Guanine-nucleotide and hormone regulation of polyphosphoinositide phospholipase C activity of rat liver plasma membranes

Bivalent-cation and phospholipid requirements

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The effect of the GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[S]) on the polyphosphoinositide phospholipases C (PLC) of rat liver was examined by using exogenous [^{3} H]phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2]. GTP[S] stimulated the membrane-bound PLC up to 20-fold, with a half-maximal effect at approx. 100 nM. Stimulation was also observed with guanosine 5'-[β -imido]triphosphate, but not with adenosine 5'-[γ -thio]triphosphate, and was inhibited by guanosine 5'-[β -thio]diphosphate. Membrane-bound PLC was entirely Ca^{2+} -dependent, and GTP[S] produced both a decrease in the Ca^{2+} requirement and an increase in activity at saturating [Ca^{2+}]. The stimulatory action of GTP[S] required millimolar Mg^{2+} . [8-arginine]Vasopressin (100 nM) stimulated the PLC activity approx. 2-fold in the presence of 10 nM-GTP[S], but had no effect in the absence of GTP[S] or at 1 μ M-GTP[S]. The hydrolysis of PtdIns(4,5) P_2 by membrane-bound PLC was increased when the substrate was mixed with phosphatidylethanolamine, phosphatidylcholine or various combinations of these with phosphatidylserine. With PtdIns(4,5) P_2 , alone or mixed with phosphatidylcholine, GTP[S] evoked little or no stimulation of the PLC activity. However, maximal stimulation by GTP[S] was observed in the presence of a 2-fold molar excess of phosphatidylserine or various combinations of phosphatidylethanolamine and phosphatidylserine. Hydrolysis of [^{3} H]phosphatidylinositol 4-phosphate by membrane-bound PLC was also increased by GTP[S]. However, [^{3} H]phosphatidylinositol was a poor substrate, and its hydrolysis was barely affected by GTP[S]. Cytosolic PtdIns(4,5) P_2 -PLC exhibited a Ca^{2+} -dependence similar to that of the membrane-bound activity, but was unaffected by GTP[S]. It is concluded that rat liver plasma membranes possess a Ca^{2+} -dependent polyphosphoinositide PLC that is activated by hormones and GTP analogues, depending on the Mg^{2+} concentration and phospholipid environment. It is proposed that GTP analogues and hormones, acting through a guanine nucleotide-binding protein, activate the enzyme mainly by lowering its Ca^{2+} requirement.

INTRODUCTION

The polyphosphoinositide phospholipase C of the plasma membrane catalyses the hormone-stimulated breakdown of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] to form the Ca^{2+} -mobilizing agent *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] and the protein kinase C activator 1,2-diacylglycerol (DAG) [1–5]. A guanine nucleotide-regulatory protein (G protein), often termed G_p , has been implicated in the coupling of receptor occupation to PtdIns(4,5) P_2 hydrolysis by studies demonstrating increased Ins(1,4,5) P_3 release, in response to GTP and its non-hydrolysable analogues, from endogenously labelled membranes and permeabilized cells from a variety of tissues [6–13]. Islet-activating protein, which inhibits the function of the inhibitory G protein of the adenylate cyclase system by ADP-ribosylation of its α subunit [14,15], also inhibits

agonist- and guanine nucleotide-stimulated phosphoinositide breakdown in some cell types (reviewed in [5]), but not in others, including liver [6].

Studies utilizing exogenous labelled PtdIns(4,5) P_2 have shown that guanine nucleotide-stimulated polyphosphoinositide breakdown is a consequence of activation of a membrane-bound phospholipase C (PLC). Thus agonist- and guanine nucleotide-dependent stimulation of PtdIns(4,5) P_2 -PLC activity has been observed in membranes from blowfly salivary glands [16], transformed mink lung epithelial cells [17], coronary-artery smooth muscle [18], fibroblasts [19] and rat liver [20]. However, these reports have not examined the effects of other phospholipids on the guanine nucleotide stimulation, and have presented diverse results with respect to its Ca^{2+} - and Mg^{2+} -dependence.

Phosphoinositide PLC activities are present in both membranous and cytosolic fractions of most cell types

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; Ins(1,4,5) P_3 , *myo*-inositol 1,4,5-trisphosphate; Ins(1,4) P_2 , *myo*-inositol 1,4-bisphosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PLC, phospholipase C; G protein, guanine nucleotide-binding protein; G_p , the putative regulatory G protein of phospholipase C; DAG, 1,2-diacylglycerol.

studied, and generally predominate in the latter fraction. Soluble phosphoinositide PLC has been purified to varying degrees from various sources, including platelets [21,22], seminal vesicles [23,24], liver [25,26] and brain [26,27]. Although these purified enzymes generally exhibit non-specificity as regards the phosphoinositide substrate, polyphosphoinositides are hydrolysed in preference to PtdIns at low Ca^{2+} concentrations [21,22,24,26,28]. However, since no membrane-associated phosphoinositide PLC has yet been purified to homogeneity, it is not known whether the soluble and particulate enzymes represent a single ambiguous activity or two (or more) distinct activities. In this respect, it is of interest that guanine nucleotide-stimulation of soluble PtdIns(4,5) P_2 -PLC from human platelets has been demonstrated [29,30].

The phospholipid composition of phosphoinositide-containing vesicles has been shown to influence markedly polyphosphoinositide hydrolysis by soluble PLC. In particular, phosphatidylethanolamine (PtdEtn) stimulates and phosphatidylcholine (PtdCho) inhibits hydrolysis of PtdIns(4,5) P_2 [22,24,31]. However, stimulation of PtdIns(4,5) P_2 hydrolysis by a partially purified membrane-bound PLC from thymocytes has been observed with both PtdEtn and PtdCho [28].

The present studies demonstrate that the stimulation of membrane-bound PtdIns(4,5) P_2 -PLC activity from liver by guanine nucleotides requires the presence of physiological concentrations of Ca^{2+} and Mg^{2+} , and that the extent of stimulation is profoundly influenced by the phospholipid composition of the PtdIns(4,5) P_2 -containing vesicles. Furthermore, under conditions in which membrane-bound PtdIns(4,5) P_2 -PLC is maximally stimulated, no effect of guanine nucleotides on soluble PtdIns(4,5) P_2 -PLC is observed.

MATERIALS AND METHODS

Materials

[^3H]PtdIns(4,5) P_2 , [^3H]PtdIns4P and [^3H]PtdIns were obtained from New England Nuclear. Guanine and adenine nucleotides were from either Boehringer Mannheim or Sigma. Phosphoinositides (bovine brain), PtdIns (bovine liver), [8-arginine]vasopressin, bovine serum albumin (fraction V) and neomycin sulphate were from Sigma. PtdCho (pig liver), PtdEtn (pig liver) and PtdSer (pig brain) were from Serdary. AG 1-X8 anion-exchange resin was from Bio-Rad, and sodium cholate was from Calbiochem.

Preparation of subcellular fractions

Plasma membranes were prepared from rat liver, in the presence of 1 mM-EGTA, by the method of Prpic *et al.* [32], and were resuspended in 0.25 M-sucrose/5 mM-Hepes (pH 7.4)/1 mM-EGTA. Cytosol was prepared by centrifugation of the 1500 g-10 min supernatant at 100 000 g for 60 min. Membranes and cytosol were stored at -70°C for no longer than 2 weeks.

Preparation of substrates

PtdIns(4,5) P_2 and PtdIns4P were prepared from mixed phosphoinositides (Sigma) by chromatography on neomycin-linked glass beads (Glycophase CPG; Pierce), essentially as described by Schacht [33]. The purity of prepared phosphoinositides was checked by t.l.c. Before use in assays, phosphoinositides in chloroform solution

were mixed with [^3H]phosphoinositides and other phospholipids, as indicated, dried under N_2 , and the required volume of 50 mM-Hepes (pH 7)/100 mM-NaCl was added. Phospholipids were dispersed by vortex-mixing for 5 min, followed by brief sonication, under N_2 , in an ultrasonic cleaning bath. Phospholipid dispersions were used in PLC assays within 2 h of preparation.

Phospholipase C assay

PLC activity was assayed in duplicate, at 37°C , in a final volume of 0.2 ml, containing (unless otherwise stated) 50 mM-Hepes, pH 7, 100 mM-NaCl, 0.5 mg of bovine serum albumin/ml, 0.1% (w/v) sodium cholate, 2.5 mM- MgCl_2 , 0.2 mM-[^3H]PtdIns(4,5) P_2 /[^3H]PtdIns4P (800–1200 c.p.m./nmol) presented as a mixture with PtdEtn and PtdSer [PtdIns(4,5) P_2 /PtdIns4P:PtdEtn:PtdSer, 1:2:2 (molar proportions)] and 25–35 μg of membrane or cytosol protein. PtdIns-PLC activity was assayed with either [^3H]PtdIns or [^3H]PtdIns:PtdSer (1:2 molar ratio). Free Ca^{2+} concentrations were maintained with Ca^{2+} /EGTA buffers with 2 mM-EGTA, as determined by the COMICS program [34]. Unless otherwise indicated, the free Ca^{2+} concentration was 3.4 μM . Assays were initiated by the addition of enzyme and terminated by the addition of 200 μl of 10% (v/v) trichloroacetic acid, followed by 100 μl of bovine serum albumin (10 mg/ml). After 10–15 min on ice, the reaction mixtures were centrifuged (4 min at 9000 g) and 0.4 ml of the supernatant was counted for radioactivity. Blank assays were performed either in the absence of Ca^{2+} or by addition of enzyme after trichloroacetic acid.

In some experiments, labelled inositol phosphates were separated by ion-exchange chromatography on AG 1-X8 as described by Berridge [35]. For analysis of labelled phosphoinositides, reactions were terminated by the addition of 1 ml of chloroform/methanol/conc. HCl (1000:1000:6, by vol.), followed by 0.3 ml of 1 M-HCl/5 mM-EGTA. Samples of the lower (chloroform) phase were run on t.l.c. plates, and bands corresponding to phosphoinositide standards were scraped off and counted for radioactivity.

Other methods

Protein was assayed with the Pierce protein assay kit, with bovine serum albumin as standard.

RESULTS

Characteristics of guanine nucleotide-stimulated plasma-membrane-bound PtdIns(4,5) P_2 -PLC

The effect of the non-hydrolysable GTP analogue GTP[S] on the activity of membrane-bound PtdIns(4,5) P_2 -PLC was examined in an assay system employing exogenously added [^3H]PtdIns(4,5) P_2 , which was presented as a mixture with PtdEtn and PtdSer in the molar proportions PtdIns(4,5) P_2 :PtdEtn:PtdSer 1:2:2. Sodium cholate (0.1%, w/v) was also included in assays, since at this concentration it stimulated PtdIns(4,5) P_2 -PLC activity by 3–5-fold, but had little effect on either the Ca^{2+} -concentration/activity profile or the percentage stimulation of activity by GTP[S] (results not shown; see also Fig. 5). The specific activities of PtdIns(4,5) P_2 -PLC obtained from different experiments and plasma-membrane preparations were found to be very similar,

Fig. 1. Time course of membrane-bound PtdIns(4,5)P₂-PLC activity

PtdIns(4,5)P₂-PLC activity was assayed as described in the Materials and methods section in the presence of 3.4 μM-Ca²⁺, with [³H]PtdIns(4,5)P₂:PtdEtn:PtdSer (1:2:2) as substrate. (a) Water (●) or 10 μM-GTP[S] (■) was added at zero time (····) or 5 min (—), and the trichloroacetic acid supernatants obtained at each time point were assayed for radioactivity. (b) PLC activity was assayed in the presence of 3 mM-2,3-diphosphoglycerate. Saline (●) or 10 μM-GTP[S] (■) was added at 0 min, and the trichloroacetic acid supernatants were assayed for total radioactivity (●, ■) or were chromatographed [35] to separate the [³H]inositol phosphates formed in the presence of GTP[S]: ◆, Ins(1,4,5)P₃; ▲, Ins(1,4)P₂. Results are means of two experiments each.

although slight variation in the magnitude of stimulation by GTP[S] was observed between some sets of experiments.

Under various assay conditions, GTP[S] stimulated membrane-bound PtdIns(4,5)P₂-PLC activity by 2–20-fold. In the absence of GTP[S], PtdIns(4,5)P₂-PLC activity was linear with respect to time for at least 15 min (Fig. 1a). Addition of 10 μM-GTP[S] at 0 min caused a marked stimulation after a slight lag (Fig. 1a). This lag is apparently due to a delay in GTP[S] action, possibly in its binding to G_p, rather than delayed enzyme–substrate accessibility, since it was still seen when the nucleotide was added after 5 min (Fig. 1a).

Analysis of the reaction products formed in the presence of 10 μM-GTP[S] and 3 mM-2,3-diphosphoglycerate, which is an inhibitor of Ins(1,4,5)P₃ phosphatase, revealed that the initial reaction product was [³H]Ins(1,4,5)P₃, with [³H]Ins(1,4)P₂ being formed at later time points (Fig. 1b). *myo*-[³H]Inositol and *myo*-[³H]inositol monophosphate were not detected. It was also evident that GTP[S]-stimulated inositol phosphate production declined after 5 min in the presence of 2,3-diphosphoglycerate (cf. Figs. 1b and 1a). This attenuation may be attributable to chelation of Mg²⁺ by 2,3-diphosphoglycerate.

T.l.c. of the phosphoinositides remaining after PLC assays in the absence or presence of 10 μM-GTP[S] and with or without 2,3-diphosphoglycerate showed that over 95% of the recovered ³H was present in PtdIns(4,5)P₂, with no radioactivity being detected in the fractions corresponding to PtdIns4P and PtdIns standards (results not shown).

Stimulation of membrane-bound PtdIns(4,5)P₂-PLC activity by GTP[S] was maximal (4–5-fold stimulation at 3.4 μM-Ca²⁺) with 1 μM-GTP[S], and half-maximal with approx. 100 nM-GTP[S]. The non-hydrolysable GDP analogue guanosine 5'-[β-thio]diphosphate (GDP[S])

Fig. 2. Inhibition of GTP[S]-stimulated PtdIns(4,5)P₂-PLC activity by GDP[S]

Membrane-bound PtdIns(4,5)P₂-PLC activity was measured as a function of GTP[S] concentration in the absence (■) or presence (●) of 0.5 mM-GDP[S]. Results are means of three separate experiments.

markedly inhibited the effect of GTP[S] (Fig. 2). PtdIns(4,5)P₂-PLC activity was also stimulated by guanosine 5'-[βγ-imido]diphosphate, although to a lesser extent than by GTP[S], whereas ATP and its non-hydrolysable analogue adenosine 5'-[γ-thio]triphosphate

Table 1. Nucleotide specificity of stimulation of membrane-bound PtdIns(4,5) P_2 -PLC activity

Membrane-bound PtdIns(4,5) P_2 -PLC activity was assayed with [^{3} H]PtdIns(4,5) P_2 :PtdEtn:PtdSer (1:2:2) as substrate in the presence of 3.4 μ M- Ca^{2+} . Guanine and adenine nucleotides were added at the indicated concentrations. PLC activities are expressed either as nmol of PtdIns(4,5) P_2 hydrolysed/min per mg of protein or as a percentage of activity in the absence of nucleotides. Results are means of three separate experiments. Abbreviations: p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate.

Nucleotide	Concn.	PtdIns(4,5) P_2 hydrolysis	
		(nmol/min per mg of protein)	(% of control)
None		2.88	100
GTP[S]	1 μ M	9.48	329
	10 μ M	8.69	302
p[NH]ppG	1 μ M	5.48	190
	10 μ M	6.51	226
GTP	10 μ M	2.45	85
	100 μ M	2.30	80
ATP[S]	1 μ M	2.73	95
	10 μ M	2.44	85
ATP	10 μ M	2.45	85
	100 μ M	2.25	78

slightly inhibited enzyme activity (Table 1). The lack of a stimulatory effect of GTP on PtdIns(4,5) P_2 -PLC activity (Table 1) is presumably due to its rapid hydrolysis by non-specific nucleoside triphosphatase activity.

Ca^{2+} - and Mg^{2+} -dependence

Membrane-bound PtdIns(4,5) P_2 -PLC activity was totally Ca^{2+} -dependent, with no activity being observed, in either the presence or the absence of GTP[S], when 2 mM-EGTA was present (Fig. 3). At concentrations of Ca^{2+} approximating to those of the intracellular environment (100 nM), PtdIns(4,5) P_2 -PLC activity was stimulated approx. 20-fold by 10 μ M-GTP[S], with maximal stimulation being attained at 1–10 μ M- Ca^{2+} (Fig. 3). However, 3-fold stimulation of PtdIns(4,5) P_2 -PLC activity by GTP[S] was still observed at 100 μ M- Ca^{2+} . Thus GTP[S] induces both an apparent decrease in the Ca^{2+} requirement of the enzyme and an increase in activity at saturating Ca^{2+} concentrations.

Since Mg^{2+} ions are essential for the binding of GTP and its analogues to G proteins [14], the effect of Mg^{2+} on the stimulation of membrane-bound PtdIns(4,5) P_2 -PLC activity by GTP[S] was examined. No effect of 10 μ M-GTP[S] on PtdIns(4,5) P_2 -PLC activity was observed in the absence of Mg^{2+} . However, maximal stimulation of activity was observed in the presence of 2.5 mM- Mg^{2+} (Fig. 4). At this Mg^{2+} concentration, there was no change in basal PtdIns(4,5) P_2 -PLC activity. However, at Mg^{2+} concentrations higher than 5 mM, activity was inhibited in both the presence and the absence of GTP[S]. With PtdIns(4,5) P_2 alone as substrate, PLC activity was inhibited at lower (< 1 mM) Mg^{2+} concentrations (results not shown). Therefore it appears likely that the inhibition

Fig. 3. Ca^{2+} -dependence of membrane-bound PtdIns(4,5) P_2 -PLC activity

PtdIns(4,5) P_2 -PLC activity was assayed as a function of Ca^{2+} concentration in the absence (■) or presence (▲) of 10 μ M-GTP[S]. Free Ca^{2+} concentrations were maintained with Ca^{2+} /EGTA buffers with 2 mM-EGTA. Results are means of two separate experiments.

Fig. 4. Mg^{2+} -dependence of GTP[S]-stimulated PtdIns(4,5) P_2 -PLC activity

Membrane-bound PtdIns(4,5) P_2 -PLC activity was assayed in the absence (■) or presence (●) of 10 μ M-GTP[S] as a function of $MgCl_2$ concentration. Results are means of three separate experiments.

of activity at $> 5 \text{ mM-Mg}^{2+}$ is due to interaction of the bivalent cation with $\text{PtdIns}(4,5)\text{P}_2$, possibly by replacement of bound Ca^{2+} .

Effects of hormones

The Ca^{2+} -mobilizing hormone vasopressin had no effect on membrane-bound $\text{PtdIns}(4,5)\text{P}_2$ -PLC activity in the absence of GTP[S] or at concentrations of GTP[S] causing maximal stimulation of activity (Fig. 5a). However, at 10 nM-GTP[S] , $100 \text{ nM-vasopressin}$ caused a

2-fold stimulation of $\text{PtdIns}(4,5)\text{P}_2$ -PLC activity and decreased the concentration of GTP[S] required for half-maximal stimulation by approx. 8-fold (Fig. 5a). Angiotensin II (100 nM) produced similar effects (results not shown). In the presence of 50 nM-GTP[S] , vasopressin increased the activity of the enzyme at physiological Ca^{2+} concentrations ($0.1\text{--}1 \mu\text{M}$) (Fig. 5b). These experiments were conducted in the absence of cholate, to preclude possible inhibition of hormone binding by the detergent.

Fig. 5. Effect of [8-arginine]vasopressin on GTP[S]-stimulated $\text{PtdIns}(4,5)\text{P}_2$ -PLC activity

Membrane-bound $\text{PtdIns}(4,5)\text{P}_2$ -PLC activity was assayed as described in the Materials and methods section, except that sodium cholate was not included. (a) Activity was measured as a function of GTP[S] concentration in the absence (■) or presence (▲) of $100 \text{ nM-[8-arginine]vasopressin}$; free Ca^{2+} concn. was fixed at $1 \mu\text{M}$. (b) Activity was measured in the presence of 50 nM-GTP[S] , without (■) or with (▲) $100 \text{ nM-[8-arginine]vasopressin}$, as a function of free Ca^{2+} concentration. Results are means of three (a) and two (b) experiments.

Table 2. Effect of phospholipids on membrane-bound $\text{PtdIns}(4,5)\text{P}_2$ -PLC activity

$\text{PtdIns}(4,5)\text{P}_2$ -PLC activity was assayed with [^3H] $\text{PtdIns}(4,5)\text{P}_2$ mixed in different molar proportions with PtdEtn , PtdSer or PtdCho , as indicated, in the presence of $3.4 \mu\text{M-Ca}^{2+}$. Results are expressed as specific activities (nmol/min per mg of protein), or (in the absence of GTP[S]) as a percentage of activity without added phospholipids, or (in the presence of $10 \mu\text{M-GTP[S]}$) as a percentage of activity without GTP[S]. The results are the means of two separate experiments.

Substrate	No GTP[S]		+ $10 \mu\text{M-GTP[S]}$	
	(nmol/min per mg)	(% of control)	(nmol/min per mg)	(% of control)
Series 1				
$\text{PtdIns}(4,5)\text{P}_2$	0.70	100	0.73	104
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn (1:1)	0.77	110	1.18	153
$\text{PtdIns}(4,5)\text{P}_2$: PtdSer (1:1)	0.59	84	1.25	212
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn : PtdSer (1:1:1)	2.11	301	10.30	488
$\text{PtdIns}(4,5)\text{P}_2$: PtdCho (1:1)	2.07	296	2.12	102
Series 2				
$\text{PtdIns}(4,5)\text{P}_2$	0.63	100	1.03	163
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn (1:2)	1.77	281	3.19	180
$\text{PtdIns}(4,5)\text{P}_2$: PtdSer (1:2)	1.01	160	5.44	539
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn : PtdSer (1:2:2)	2.82	448	14.10	500
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn : PtdSer : PtdCho (1:1:1:1)	2.32	368	10.82	466
$\text{PtdIns}(4,5)\text{P}_2$: PtdEtn : PtdSer : PtdCho (1:2:2:2)	1.32	210	3.72	282

Effects of other phospholipids

The hydrolysis of polyphosphoinositides by soluble and membrane-bound PLC activities has been shown to be influenced by the presence of other phospholipids, in particular PtdEtn, PtdCho and PtdSer [22,24,28,31]. The effects of these phospholipids on membrane-bound PtdIns(4,5) P_2 -PLC activity and its stimulation by GTP[S] were therefore examined. Hydrolysis of PtdIns-(4,5) P_2 by membrane-bound PLC activity was increased in the presence of an equimolar concentration of PtdCho, an excess of PtdEtn, or combinations of these with PtdSer (Table 2). With PtdIns(4,5) P_2 alone, or PtdIns-(4,5) P_2 in the presence of PtdCho, as substrate, GTP[S] (10 μ M) had little or no effect. However, a 5-fold stimulation of activity by 10 μ M-GTP[S] was observed when the substrate was presented with either a molar excess of PtdSer or combinations of PtdEtn and PtdSer (Table 2). PtdCho was unable to block the stimulatory action of GTP[S] when present in a phospholipid mixture containing PtdEtn and PtdSer.

Phosphoinositide specificity

The phosphoinositide specificity of the GTP[S]-sensitive PLC activity was assessed with [^{3} H]PtdIns4P and [^{3} H]PtdIns. The hydrolysis of [^{3} H]PtdIns4P by membrane-bound PLC activity, when assayed under the same conditions (in the presence of PtdEtn and PtdSer), exhibited a similar Ca^{2+} -dependence and was stimulated by 10 μ M-GTP[S] in a manner comparable with that of PtdIns(4,5) P_2 -PLC activity (Fig. 6). The specific activity of membrane-bound PtdIns4P-PLC was approximately half that of PtdIns(4,5) P_2 -PLC activity. [^{3} H]PtdIns served as a poor substrate for membrane-bound PLC activity and, as previously reported [36], PtdIns-PLC

activity was strongly inhibited by both PtdEtn and PtdSer, which produced an additive inhibition in combination (Table 3, and results not shown). At 10 μ M, GTP[S] had a negligible effect on the hydrolysis of [^{3} H]PtdIns at either 1 μ M- or 1 mM- Ca^{2+} (Table 3), in either the presence or the absence of PtdSer (PtdIns-PLC activity at 1 μ M- Ca^{2+} in the presence of PtdSer was undetectable). It therefore appears that the GTP[S]-stimulated PLC activity is specific for the polyphosphoinositides.

Soluble PtdIns(4,5) P_2 -PLC

It has been reported that guanine nucleotides are able to stimulate a soluble phosphoinositide-PLC activity

Table 3. Effect of GTP[S] on membrane-bound PtdIns-PLC activity

The hydrolysis of [^{3} H]PtdIns by membrane-bound PLC activity was assayed in the absence or presence of 10 μ M-GTP[S], at 1 μ M- or 1 mM- Ca^{2+} . PtdIns-PLC activity was assayed with [^{3} H]PtdIns or with [^{3} H]PtdIns:PtdSer (1:2). Results are expressed as specific activities (nmol/min per mg of protein) and are means of two separate experiments.

Substrate	[Ca^{2+}]	PtdIns-PLC activity	
		No GTP[S]	+ 10 μ M-GTP[S]
PtdIns	1 μ M	0.22	0.25
	1 mM	0.92	1.13
PtdIns:PtdSer (1:2)	1 μ M	*	*
	1 mM	0.42	0.57

* No activity detected.

Fig. 6. Effect of GTP[S] on membrane-bound PtdIns4P-PLC activity

PtdIns4P-PLC activity was assayed as described in the Materials and methods section, with [^{3} H]-PtdIns4P:PtdEtn:PtdSer (1:2:2) as substrate, as a function of Ca^{2+} concentration, in the absence (●) or presence (▲) of 10 μ M-GTP[S]. Results are means of two separate experiments.

Fig. 7. Effect of GTP[S] on soluble PtdIns(4,5) P_2 -PLC activity

The activity of cytosolic PtdIns(4,5) P_2 -PLC was assayed under the same conditions as the membrane-bound enzyme, as a function of Ca^{2+} concentration and in the absence (■) or presence (▲) of 10 μ M-GTP[S]. Results are means of two separate experiments.

from human platelets when [^3H]PtdIns(4,5) P_2 -labelled lipid vesicles prepared from total platelet lipids are used as substrates [29,30]. When assayed under the conditions described by Deckmyn *et al.* [30], with [^3H]PtdIns(4,5) P_2 -labelled vesicles prepared from liver plasma-membrane lipids, liver cytosolic PtdIns(4,5) P_2 -PLC activity was barely detectable, and various concentrations of GTP[S] had no effect on activity (results not shown). With PtdIns(4,5) P_2 :PtdEtn:PtdSer (1:2:2) as substrate, soluble PLC activity exhibited a similar Ca^{2+} -concentration/activity profile to the membrane-bound activity. However, when assayed under conditions producing maximal stimulation of membrane-bound activity, 10 μM -GTP[S] had no effect on, or slightly inhibited, the soluble PtdIns(4,5) P_2 -PLC activity of rat liver (Fig. 7). GTP[S] also had no effect on soluble PLC activity with PtdIns(4,5) P_2 alone as substrate (results not shown).

DISCUSSION

The involvement of a regulatory G protein, termed G_p , in the signal-transduction mechanism of agonists stimulating the turnover of phosphoinositides has been strongly implicated in several cell types by studies demonstrating increased polyphosphoinositide breakdown and Ins(1,4,5) P_3 release from endogenously labelled membranes and permeabilized cells incubated with guanine nucleotides [6–13]. In some tissues, exogenous phosphoinositides have been utilized as enzyme substrates to show that the site of action of guanine nucleotides is a membrane-associated polyphosphoinositide-PLC [16–20]. In agreement with these findings, stimulation of polyphosphoinositide-PLC activity by GTP[S] in liver plasma membranes was found to be characteristic of regulation via a stimulatory G protein, i.e. it was specific for micromolar concentrations of GTP analogues (Table 1) and was inhibited by GDP[S] (Fig. 2). It was also enhanced synergistically by vasopressin at sub-saturating concentrations of GTP[S], and was dependent on the presence of Mg^{2+} ions. The ultimate site of action of guanine nucleotides was shown to be a PLC activity, as demonstrated by the initial formation of Ins(1,4,5) P_3 from PtdIns(4,5) P_2 , although Ins(1,4) P_2 was subsequently formed as a result of Ins(1,4,5) P_3 phosphatase action. The guanine nucleotide-sensitive PLC activity was shown to be specific for PtdIns(4,5) P_2 and PtdIns4 P , with PtdIns hydrolysis being negligibly affected by guanine nucleotides.

Certain discrepancies exist in the literature about the Ca^{2+} -dependence of membrane-bound guanine nucleotide-sensitive PLC activity. Thus, in coronary-artery smooth-muscle membranes [18] and fibroblast membranes [19], activity was reported to be Ca^{2+} -dependent, and agonist and guanine-nucleotide stimulation of activity was observed at nanomolar Ca^{2+} concentrations. On the other hand, in transformed epithelial-cell membranes [17] and salivary-gland membranes [16], polyphosphoinositide-PLC activity and its stimulation by guanine nucleotides were apparently Ca^{2+} -independent. In liver plasma membranes, Melin *et al.* [20] demonstrated slight stimulation of PtdIns(4,5) P_2 -PLC activity at $> 300 \text{ nM}$ - Ca^{2+} , but inhibition of activity by guanosine 5'-[β -methylene]triphosphate at lower Ca^{2+} concentrations.

The present studies indicate that the activity of rat liver plasma-membrane polyphosphoinositide-PLC is totally dependent on the presence of Ca^{2+} ions, as is stimulation of activity by guanine nucleotides. At 100 nM - Ca^{2+} and 2.5 mM - Mg^{2+} , concentrations approximating to those that pertain in the cytosol of unstimulated cells *in vivo*, membrane-bound polyphosphoinositide-PLC activity was very low, but was greatly stimulated (approx. 20-fold) by GTP[S] (Fig. 3). Raising the free Ca^{2+} concentration to 1 μM increased the basal polyphosphoinositide-PLC activity, and further increased the GTP[S]-stimulated activity approx. 2-fold (Fig. 3). These results raise the possibility that polyphosphoinositide-PLC activity may be increased secondarily to an increase in cytosolic Ca^{2+} *in vivo*. However, Fig. 3 shows that such an increase would be very small compared with that mediated via G_p . In any case, it should be recognized that caution is needed when extrapolating from isolated-membrane data to the intact cell, in which many factors could play a role, such as a limitation of substrate supply and alterations of receptor-G protein-polyphosphoinositide-PLC coupling, e.g. by protein kinase C [37,38].

In the present studies, membranes were prepared and assays were conducted in the presence of millimolar EGTA. In other studies in which Ca^{2+} chelators were used, a similar Ca^{2+} -dependence of guanine-nucleotide stimulation of PLC activity was observed [18,19]. It therefore seems possible that the Ca^{2+} -independent guanine-nucleotide stimulation of PLC activity observed in some studies may have been due to the presence of residual membrane-bound Ca^{2+} . Previous studies of the Ca^{2+} requirements for guanine-nucleotide stimulation of Ins(1,4,5) P_3 release from endogenously labelled membranes [6,39,40] have yielded findings very similar to those of the present study.

The present data also demonstrate that GTP[S] stimulation of polyphosphoinositide-PLC activity requires millimolar Mg^{2+} , presumably as a consequence of the Mg^{2+} -dependency of guanine-nucleotide binding to G proteins [14]. Given such a Mg^{2+} requirement, it is probable that, in previous studies demonstrating guanine-nucleotide effects in the absence of Mg^{2+} , sufficient protein- and/or lipid-bound Mg^{2+} was carried over into assays to enable guanine nucleotide binding. Uhing *et al.* [6] demonstrated that maximal stimulation of Ins(1,4,5) P_3 release from endogenously labelled membranes by GTP[S] required millimolar Mg^{2+} concentrations.

Irvine *et al.* [31] demonstrated that presentation of PtdIns(4,5) P_2 in a non-bilayer configuration in the presence of excess PtdEtn enabled its hydrolysis by soluble brain PLC activity under conditions of physiological Ca^{2+} and Mg^{2+} concentrations, whereas PtdCho inhibited this hydrolysis. Similar results have been obtained with other enzyme sources [22,24,28]. The present data demonstrated that membrane-bound PtdIns(4,5) P_2 -PLC activity was stimulated both by PtdEtn, in excess, and by PtdCho. This is in agreement with the findings of Wang *et al.* [28]. In the presence of PtdEtn and PtdSer, the latter of which alone had little effect on membrane-bound PLC activity, a synergistic stimulation of PtdIns(4,5) P_2 -PLC activity was observed (Table 2). Furthermore, the presence of PtdSer or of PtdEtn and PtdSer was necessary for the full expression of GTP[S]-stimulated polyphosphoinositide-PLC activity. It is therefore possible that PtdEtn may enhance

PtdIns(4,5) P_2 hydrolysis by presentation of the substrate in a suitable conformation, and that PtdSer may influence polyphosphoinositide-PLC activity and its stimulation by guanine nucleotides by virtue of its ability to form complexes with Ca^{2+} and other phospholipids. When PtdIns(4,5) P_2 -PLC activity was assayed with PtdIns(4,5) P_2 alone as substrate, it was inhibited at much lower Mg^{2+} concentrations than those required to inhibit activity in the presence of PtdEtn and PtdSer. Thus the effect of PtdSer may be to increase the apparent affinity for Ca^{2+} of either substrate or enzyme (or both) relative to Mg^{2+} .

One might speculate that, on interaction with the α subunit of G_p , polyphosphoinositide-PLC undergoes a conformational change such that it becomes activatable by a Ca^{2+} -PtdSer complex, perhaps involving other lipids, the result being an increase in the apparent affinity of polyphosphoinositide-PLC for Ca^{2+} . Such a mechanism of activation would be analogous to that of protein kinase C, whereby DAG increases the affinity of protein kinase C for Ca^{2+} by formation of a (PtdSer) $_4$ - Ca^{2+} -DAG complex [3,41]. Interestingly, PtdEtn enhances activation of protein kinase C by PtdSer and DAG, whereas PtdCho exerts an inhibitory effect [42]. In the present studies, PtdEtn increased GTP[S]-stimulated polyphosphoinositide-PLC activity in the presence of PtdSer, whereas PtdCho did not support such a stimulation. Whatever the mechanisms of action of these phospholipids, it is evident that the local lipid environment of the membrane-bound polyphosphoinositide-PLC and perturbations of this environment have profound effects on the receptor-mediated hydrolysis of PtdIns(4,5) P_2 .

Two reports [29,30] have demonstrated that a cytosolic polyphosphoinositide-specific PLC from human platelets can be activated by guanine nucleotides in an apparently Ca^{2+} -independent manner. Deckmyn *et al.* [30] also demonstrated pertussis-toxin-induced ADP-ribosylation of a 41 kDa protein, possibly the inhibitory G protein of adenylate cyclase, in platelet cytosol. However, the stimulation of soluble PLC by GTP[S] was unaffected by this toxin, suggesting that agonist-stimulated polyphosphoinositide turnover in platelets might involve a soluble G protein distinct from those regulating adenylate cyclase. On the other hand, Rock & Jackowski [43] have demonstrated that, in a different assay system, platelet soluble PtdIns(4,5) P_2 -PLC activity is non-specifically stimulated by several nucleotides. In the present studies, no stimulation of liver cytosolic polyphosphoinositide-PLC activity by guanine nucleotides was observed under conditions allowing optimal stimulation of the membrane-bound activity or under conditions reported to allow stimulation of platelet soluble PLC [30]. This suggests that one or more component of the G_p -PLC complex is absent or non-functional in liver cytosol.

There is evidence that there are differences in the G-protein-coupled stimulation of polyphosphoinositide breakdown in liver, platelets and other tissues. Most strikingly, pertussis toxin does not inhibit the ability of Ca^{2+} -mobilizing hormones to activate phosphorylase in hepatocytes [44] or to stimulate Ins(1,4,5) P_3 release from liver plasma membranes in the presence of GTP[S] [6], whereas the toxin has been shown to inhibit thrombin-stimulated phosphatidate formation and $^{45}Ca^{2+}$ release in permeabilized platelets [45]. Other factors possibly responsible for the differences in guanine-nucleotide

effects on soluble PLC activity observed in liver and platelets include differences in the method of cell disruption and the preparation of fractions.

In summary, the present data lend further support to the notion that a membrane-bound polyphosphoinositide-specific PLC activated by a specific G protein is responsible for the receptor-mediated formation of the intracellular messengers Ins(1,4,5) P_3 and DAG.

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