Effect of guanine nucleotides on polyphosphoinositide synthesis in rat liver plasma membranes

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The effect of guanosine 5'-[γ -thio]triphosphate (GTP[S]) on PtdIns and PtdIns(4)*P* kinase activities was measured in rat liver plasma membranes. The addition of [32 P]ATP resulted in the rapid incorporation of 32 P into PtdIns(4)*P* and PtdIns(4,5)*P*₂, with maximal levels reached within 30 s. GTP[S] (25–500 μ M) increased the rate and magnitude of [32 P]PtdIns(4)*P* and [32 P]PtdIns(4,5)*P*₂ formation by 50 and 120% respectively. Similar stimulatory effects were induced by guanosine 5'-[β -imido]triphosphate, GTP, GDP and guanosine 5'-[β -thio]diphosphate. The stimulation of PtdIns phosphorylation by GTP[S] occurred in the presence of 2 mM-EGTA, a condition which fully inhibited phosphoinositide-specific phospholipase C. GTP[S] did not stimulate phosphomonoesterase activity, and its action was not due to the binding of magnesium. However, the overall ATP-hydrolysing activity of the membrane preparation was inhibited by GTP[S] and the other guanine nucleotides. There was a direct correlation between the extent of this inhibition and the stimulation of polyphosphoinositide formation. The results indicate that stimulation of polyphosphoinositide formation by guanine nucleotides in rat liver plasma membranes can be accounted for by an inhibition of ATP hydrolysis. These data are inconsistent with a specific GTP-binding protein (G-protein)-mediated stimulation of PtdIns or PtdIns(4)*P* kinase.

INTRODUCTION

The effects of a wide range of Ca²⁺-dependent hormones are brought about by the activation of phosphoinositide-specific phospholipase C and the formation of the second messenger products InsP₃ and 1,2-diacylglycerol (1,2-DG) [1,2]. The resultant mobilization of intracellular Ca²⁺ and the activation of protein kinase C, respectively, lead to co-ordinated cellular responses. Numerous studies have demonstrated that the activation of phospholipase C is mediated by a GTP-binding protein (G-protein) in a manner similar to that associated with the activation of adenylate cyclase [2,3]. The evidence for such a pathway in hepatocytes derives from studies of the effect of GTP and non-hydrolysable GTP analogues on phospholipase C activation in membranes and permeabilized cell preparations [4–6]. In these models, GTP analogues stimulate phospholipase C independently or can act synergistically with physiological agonists to activate the enzyme. A role for G-proteins has also been implicated in the activation of phospholipase A₂ [7,8], phospholipase D [9] and ion channels [10].

The levels of the polyphosphoinositides in hormone-responsive cells are tightly regulated by specific phosphokinases and phosphomonoesterases [11]. Several forms of phosphoinositide kinase have been identified which vary with respect to substrate specificity [12,13]. Specific phosphoinositide kinases have also been found in association with the platelet-derived growth factor (PDGF) receptor [14] and several oncogene products [15,16]. Coincident with the activation of phospholipase C is the stimulation of PtdIns and PtdIns(4)*P* kinases, an action which replenishes polyphosphoinositide substrates [11]. For example, in rat hepatocytes, the levels of the polyphosphoinositides initially decrease upon stimulation with vasopressin, but then stabilize or may actually increase, despite continued activation of phospholipase C [17]. This phenomenon appears to be exaggerated in rat hepatocytes [18] and human platelets [19] following the

activation of phospholipase C by the acute administration of ethanol. Similarly, the activities of the phosphoinositide kinases in human neutrophils have been shown to be increased on binding of receptors by the *N*-formylated peptide chemo-attractant fMetLeuPhe [20].

The mechanism by which polyphosphoinositide synthesis is stimulated is not known. The kinases could be activated by the phospholipase C-mediated depletion of PtdIns(4,5)*P*₂ and PtdIns(4)*P* (i.e. removal of feedback inhibition). Alternatively, more direct regulatory mechanisms may exist, such as an inhibition of the phosphomonoesterases, or, as has been suggested in several cell types, regulation of the kinases by protein kinase C [21], cyclic AMP-dependent protein kinase [22] or tyrosine kinases [2,23]. In a recent study [24], it was reported that the addition of the non-hydrolysable GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]) to rat liver plasma membranes could stimulate the incorporation of 32 P from [32 P]ATP into PtdIns(4,5)*P*₂. Pretreatment of the membranes with cholera toxin abolished this response. These data were interpreted as evidence for the direct stimulation of PtdIns(4)*P* kinase by a G-protein. Similar results have been reported in membranes prepared from human placenta [25] and rat brain [26].

In the present study, we examined the effect of GTP[S] and other guanine nucleotide analogues on PtdIns and PtdIns(4)*P* kinases in rat liver plasma membranes. GTP[S] enhanced the formation of both [32 P]PtdIns(4,5)*P*₂ and [32 P]PtdIns(4)*P*. However, we demonstrate that this effect was due to a protective effect of guanine nucleotides on ATP hydrolysis. Moreover, the data do not support a role for a specific G-protein in regulation of the phosphoinositide kinases in this preparation.

EXPERIMENTAL

Materials

[32 P]ATP (3000 Ci/mmol) was purchased from Amersham.

Abbreviations used: G-protein, GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; App[NH]p, adenosine 5'-[β -imido]triphosphate; PDGF, platelet-derived growth factor.

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myo-[2-³H]inositol (15 Ci/mmol in 95% ethanol) was purchased from American Radiolabeled Chemicals. Before use, the ethanol was removed by evaporation and the sample was treated with Dowex AG 1-X8 resin (Bio-Rad) to remove negatively charged impurities. All nucleotides and non-hydrolysable nucleotide analogues were obtained from Boehringer Mannheim. All other chemicals and biochemicals, of the highest purity commercially available, were purchased from Sigma or Fisher Scientific.

Preparation of rat liver plasma membranes

The liver from a 250–300 g Sprague–Dawley rat was perfused for 15 min with oxygenated Krebs–Ringer buffer: 120 mM-NaCl, 24 mM-NaHCO₃, 4.8 mM-KCl, 1.2 mM-MgSO₄, 1.2 mM-KH₂PO₄, 10 mM-Hepes, pH 7.4, 5 mM-glucose and 1.3 mM-CaCl₂. For labelling with [³H]inositol, the perfusion was then continued for 45 min with Krebs–Ringer buffer containing 1 mM-GSH, 2 mM-L-glutamine, 2 mM-sodium lactate, a mixture of amino acids (1:50 dilution of minimal essential medium amino acids; Gibco), 1.3 mM-CaCl₂, 0.2% BSA, and, where indicated, 0.02 mCi of *myo*-[³H]inositol/ml. The liver was then perfused for an additional 15 min with Krebs–Ringer buffer alone. After perfusion, the liver was placed in ice-cold homogenization buffer consisting of 250 mM-sucrose, 2 mM-EGTA, 5 mM-KOH/Hepes, pH 7.5, and 0.5 µg each of antipain, pepstatin, aprotinin and leupeptin/ml. Liver plasma membranes were then prepared by Percoll gradient centrifugation according to the method described by Prpic *et al.* [27]. The membranes were suspended at a protein concentration of 5–10 mg/ml in resuspension buffer consisting of 250 mM-sucrose and 50 mM-Tris/HCl (pH 7.8). The final specific activity of 5'-nucleotidase was increased by 18–24-fold over that in the initial homogenate.

Assay of PtdIns and PtdIns(4)P kinase activities

Membrane portions (0.1 ml) were preincubated at 30 °C for 2 min before mixing with an equal volume of pre-warmed phosphorylation buffer consisting of 4 mM-MgCl₂, 50 mM-Tris/HCl, pH 7.5, and 0.8 mM-[³²P]ATP (100 c.p.m./pmol). Unless otherwise indicated, the final Ca²⁺ concentration of the incubation buffer was buffered with an equimolar mixture of 0.5 mM-EGTA and 0.5 mM-CaEGTA. At the indicated times, the samples were quenched with 3.75 vol. of chloroform/methanol/HCl (40:20:1, by vol.). The phases were separated by addition of 1.25 vol. of each of 2 M-KCl/10 mM-EDTA and chloroform. The samples were then centrifuged for 2 min at 800 g, and the chloroform layers were removed. The remaining aqueous phases were washed with 2.5 vol. of chloroform, and the chloroform layers were combined. The chloroform samples were dried in a Rotovac evaporator, and the lipids were separated by t.l.c. on oxylate-treated silica-gel 60 plates (EM Reagents) with a solvent system of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) [28]. The location of the polyphosphoinositides was verified using authentic phospholipid standards. Following autoradiography, the ³²P radioactivity of the individual phospholipids was quantified by liquid-scintillation counting.

Determination of [³H]inositol phosphates

The aqueous phases of the chloroform/methanol/HCl extract were neutralized to pH 7.0 with 2 M-KOH/10 mM-Mops, pH 7.0, and diluted in 10 ml of water. Inositol phosphates were quantified by anion-exchange chromatography on 0.75 ml Dowex 1-X8 anion-exchange resin columns (formate form; 200–400 mesh). Free *myo*-inositol and glycerophosphoinositol were eluted with 15 ml of 25 mM-sodium formate/5 mM-sodium borate, and total inositol phosphates were eluted with 4 ml of 1 M-ammonium

formate/0.1 M-formic acid. The ³H content of the total inositol phosphate fraction was determined by liquid-scintillation counting.

Assay of [³²P]ATP hydrolysis

Membranes were incubated in phosphorylation buffer as described above. At the indicated times, the reaction was stopped by the addition of 4% HClO₄ and 10 mg of activated charcoal. The samples were allowed to sit at room temperature for 10 min and were then centrifuged at 800 g for 2 min, and the radioactivity in the supernatants was determined by liquid scintillation counting. In some experiments the HClO₄ pellets were resuspended in chloroform/methanol/HCl (40:20:1, by vol.), and the phospholipids were extracted as described above.

Statistics

Each experiment was performed using at least three plasma membrane preparations. Statistical significance was determined by Student's *t* test.

RESULTS

Incubation of rat liver plasma membranes with [³²P]ATP resulted in the rapid incorporation of ³²P into PtdIns(4)P and PtdIns(4,5)P₂ (Fig. 1). Maximal ³²P incorporation was reached within 15–30 s for both phospholipids. In separate experiments, the rate of [³²P]polyphosphoinositide formation was linear up to

Fig. 1. Effect of GTP[S] on the incorporation of ³²P from [³²P]ATP into polyphosphoinositides in rat liver plasma membranes

Rat liver plasma membranes were incubated at 30 °C with 0.4 mM-[³²P]ATP in the absence (○, □) or presence (●, ■) of 100 µM-GTP[S]. At the indicated times, samples were taken for measurement of the radioactivity in [³²P]PtdIns(4)P (a) and [³²P]PtdIns(4,5)P₂ (b), as described in the Experimental section. The data are the means ± S.E.M. of triplicate determinations from a representative experiment; **P* < 0.02 compared with control.

Fig. 2. Concentration-dependence for GTP[S] of the stimulation of $[^{32}\text{P}]$ polyphosphoinositide formation

Membranes were incubated with the indicated concentrations of GTP[S] in the presence of 0.4 mM- $[^{32}\text{P}]\text{ATP}$, as described for Fig. 1. At the end of 2 min, samples were taken for determination of the radioactivity within $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ (●) and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (■). The data are the means $\pm$ S.E.M. of values obtained from three separate experiments, with individual measurements expressed as a percentage of the value obtained in the absence of GTP[S] within that experiment.

Table 1. Effect of nucleotides and non-hydrolysable nucleotide analogues on $[^{32}\text{P}]$ polyphosphoinositide formation

Plasma membranes were phosphorylated for 2 min in the absence or presence of the indicated nucleotides. The data are expressed as the percentage increase over control of the radioactivity within $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$, and are the means of duplicate determinations from a representative experiment, with values varying by less than 10%.

Addition	Increase in $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ (%)	Increase in $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (%)
GTP[S] (100 μM)	91	165
GTP[S] (1 mM)	284	372
Gpp[NH]p (100 μM)	74	112
Gpp[NH]p (1 mM)	265	296
GTP (100 μM)	23	52
GTP (1 mM)	52	50
GDP (100 μM)	17	10
GDP (1 mM)	75	32
GDP[S] (100 μM)	23	32
GDP[S] (1 mM)	134	92
GDP[S] (1 mM)	197	163
+ GTP[S] (100 μM)		
App[NH]p (100 μM)	-7	3
App[NH]p (1 mM)	23	57
ADP (100 μM)	-18	-8
ADP (1 mM)	-29	5
CTP (100 μM)	-35	-11
CTP (1 mM)	-38	-42
UTP (100 μM)	-3	18
UTP (1 mM)	-15	-8

10 s (results not shown). The level of $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ had declined by 25% at 5 min, while $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ levels declined more slowly. GTP[S] (100 μM) increased the ^{32}P labelling of $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ by 50% and 120% respectively at 15 s. In the presence of GTP[S], $[^{32}\text{P}]$ polyphosphoinositide levels remained elevated above control values for 2 min. However, the effect of GTP[S] subsequently declined and was not significant at 5 min. Fig. 2 illustrates the concentration-dependence for GTP[S] of the stimulation of $[^{32}\text{P}]$ polyphosphoinositide formation. GTP[S] enhanced ^{32}P incorporation at concentrations between 25 and 1000 μM , with maximal stimulation occurring at approx. 500 μM -GTP[S].

The specificity of the action of GTP[S] was examined in the experiment in Table 1. Other guanine nucleotides also stimulated the formation of $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ with a relative potency of GTP[S] > guanosine 5'-[β -imido]triphosphate (Gpp[NH]p) > guanosine 5'-[β -thio]diphosphate (GDP[S]) > GDP $\geq$ GTP. GDP[S] (1 mM), which caused a partial enhancement on its own, did not reverse the response to GTP[S]. ADP, CTP and UTP did not enhance ^{32}P incorporation into phospholipids, but 1 mM-App[NH]p had a slight stimulatory effect.

The stimulation of ^{32}P incorporation into the polyphosphoinositides by GTP[S] could be due to a direct activation of PtdIns and $\text{PtdIns}(4)\text{P}$ kinases. Alternatively, such effects might occur on activation of phospholipase C by GTP[S], thereby resulting in the removal of feedback inhibition by the product lipids. To test this hypothesis, the formation of inositol phosphates in $[^3\text{H}]$ inositol-prelabelled membranes was used as a measure of phospholipase C activity (Fig. 3). Maximal stimulation of $[^3\text{H}]$ inositol phosphate formation was observed at 1 μM -GTP[S]. This concentration of GTP[S] was below the threshold for activation of $[^{32}\text{P}]$ polyphosphoinositide formation (see Fig. 2).

Fig. 3. Effect of GTP[S] on inositol phosphate formation in rat liver plasma membranes

$[^3\text{H}]$ Inositol-labelled membranes were incubated at 30 $^{\circ}\text{C}$ with the indicated concentrations of GTP[S] in the absence (○) or presence (●) of 2 mM-EGTA. After 2 min, samples were obtained for analysis of total $[^3\text{H}]$ inositol phosphates as described in the Experimental section. Data are expressed as percentages of basal control values obtained in the absence of GTP[S] and are the means $\pm$ S.E.M. of values obtained from three separate experiments.

Table 2. Effect of EGTA on the stimulation of [32 P]polyphosphoinositide formation by GTP[S]

Plasma membranes were incubated with 0.4 mM- $[^{32}\text{P}]\text{ATP}$ at 30 °C for 2 min in the presence of either 0.5 mM-EGTA/0.5 mM-EGTA/ CaCl_2 (+ Ca^{2+}) or 2 mM-EGTA alone ($-\text{Ca}^{2+}$). Where indicated, 100 μM -GTP[S] was included in the incubation buffer. Samples were obtained for determination of the radioactivity (c.p.m.) in [^{32}P]PtdIns(4) P and [^{32}P]PtdIns(4,5) P_2 as described in the Experimental section. The data are the mean $\pm$ S.E.M. of triplicate determinations from a representative experiment; * $P < 0.01$ compared with control.

	[^{32}P]PtdIns(4) P		[^{32}P]PtdIns(4,5) P_2	
	(c.p.m.)	(% stimulation)	(c.p.m.)	(% stimulation)
+Ca^{2+}				
Control	2377 $\pm$ 306		781 $\pm$ 130	
GTP[S]	3708 $\pm$ 859*	56	1777 $\pm$ 367*	127
-Ca^{2+}				
Control	1198 $\pm$ 342		367 $\pm$ 79	
GTP[S]	1791 $\pm$ 556*	50	803 $\pm$ 164*	118

Fig. 4. Mg^{2+} -dependence of the stimulation of polyphosphoinositide synthesis by GTP[S]

Rat liver plasma membranes were incubated with 0.4 mM- $[^{32}\text{P}]\text{ATP}$ for 2 min at 30 °C with the indicated concentrations of MgCl_2 in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 100 μM -GTP[S]. (a) [^{32}P]PtdIns(4) P ; (b) [^{32}P]PtdIns(4,5) P_2 . Each point represents the mean of duplicate determinations from a representative experiment, with the differences varying by less than 10%.

Inositol phosphate formation in response to GTP[S] was completely inhibited in the presence of 2 mM-EGTA (Fig. 3). The effect of EGTA on the stimulation of [^{32}P]polyphosphoinositide formation by GTP[S] is shown in Table 2. In the presence of

EGTA, the levels of [^{32}P]PtdIns(4) P and [^{32}P]PtdIns(4,5) P_2 were both decreased by approx. 50%. However, the potency of GTP[S] for the stimulation of ^{32}P incorporation into both phospholipids was unchanged by removal of Ca^{2+} . Thus, taken together with the differences in the GTP[S] dose responses, these data suggest that the stimulatory effect of GTP[S] on [^{32}P]polyphosphoinositide formation occurs independently of the stimulation of phospholipase C.

The rates of [^{32}P]polyphosphoinositide accumulation could be influenced by the activity of the phosphomonoesterases which dephosphorylate PtdIns(4) P and PtdIns(4,5) P_2 . The activity of phosphomonoesterases is influenced by magnesium [29]. Therefore the stimulation of [^{32}P]polyphosphoinositide synthesis by GTP[S] could be due to competition for magnesium. Fig. 4 illustrates that [^{32}P]polyphosphoinositide formation increased directly with magnesium concentration in the range 0.2–2 mM. GTP[S] increased ^{32}P incorporation into the phospholipids at MgCl_2 concentrations of 0.4 mM and above, a concentration that was equivalent to the added ATP concentration. The percentage stimulation by GTP[S] was unchanged over the range of Mg^{2+} concentrations of 0.4–2 mM. Thus the effect of GTP[S] was not overcome at higher concentrations of Mg^{2+} . For a more direct measurement of phosphomonoesterase activity, membranes were first phosphorylated with [^{32}P]ATP for 2 min, followed by the rapid removal of the ATP with glucose and hexokinase (Fig. 5). Under these conditions, the maximum decrease in the level of [^{32}P]PtdIns(4) P was only 30%, and the amount of [^{32}P]PtdIns(4,5) P_2 was unchanged. GTP[S] had no effect on phosphomonoesterase activity assayed in this manner.

As shown in Fig. 1, the levels of the polyphosphoinositides reached a plateau and even declined slowly after reaching peak levels at 15–30 s. This could be due to phosphomonoesterase activity coming into balance with kinase activity. However, phosphomonoesterase activity is very low under these conditions (Fig. 5). An alternative explanation could be that the removal of

Fig. 5. Effect of GTP[S] on phosphomonoesterase activity in rat liver plasma membranes

Plasma membranes were phosphorylated with [^{32}P]ATP in the presence of 2 mM-EGTA in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 100 μM -GTP[S]. After 2 min, [^{32}P]ATP was removed by the addition of 10 mM-glucose/hexokinase (10 units/ml) at time zero. At the indicated times thereafter, samples were obtained for analysis of [^{32}P]PtdIns(4) P ($\circ$, $\bullet$) and [^{32}P]PtdIns(4,5) P_2 ($\square$, $\blacksquare$), as described in the Experimental section. Data are the means $\pm$ S.E.M. of triplicate determinations from a representative experiment.

Fig. 6. Effect of GTP[S] on ATP hydrolysis by rat liver plasma membranes

Plasma membranes were incubated with 0.4 mM- $[^{32}\text{P}]\text{ATP}$ in the absence (●) or presence of 100 μM -GTP[S] (■) or 500 μM -GTP[S] (▲). At the indicated times, samples were removed and quenched in HClO_4 for quantification of release of $[^{32}\text{P}]\text{P}_i$, as described in the Experimental section. The data are the means $\pm$ S.E.M. of triplicate determinations from a representative experiment.

Fig. 7. Effect of ATP concentration of the stimulation of $[^{32}\text{P}]\text{polyphosphoinositide}$ synthesis by GTP[S]

Plasma membranes were phosphorylated with the indicated concentrations of $[^{32}\text{P}]\text{ATP}$ (100 000 c.p.m./assay) for 1 min in the absence (○, □) or presence (●, ■) of 100 μM -GTP[S]. ○, ●, $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$; □, ■, $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$. The data are the means $\pm$ S.E.M. of triplicate determinations from a representative experiment.

ATP by various ATPases may limit its use as a substrate for the phosphoinositide kinases. The hydrolysis of ATP by liver membranes was determined by the measurement of $[^{32}\text{P}]\text{P}_i$ release (Fig. 6). In the absence of GTP[S], ATP hydrolysis was rapid, with maximal $^{32}\text{P}[\text{P}_i]$ release observed between 30 and 60 s. This

amounted to an approx. 75% decrease in the initial concentration of ATP (results not shown). Thus inositol lipid phosphorylation appears to be limited by the availability of ATP in this membrane preparation. Furthermore, the hydrolysis of ATP was inhibited by GTP[S] in a concentration-dependent manner. At 30 s, 100 μM - and 500 μM -GTP[S] inhibited ATP hydrolysis by 35% and 70% respectively. However, at later time points, the total release of $[^{32}\text{P}]\text{P}_i$ in the presence of GTP[S] approached control values. This suggests that the present effects of GTP[S] could be accounted for by a decrease in the rate of non-specific ATP hydrolysis.

If the sparing of ATP by GTP[S] allows for increased $[^{32}\text{P}]\text{polyphosphoinositide}$ formation, it would be expected that the inclusion of excess ATP would lower the potency of GTP[S]. Indeed, the experiment shown in Fig. 7 demonstrates that the stimulation of $[^{32}\text{P}]\text{polyphosphoinositide}$ synthesis by GTP[S] could be overcome by increasing the concentration of ATP. At 5 mM-ATP, the stimulatory effect of 100 μM -GTP[S] was abolished when measured at 1 min. It is of note that the formation of $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ proceeded linearly for up to 1 min in the presence of 5 mM-ATP, and was not affected by

Fig. 8. Correlation between the inhibition of ATP hydrolysis and enhanced synthesis of $[^{32}\text{P}]\text{polyphosphoinositides}$

Liver plasma membranes were incubated with $[^{32}\text{P}]\text{ATP}$ in the absence or presence of the indicated nucleotides (500 μM each). After 30 s, samples were taken for analysis of $[^{32}\text{P}]\text{P}_i$ release and the formation of $[^{32}\text{P}]\text{PtdIns}(4)\text{P}$ (a) and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (b). The data represent the means of duplicate determinations from a representative experiment with the differences varying by less than 10%, and are expressed as percentages of control values for samples incubated in the presence of $[^{32}\text{P}]\text{ATP}$ alone.

GTP[S] (results not shown). However, at 5 min, the levels of PtdIns(4)P and PtdIns(4,5)P₂ were enhanced in the presence of GTP[S] by 42 % and 160 % respectively. Thus these data indicate that GTP[S] does not stimulate the initial rates of polyphosphoinositide formation, but increases the final phospholipid levels by preserving substrate levels of ATP.

In Fig. 8, the effects of various guanine nucleotides on ATP hydrolysis are compared with their effects on [³²P]polyphosphoinositide formation. There was a good correlation between the ability of the guanine nucleotides to lower ATP hydrolysis and to enhance [³²P]polyphosphoinositide formation. App[NH]p and ADP (each 500 μM) also inhibited ATP hydrolysis by approx. 20–25 %. However, neither nucleotide increased ³²P incorporation into phospholipid. A lower concentration of GTP[S] (10 μM) did not inhibit the initial linear rates of ATP hydrolysis (within 10 s), and was without effect on [³²P]phospholipid formation over the same time period. Lastly, it has been demonstrated in several systems [30] that the activation of G-proteins by non-hydrolysable GTP analogues is maximal following a lag period. However, in the present membrane preparation, a 2 min preincubation with 10 μM-GTP[S] was ineffective in enhancing [³²P]polyphosphoinositide formation.

DISCUSSION

In the present study, we have investigated the hypothesis that the kinases responsible for the synthesis of the polyphosphoinositides from PtdIns are regulated by G-protein. In agreement with a previous report [24], the data demonstrate that GTP[S] can enhance the ATP-dependent synthesis of PtdIns(4,5)P₂. Moreover, in this study, GTP[S] also increased the synthesis of PtdIns(4)P. However, the data do not support a role for a specific G-protein in this response. The concentrations of GTP[S] required to elicit these effects (25–500 μM) far exceed those required for other G-protein-dependent processes in rat liver plasma membranes [4,5,9]. In particular, phospholipase C activity in the present membrane preparation was maximal at 1 μM-GTP[S] (Fig. 3). Moreover, the action of GTP[S] was not specific, as other guanine nucleotides also stimulated polyphosphoinositide synthesis. GDP[S], an agent that generally blocks G-protein-mediated events [30], was also effective in stimulating polyphosphoinositide formation and did not prevent such actions by GTP[S].

Another explanation for the action of GTP[S] is its phospholipase C-mediated depletion of the polyphosphoinositides, thereby leading to removal of feedback inhibition of the kinases. The present data are inconsistent with this notion. The ability of GTP[S] to stimulate ³²P incorporation into the polyphosphoinositides was unaffected in the absence of phospholipase C activity (Table 2). Moreover, GTP[S] did not stimulate ³²P incorporation into phospholipid at the relatively low concentrations which maximally activated phospholipase C (Figs. 1 and 3). The data presented here also demonstrate that the action of GTP[S] cannot be explained on the basis of an inhibition of phosphomonoesterase either directly (Fig. 5) or by binding to Mg²⁺ (Fig. 4).

As an alternative mechanism for the action of guanine nucleotides, we have analysed the relationship between ATP and polyphosphoinositide levels. ATP hydrolysis by the plasma membranes occurred rapidly and was maximal within 60 s. The loss of ATP corresponded closely with the cessation of polyphosphoinositide formation. However, only a small portion of the ATP hydrolysis can be accounted for on the basis of the phosphorylation of PtdIns and PtdIns(4)P. Under the current experimental conditions (e.g. Fig. 1), approx. 20 μmol of ATP was hydrolysed in 15 s. This contrasts with polyphosphoinositide

formation of less than 50 pmol over the same time period (based on the specific radioactivity of [³²P]ATP). Several lines of evidence support the conclusion that the sparing of ATP hydrolysis by guanine nucleotides accounts for their stimulatory effect on polyphosphoinositide formation. First, the potency of the various guanine nucleotides was directly proportional to their ability to inhibit ATP hydrolysis (Fig. 8). Secondly, the stimulatory effect of GTP[S] was abolished by increasing the initial concentration of ATP (Fig. 7). Thirdly, a low concentration of GTP[S] (10 μM), which was maximally effective in stimulating phospholipase C, neither inhibited ATP hydrolysis nor stimulated polyphosphoinositide synthesis. Both App[NH]p and ADP also inhibited ATP hydrolysis, yet did not stimulate polyphosphoinositide formation. A possible explanation for this discrepancy is that these adenine nucleotides may be competitive inhibitors of ATP for the kinase [31]. Indeed, it is probably the decrease in the ATP/ADP ratio, rather than the absolute fall in ATP levels, which limits polyphosphoinositide formation as ATP is hydrolysed. It is of note that Coco & Macara [32] have demonstrated that the stimulation of polyphosphoinositide formation in rat lymphocyte membranes by the catalytic subunit of protein kinase A may also be due to the sparing of ATP hydrolysis. Furthermore, in related studies, 10 μM-GTP[S] did not increase the activity of PtdIns kinase in platelet plasma membrane preparations (R. Rubin, unpublished work).

The major ATPase in rat liver plasma membranes is a Ca²⁺- or Mg²⁺-stimulated ecto-ATPase (unrelated to the plasma membrane Ca²⁺ pump) [33,34]. The function of this enzyme complex is not known, but it may regulate P₂-purinergic receptor activation by degrading extracellular ATP and thereby terminating its agonist effects. The enzyme has been reported to possess broad specificity with respect to nucleotides, and GTP can effectively replace ATP as a substrate for both the Ca²⁺- and Mg²⁺-dependent activities [33]. Thus it is possible that the non-hydrolysable guanine nucleotide analogues used in the present study interfere with this enzyme activity, preserving ATP levels for inositol lipid phosphorylation.

The mechanism by which the phosphoinositide kinases are stimulated in response to hormones in intact cells remains in question. The stimulation of polyphosphoinositide formation by high concentrations of GTP[S] in rat liver plasma membranes has been reported previously by Urumow & Wieland [24]. In their study, cholera toxin reversed the action of GTP[S], an effect which was interpreted as evidence for a role for a G-protein. However, cholera toxin also markedly inhibited the basal phosphorylation of PtdIns and PtdIns(4)P. Similar inhibitory effects of cholera toxin on polyphosphoinositide formation were noted earlier by Biffen & Martin [35], and were shown to be unrelated to changes in ATP hydrolysis.

In a recent report [26], the synthesis of PtdIns(4,5)P₂ in rat brain membranes was also increased in the presence of GTP[S]. In that model, the concentration-dependence of GTP[S] was within the range reported for other G-protein-mediated events, with maximal activity reported between 1 and 10 μM. Moreover, in contrast with the findings in our study, GDP[S] partially inhibited the action of GTP[S] in brain membranes. Therefore it remains a possibility that a specific G-protein can regulate the PtdIns(4)P kinase in some systems. It is of note that these studies differed significantly from ours in that the brain membrane kinase was measured in detergent-solubilized preparations, and PtdIns(4)P was presented as exogenous lipid. ATP hydrolysis was measured only in the presence of 10 μM-GTP[S]. Treatment of rat liver plasma membranes with Triton X-100 enhanced the rate of ATP hydrolysis, but 10 μM-GTP[S] was still without effect on the formation of polyphosphoinositides (C. Benistant, unpublished work).

Recent studies in a variety of cell types have demonstrated that a specific PtdIns kinase may be associated with receptors for PDGF and oncogene products [14–16]. Moreover, there is evidence that this kinase may be a substrate for tyrosine phosphorylation, an event which could directly increase the activity of the enzyme [2,22]. It is of particular interest that the kinase involved in these pathways, kinase I, specifically phosphorylates PtdIns to the novel polyphosphoinositide, PtdIns(3)*P* [12,13]. The regulation of phosphoinositide kinases by phosphorylation has also been suggested to be mediated in some systems by protein kinase C [21] and protein kinase A [22]. It remains to be determined whether the direct phosphorylation of the kinases responsible for the formation of PtdIns(4)*P* and PtdIns(4,5)*P*₂ (kinase II) can also lead to increases in activity.

In summary, we have demonstrated that high concentrations of guanine nucleotides can interfere with the non-specific hydrolysis of ATP by liver plasma membranes, leading to increased availability of ATP for inositol lipid phosphorylation. These studies should raise concerns about the use of these agents in ATP-dependent processes in membranes and permeable cell preparations. Similar problems with guanine nucleotides could also occur in intact cells. For example, Krishnamurthi *et al.* [36] have demonstrated that GDP[S] and other non-hydrolysable analogues inhibit platelet activation in response to a variety of physiological agonists, an effect which can be separated from its inhibitory effect on phospholipase C.

We gratefully acknowledge the excellent technical assistance of Mr. Stephen Gross. This work was supported by U.S. Public Health Service Grants AA 07309, AA 07186 and DK 38422.

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Received 9 May 1990; accepted 29 June 1990