture medium [18]. Differentiated cells were pelleted by centrifugation [18] ($\sim 2 \times 10^{10}$ cells, ~ 30 ml packed cells), resuspended in 200 ml of lysis buffer, containing 0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.5 at 20 °C, 1.5 mM-$MgCl_2$, 1 mM-ATP, 3 mM-benzamidine, 1 μM-leupeptin, 1 mM-phenylmethanesulphonyl fluoride and 2 μg of soybean trypsin inhibitor/ml. Cells were homogenized by nitrogen cavitation [17,18]; the product was supplemented with 1.25 mM-EGTA. Unbroken cells and nuclei (~ 10 ml) were removed by low-speed centrifugation [18], and cytosol was prepared from the post-nuclear supernatant by sequential centrifugation in a Beckman type JA-20 rotor ($r_{av.} = 7.0$ cm) at 15000 rev./min for 20 min at 4 °C and in a Beckman type Ti 60 rotor ($r_{av.} = 6.3$ cm) at 40000 rev./min for 60 min at 4 °C. In some cases, cytosolic proteins were concentrated by pressure filtration in a stirred cell equipped with an Amicon PM 10 membrane. The resulting supernatant or concentrate was passed through 0.45 μm-pore-size Millipore Millex HA filters, snap-frozen in liquid N_2 and stored at −70 °C.

Preparation of lipid substrate

A mixture of phosphatidylethanolamine, PtdIns(4,5)P_2 and [^{3}H]PtdIns(4,5)P_2 was evaporated to dryness under a stream of N_2 at 4 °C, in a 14 mm × 100 mm Pyrex tube. Lipids were resuspended by vortex-mixing for 30 min at 20 °C in a mixture (40 μl/assay) containing 87.5 mM-Tris/maleate buffer (adjusted to pH 7.3 at 20 °C with NaOH [19]), 140 mM-KCl, 17.5 mM-LiCl, 0.07% (w/v) sodium deoxycholate and 4.8 mM-EGTA. This suspension was sonicated for 15 min at 20 °C in a bath-type sonicator (Sonorex RK 102; Bandelin, Berlin, Germany) and directly used for determination of phospholipase C activity.

Phospholipase C assay

Phospholipase C activity was assayed for 20 min at 25 °C in a mixture (70 μl) consisting of 40 μl of lipid substrate, 15 μl of cytosol, 10μl of the nucleotides specified in the Figure legends and 5 μl of $CaCl_2$ to adjust the concentration of free Ca^{2+} to the desired value. The concentrations of $CaCl_2$ were calculated as described in [20] by using the stability constants for EGTA given in [21]. The final concentrations of phosphatidylethanolamine and [^{3}H]PtdIns(4,5)P_2 were 280 and 28 μM (5 Ci/mol) respectively. When samples were analysed for individual inositol phosphates, the incubation medium was supplemented with 10 mM-2,3-bisphosphoglycerate to inhibit degradation of Ins(1,4,5)P_3 [22]. The reaction was started by the addition of cytosol and terminated by adding 350 μl of chloroform/methanol/conc. HCl (500:500:3, by vol.). Samples were incubated for 30 min at 20 °C and then supplemented with 100 μl of 1 M-HCl containing 5 mM-EGTA. Phase separation was accelerated by centrifugation for 1 min in an Eppendorf microcentrifuge. A 200 μl portion of the aqueous (upper) phase was supplemented with 4 ml of scintillation fluid (Quicksafe A; Zinsser Analytic, Frankfurt, Germany), and radioactivity was measured in a liquid-scinitillation counter. The specific activity of soluble phospholipase C was critically dependent on the protein concentration used in the assay (see below), and ranged from $\leqslant 10$ to $\geqslant 700$ pmol of inositol phosphates formed/min per mg of protein when assayed under standard conditions (0.1 μM free Ca^{2+}, 0.04% (w/v) deoxycholate, no GTP[S]) at high ($\geqslant 9.6$ mg/ml) or low ($\leqslant 0.2$ mg/ml) protein concentration respectively.

Inositol phosphate analysis

Inositol phosphates were analysed by using a slightly modified form of the procedure described in [23,24]. In brief, 200 μl of the aqueous (upper) phase obtained by phospholipid extraction was supplemented with liquid indicator (Riedel-de Haën, Seelze, Germany), neutralized with ~ 40 μl of 1.5 M-KOH containing 75 mM-Hepes, and applied to columns (0.6 cm × 1.5 cm) of Dowex-1 resin (Cl^-form; Sigma 1 × 8−200), which had been converted into the formate form by washing with 1 M-NaOH, followed by washing with 1 M-formic acid and equilibration with water in accordance with ref. [25]. Inositol was eluted with 3 ml of water, glycerophosphoinositol with 3 ml of 60 mM-sodium formate/5 mM-sodium tetraborate, Ins$P$ with 3 ml of 0.2 M-ammonium formate/0.1 M-formic acid, InsP_2 with 3 ml of 0.4 M-ammonium formate/0.1 M-formic acid, and InsP_3 with 3 ml of 1 M-ammonium formate/0.1 M-formic acid. Each fraction was supplemented with 10 ml of scintillation fluid (Instant Scint. Gel; Packard, Frankfurt, Germany), and radioactivity was determined in a liquid-scintillation counter. The columns were re-used after regeneration by sequential washing with 8 ml of 2 M-ammonium formate/0.1 M-formic acid and 10 ml of water.

Inositol phospholipid analysis

Inositol phospholipids were analysed by t.l.c. on precoated HPTLC silica-gel 60 plates (Merck, Darmstadt, Germany) as described in [26,27]. In brief, the plates were impregnated with 1.2% (w/v) potassium oxalate in methanol/water (2:3, v/v) and activated by incubation for 15 min at 120 °C before spotting. Portions (140 μl) of the organic (lower) phase obtained by phospholipid extraction were evaporated to dryness, resuspended in 120 μl of chloroform, supplemented with 3 μg each of non-radioactive PtdIns, PtdIns4P and PtdIns(4,5)P_2/tube, and applied to the activated plates. The individual phospholipids were detected by iodine vapour, and the radioactivity in the spots corresponding to PtdIns, PtdIns4P and PtdIns(4,5)P_2 was determined by scraping and liquid-scintillation counting in 4.5 ml of scintillation fluid (Quicksafe A).

Data presentation

All experiments were performed at least three times. Data from representative experiments are shown as means of triplicate observations. The S.D. of the means shown in the Figures was typically less than 10% and generally less than 15%.

Miscellaneous

Protein was determined as described by Bradford [28], with bovine IgG as standard.

RESULTS

Fig. 1 shows that GTP[S] markedly stimulates the ability of HL-60-cell cytosol to generate inositol phosphates from phospholipid vesicles containing PtdIns(4,5)P_2. Half-maximal and maximal stimulation were observed at approx. 1.5 μM- and 30 μM-GTP[S] respectively. The data shown in Table 1 demonstrate that stimulation of inositol phosphate formation was specific for GTP[S]. Neither UTP, ATP or ATP[S], nor GMP, GDP, or GDP[S] (all tested at 100 μM), enhanced inositol phospholipid hydrolysis. p[NH]ppG led to an only marginal accumulation of inositol phosphates, and GTP had no effect.

The stimulation of inositol phosphate formation by GTP[S] is specifically decreased by GDP. As shown in Fig. 2, the stimulation

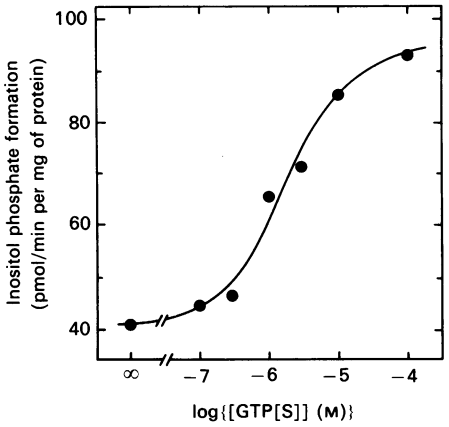

Fig. 1. Stimulation of inositol phosphate formation by GTP[S]

HL-60-cell cytosol (440 μg/tube) was incubated with phospholipid vesicles containing PtdIns(4,5)P_2 and increasing concentrations of GTP[S] as described in the Materials and methods section. The incubation was performed for 20 min in the presence of 0.1 μM free Ca^{2+}. The reaction was terminated and analysed for inositol phosphates as described in the Materials and methods section. Each value represents the mean of triplicate determinations.

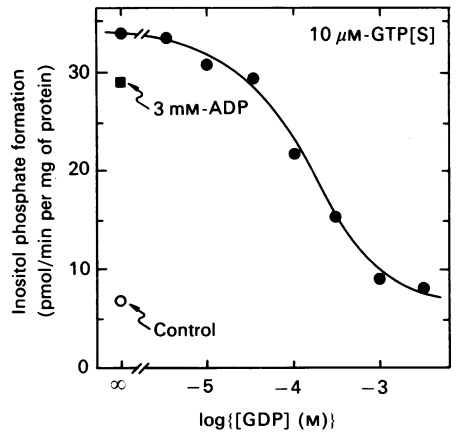

Fig. 2. Inhibition of GTP[S]-stimulated inositol phosphate formation by GDP

HL-60-cell cytosol (669 μg/tube) was incubated with phospholipid vesicles containing PtdIns(4,5)P_2 in the presence of 10 μM-GTP[S] and increasing concentrations of GDP as described in the Materials and methods section. The incubation was performed for 20 min in the presence of 0.1 μM free Ca^{2+}. The symbols (○, ■) illustrate the inositol phosphate formation obtained in the absence of GTP[S] and GDP (○) or in the presence of 10 μM-GTP[S] and 3 mM-ADP (■) respectively.

Table 1. Nucleotide specificity of the stimulation of inositol phosphate formation by GTP[S]

HL-60-cell cytosol (110 μg/tube) was incubated with phospholipid vesicles containing PtdIns(4,5)P_2 in the absence (control) or presence of 100 μM of the indicated nucleotides as described in the Materials and methods section. The incubation was performed for 20 min in the presence of 30 nM free Ca^{2+}. Each value represents the mean ± S.D. of triplicate determinations.

Nucleotide	Inositol phosphate formation (pmol/min per mg of protein)
Control	94.9±7.5
GMP	93.0±6.8
GDP	100.8±9.1
GDP[S]	82.9±15.9
GTP	110.0±18.2
p[NH]ppG	140.9±21.6
GTP[S]	234.2±9.4
ATP	103.2±17.4
ATP[S]	100.1±12.5
UTP	80.4±14.6

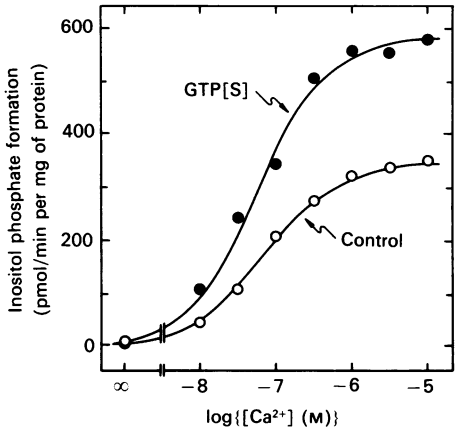

Fig. 3. Concentration-dependence on Ca^{2+} of the stimulation of inositol phosphate formation by GTP[S]

HL-60-cell cytosol (110 μg/tube) was incubated with phospholipid vesicles containing PtdIns(4,5)P_2 at increasing concentrations of free Ca^{2+} as described in the Materials and methods section. The incubation was performed for 10 min in the presence (●) or absence (○) of 100 μM-GTP[S].

obtained by addition of 10 μM-GTP[S] was half-maximally inhibited by ~ 150 μM-GDP and was completely abolished in the presence of 3 mM-GDP. ADP (3 mM) led to only a minor decrease in the GTP[S]-stimulated inositol phosphate formation.

Fig. 3 illustrates that both basal and GTP[S]-stimulated inositol phosphate formation was absolutely dependent on and markedly stimulated by free Ca^{2+}. Note that the concentrations of free Ca^{2+} required to stimulate inositol phosphate formation half-maximally and maximally were similar (i.e. ~ 0.1 μM and ~ 1 μM respectively) regardless of whether GTP[S] was absent or present in the incubation medium.

Many mammalian inositol-phospholipid-specific phospholipase C isoenzymes are stimulated by anionic detergents such as deoxycholate (see ref. [7] for a recent review). Therefore the effect of deoxycholate was studied on basal and GTP[S]-stimulated inositol phosphate accumulation by HL-60-cell cytosol. The data shown in Table 2 demonstrate that deoxycholate markedly stimulates inositol phosphate formation both in the absence and in the presence of GTP[S]. However, there was a biphasic response to deoxycholate of both the absolute and the relative GTP[S]-dependent increase in inositol phosphate formation, the maximal effect of GTP[S] being observed at 0.04% (w/v) deoxycholate.

Inositol-phospholipid-specific phospholipase C isoenzymes display two pH optima for enzyme activity, 5.0–5.5 and 6.5–7.0 [7]. Therefore, basal and GTP[S]-stimulated inositol phosphate formation was studied as a function of varying pH (results not shown). Only pH values between 6.8 and 8.0 were examined, as deoxycholate ($pK = 6.58$) precipitates below pH 6.8. Maximal activities for basal and GTP[S]-dependent inositol phosphate formation were observed at pH values between 7.0 and 7.4.

Table 2. Effect of deoxycholate on basal and GTP[S]-stimulated formation of inositol phosphates

HL-60-cell cytosol (110 μg/tube) was incubated with phospholipid vesicles containing PtdIns(4,5)P_2 in the absence or presence of 100 μM-GTP[S] as described in the Materials and methods section. Deoxycholate was present at the indicated concentrations. The incubation was performed for 20 min in the presence of 30 nM free Ca^{2+}. Each value represents the mean ± S.D. of triplicate determinations. The fold stimulation of inositol phosphate formation induced by 100 μM-GTP[S] is given in parentheses.

Deoxycholate (%, w/v)	Inositol phosphate formation (pmol/min per mg of protein)	
	Control	+100 μM-GTP[S]
0.01	84.2±10.8	136.6±8.1 (1.6)
0.02	84.4±20.8	142.8±1.3 (1.7)
0.03	83.7±19.9	170.8±8.9 (2.0)
0.04	205.8±50.7	443.6±43.1 (2.2)
0.06	274.2±26.3	479.0±28.6 (1.7)
0.08	337.5±25.6	412.3±29.0 (1.2)

Table 3. Effect of GTP[S] on the levels of inositol phospholipids and on formation of inositol phosphates

HL-60-cell cytosol (669 μg/tube) was incubated in the presence or absence of 100 μM-GTP[S] with phospholipid vesicles containing PtdIns(4,5)P_2 as described in the Materials and methods section, except that 10 mM-2,3-bisphosphoglycerate was present in the incubation medium. The incubation was performed for 20 min in the presence of 1 μM free Ca^{2+}. Samples were analysed for inositol phospholipids and inositol phosphates as described in the Materials and methods section. Each value represents the mean ± S.D. of triplicate determinations. Values obtained for PtdIns, inositol, Ins$P$ and glycerophosphoinositol were not significantly different from zero and are not shown.

	Inositol phospholipids (pmol)		Inositol phosphates (pmol)	
	PtdIns$P$	PtdInsP_2	InsP_2	InsP_3
Control	100±15	1247±21	14±8	123±32
GTP[S]	553±66	448±40	52±4	453±14

Several reports indicate that GTP[S] may stimulate hydrolysis of inositol phosphates, at least in part, by stimulating a PtdIns$P$ kinase [29,30]. In addition, evidence has been provided that GTP[S] may stimulate phospholipase D activity in HL-60-cell homogenates [31]. It therefore seemed mandatory to us to examine the levels of inositol phospholipids and of the individual inositol phosphates after incubation of HL-60-cell cytosol with phospholipid vesicles containing PtdIns(4,5)P_2 and to analyse the changes induced by addition of GTP[S]. Table 3 shows that InsP_3 was the major inositol phosphate metabolite of PtdIns(4,5)P_2 in the absence of GTP[S], and that the GTP[S]-induced increase in inositol phosphate accumulation was mainly due to an increase in InsP_3. Analysis of inositol phospholipids showed that GTP[S] markedly decreased the level of PtdIns(4,5)P_2. Note, however, that this loss was only partially accounted for by formation of water-soluble inositol phosphates, since GTP[S] also increased the hydrolysis of PtdIns(4,5)P_2 to PtdIns$P$. The mechanism by which GTP[S] elicits this increase is at present unknown. However, the effect appears to be specific for GTP[S], as it was not observed upon addition of ATP[S] (results not shown).

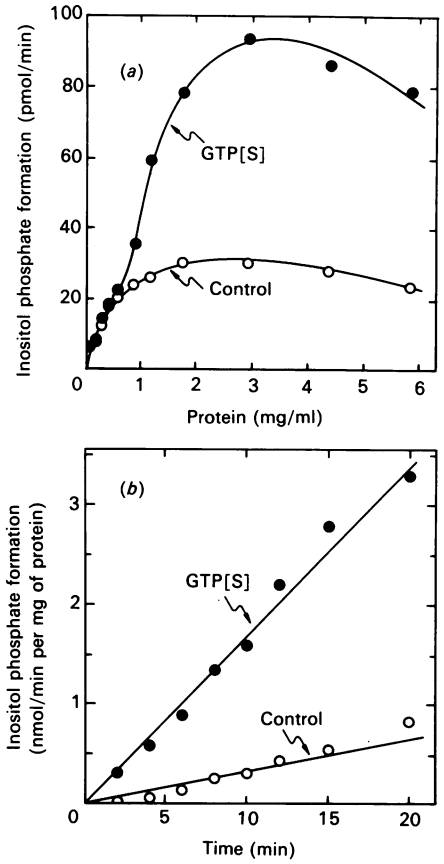

Fig. 4. Effect of protein concentration on basal and GTP[S]-stimulated inositol phosphate formation

In (*a*), HL-60-cell cytosol was incubated at increasing protein concentrations with phospholipid vesicles containing PtdIns(4,5)P_2 as described in the Materials and methods section. The incubation was performed for 15 min in the absence (○) or presence (●) of 100 μM-GTP[S]. Free Ca^{2+} was present at 0.1 μM. (*b*) shows a time course of the inositol phosphate accumulation obtained at the highest protein concentration used in (*a*) (5.8 mg/ml).

During the course of investigating the hydrolysis of PtdIns(4,5)P_2 by cytosolic preparations obtained from different batches of HL-60 cells, we noticed that both the specific activity of soluble phospholipase C and the ability of GTP[S] to stimulate this activity were critically dependent on the protein concentration used in the assay. This prompted us to study the effect of protein concentration on PtdIns(4,5)P_2 hydrolysis in more detail. As the precise composition of HL-60-cell cytosol is essentially unknown, the cytosolic preparation was concentrated by pressure filtration in a stirred cell equipped with a M_r-10000-cut-off membrane to generate an appropriate control buffer for the dilution experiment shown in Fig. 4(*a*). Several aspects of this experiment are significant. First, the accumulation of inositol phosphates was linear with protein only when low protein concentrations ($\leq$ 0.6 mg/ml) were used in the assay. Note, however, that GTP[S] led to little or no stimulation of enzyme activity over this range of low protein concentrations. Second, increasing the protein concentration above approx. 0.6 mg/ml caused only a small further increase in inositol phosphate formation in the absence of GTP[S]. This increase in protein concentration did, however, markedly enhance the stimulatory effect of GTP[S]. Specifically, the stimulation by addition of 100 μM-GTP[S] increased from 9% to 310% when the protein concentration was raised from 0.6 to 2.9 mg/ml. Finally, no further increase in inositol phosphate formation was obtained

even in the presence of GTP[S] at protein concentrations higher than ~ 3 mg/ml.

To examine whether the plateaus of inositol phosphate formation observed at high protein concentrations in the experiment shown in Fig. 4(*a*) were due to limiting amounts of substrate, a time course of inositol phosphate formation was performed at the highest concentration (5.8 mg/ml) used in this experiment. Fig. 4(*b*) demonstrates that generation of inositol phosphates was linear with time for up to 20 min, regardless of whether GTP[S] was absent or present in the incubation medium. Note that the experiment shown in Fig. 4(*a*) was performed for only 15 min. Consumption of substrate is therefore unlikely to be the cause for the plateaus observed in Fig. 4(*a*).

DISCUSSION

The results presented in this paper provide direct evidence that guanine nucleotides stimulate the hydrolysis of PtdIns(4,5)P_2 by a phospholipase C contained in cytosolic preparations of myeloid differentiated HL-60 cells. Our results differ from several other reports on guanine nucleotide regulation of cytosolic phospholipase C activities in that the cytosolic HL-60 enzyme was specifically stimulated by GTP[S], but not by other nucleoside triphosphates or guanine nucleoside diphosphates (cf. refs. [14,32–34]). Furthermore, neither pyrophosphate nor imidodiphosphate (100 μM), which reportedly stimulate hydrolysis of exogeneous PtdIns(4,5)P_2 by rat liver plasma membranes [35], had any effect on inositol phosphate formation by HL-60-cell cytosol (results not shown).

The observation that InsP_3 is the major inositol phosphate produced by GTP[S]-stimulated inositol phospholipid hydrolysis, together with the finding that GTP[S] leads to increased formation of PtdIns*P* from PtdIns(4,5)P_2, has several important implications for the mechanism of GTP[S]-induced inositol phosphate formation by HL-60-cell cytosol. First, it is unlikely that GTP[S] stimulates inositol phosphate formation indirectly by stimulating a PtdIns*P* kinase. This mechanism has previously been suggested for human placental and rat brain membranes [29,30], and has gained additional support from the very recent observation that chemoattractants may stimulate PtdIns*P* kinase activity in intact human polymorphonuclear leucocytes [36]. Although the precise mechanism(s) by which GTP[S] enhances the level of PtdIns*P* is unclear at present and requires further investigation, this finding clearly argues against an involvement of PtdIns*P* kinase in the effects of GTP[S] on inositol phosphate formation reported here. Second, the finding that GTP[S] leads to a major increase in InsP_3, but only minimally increases InsP_2, strongly suggests that the phospholipase activated by GTP[S] is of the C-type rather than the D-type. The latter possibility was raised by a report on the existence of a phosphatidylinositol-specific phospholipase D in post-nuclear fractions of human neutrophils [37]. Finally, the observation that only little InsP_2 is produced in response to GTP[S], although there is significant build-up of PtdIns*P* during the assay, argues strongly, but does not prove, that the phospholipase C stimulated by GTP[S] is relatively specific for PtdIns(4,5)P_2.

The observation of a GTP[S]-stimulated phospholipase C activity in HL-60-cell cytosol raises the important question to what extent does this activity participate in transmembrane signalling in intact granulocytes. Several previous findings on chemotactic-peptide- or GTP[S]-stimulated inositol phosphate formation in intact or permeabilized neutrophils or HL-60 cells [38–41] are pertinent to this issue. First, the finding that fMet-Leu-Phe does not stimulate inositol phosphate production in intact granulocytes when $[Ca^{2+}]_i$ is maintained at values lower than ~ 10–30 nM [38,39] is very consistent with the fact that little or no GTP[S] stimulation of inositol phosphate formation by HL-60-cell cytosol is observed at Ca^{2+} concentrations $\leqslant$ 10 nM (Fig. 3). Second, the Ca^{2+}-sensitivity of GTP[S]-stimulated inositol phosphate formation reported here for HL-60-cell cytosol is very similar to the values reported for GTP[S]-stimulated InsP_3 formation by permeabilized neutrophils or HL-60 cells [40,41]. However, markedly less Ca^{2+} appears to be required for stimulation of basal phospholipase C activity in HL-60-cell cytosol, compared with permeabilized neutrophils [40] or HL-60 cells [41]. The reasons for this discrepancy are not clear at present. Finally, it is noteworthy that the extent of GTP[S]-stimulated inositol phospholipid hydrolysis has been reported to be about 10-fold higher in permeabilized HL-60 cells than in HL-60-cell membranes [5,42]. Interestingly, addition of pig lymphocyte cytosol to HL-60-cell membranes restored the extent of inositol phospholipid hydrolysis to levels observed in permeabilized cells [5,42]. It is thus tempting to speculate that cytosolic phospholipase C enzymes may contribute significantly to GTP[S]-stimulated inositol phospholipid hydrolysis in permeabilized preparations, and may also be important for chemotactic-peptide-stimulated inositol phosphate formation in intact phagocytes.

Whether or not GTP[S] stimulates inositol phospholipid hydrolysis by HL-60-cell cytosol by direct interaction with soluble phospholipase C enzyme or by acting via soluble GTP-binding protein is at present unknown. Of interest in this regard, cytosolic preparations of myeloid differentiated HL-60 cells contain ample amounts of an ~ 40 kDa pertussis-toxin substrate (M. Camps & P. Gierschik, unpublished work). As stimulation of phospholipase C in intact HL-60 cells is well known to involve a pertussis-toxin-sensitive G-protein(s) [43–45] and agonist-activated formyl-peptide receptors have recently been shown to interact with G_{i2} and G_{i3} [12], i.e. two G-proteins with α-subunits with molecular masses in the 40 kDa range, it is likely that one of these α-subunits is also present in HL-60-cell cytosol to stimulate phospholipase C in this subcellular compartment. However, HL-60-cell cytosol also contains a variety of low-molecular-mass GTP-binding proteins (D. Sidiropoulos & P. Gierschik, unpublished work). Interestingly, low-molecular-mass GTP-binding proteins (M_r ~ 29000 and 25000) have recently been suggested to stimulate cytosolic phospholipase C activity in human platelets and calf thymocytes respectively [46–48]. Thus further experimentation will be required to determine whether α-subunits of heterotrimeric high-molecular-mass G-proteins or low-molecular-mass GTP-binding proteins are involved in regulating phospholipase C activity in HL-60-cell cytosol.

The observation that the specific activity of the cytosolic phospholipase C is markedly decreased at high protein concentrations, together with the finding that this decrease leads to a marked increase in the ability of GTP[S] to stimulate the enzyme, is most intriguing and requires further discussion. First, it is noteworthy that even the highest protein concentration used here (~ 10 mg/ml) is still substantially lower than that found in the cytosol of intact HL-60 cells. This is based on the fact that the non-concentrated cytosol preparations used here are diluted about 10-fold during cell homogenization and contain soluble proteins at concentrations of 5–10 mg/ml. It is unlikely that the plateau of enzymic activity observed even at protein concentrations in excess of 0.6 mg/ml in the absence of GTP[S] is due to consumption of substrate, because this should not allow for the marked stimulation of inositol phosphate formation by GTP[S] observed at these high protein concentrations. The linear time course of inositol phosphate formation observed even at the highest protein concentration for both basal and GTP[S]-stimulated activity also argues against this possibility. Thus the possibility has to be considered that the formation of inositol phosphates by HL-60-cell cytosol is under some inhibitory

constraint, which is only operative when the relevant components are present at the appropriate concentrations. The fact that GTP[S] appears to stimulate the phospholipase C enzyme only in its inhibited form suggests that GTP[S] acts via removal of this inhibitory constraint. Interestingly, a similar model for regulation of phospholipase C has recently been put forward on the basis of the following considerations [6]. First, the specific activity of all phospholipase C isoenzymes purified to date suffices to hydrolyse the total cellular PtdIns(4,5)P_2 within 2–20 s, even without stimulation. Second, none of the purified phospholipase C isoenzymes can be activated by reconstitution with a variety of purified G-proteins, suggesting that a negative regulator may have been lost during purification. Recently, Meisenhelder *et al.* [49] reported that immunoprecipitates of NIH 3T3 cells obtained by using anti-PLC-γ antibodies contained two proteins (M_r 47000 and 100000) in addition to the PLC-γ polypeptide. These two proteins were not related to PLC-γ by peptide mapping, and were found to be associated with PLC-γ in sucrose-density-gradient centrifugation experiments too. It is thus tempting to speculate that the relevant phospholipase C isoenzyme(s) of HL-60-cell cytosol may be associated with similar proteins, and that these proteins may be important for the inhibition of phospholipase C relieved by GTP[S].

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