Stimulation of phospholipase D in rabbit platelet membranes by nucleoside triphosphates and by phosphocreatine: roles of membrane-bound GDP, nucleoside diphosphate kinase and creatine kinase

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Previous work has shown that guanosine 5'-[γ -thio]triphosphate (GTP[S]) and GTP stimulate phospholipase D (PLD) in rabbit platelet membranes and that these effects are greatly enhanced by pretreatment of platelets with phorbol esters that activate protein kinase C [Van der Meulen and Haslam (1990), *Biochem. J.* 271, 693–700]. In the present study, the effects of Mg^{2+} , various nucleoside triphosphates and phosphocreatine (PCr) were investigated. Platelet membranes containing phospholipids labelled with [3H]glycerol were assayed for PLD in the presence of an optimal Mg^{2+} concentration (10 mM) by measuring [3H]phosphatidylethanol formation in incubations that included 300 mM ethanol. In membranes from phorbol-ester-treated platelets, the same maximal increases in PLD activity (5-fold) were seen with 1 μM GTP[S] and 100 μM GTP. Addition of adenosine 5'-[γ -thio]triphosphate (ATP[S]), ITP, XTP, UTP and CTP had similar stimulatory effects, but only at ≥ 1 mM. In contrast, ATP had a biphasic action, causing a maximal (2-fold) stimulation at 10 μM and smaller effects at higher concentrations; the inhibitory component of the action of

ATP was blocked by 2 μM staurosporine. Guanosine 5'-[β -thio]diphosphate decreased the stimulatory effects of ATP and ATP[S]. UDP, which can inhibit nucleoside diphosphate kinase (NDPK), decreased the activation of PLD by ATP[S], ATP, XTP, CTP and to a lesser extent ITP, but had no effect on the actions of GTP[S] and GTP. Rabbit platelet membranes contained NDPK and addition of [γ - ^{32}P]ATP led to the formation of [^{32}P]GTP in amounts sufficient to explain most or all of the activation of PLD; UDP prevented GTP formation. PCr (0.04–1 mM) also stimulated membrane PLD activity, an effect that was dependent on endogenous membrane-bound creatine kinase (CK). UDP and guanosine 5'-[β -thio]diphosphate each inhibited this effect of PCr. The results show that in rabbit platelet membranes, CK, NDPK and the GTP-binding protein that activates PLD can be functionally coupled. However, assay of membrane preparations at increasing dilutions showed that stimulation of PLD by the compounds studied, with the partial exception of ATP[S], involved diffusible rather than protein-bound intermediates.

INTRODUCTION

Increasing evidence indicates that the hydrolysis of phosphatidylcholine by PLD plays an important role in signal transduction in many cells, and that one of its products, phosphatidate, acts either as a second messenger in its own right or as a source of another second messenger, diacylglycerol [1,2]. Work with isolated membranes and permeabilized cells has provided some support for the view that PLD can be directly activated by a receptor coupled to a G-protein [3–5] and has shown that guanine nucleotides alone, particularly guanosine 5'-[γ -thio]triphosphate (GTP[S]), can often stimulate PLD activity [3–11]. However, in many cells, phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are also potent activators of this enzyme, suggesting that physiological increases in PLD activity may be secondary effects of the activation of phospholipase C and that protein kinase C (PKC) may play an important role in the regulation of PLD [7,12–15]. In some cells, inhibitors of PKC have been shown to block the activation of PLD by phorbol esters [12–14,16], whereas in other cell types they have been ineffective or only partially effective [7,17,18]. In accord with the latter results, it has been reported that purified PKC can activate PLD by a phosphorylation-independent mechanism [19]. In

neutrophils and U937 cells, tyrosine kinase activity appears to be required for the receptor- or GTP[S]-dependent activation of PLD, but not for the effect of phorbol ester [16,20]. Finally, increases in Ca^{2+} in the physiological range can increase the activity of PLD from some cells [7,9]. Although in principle these varied factors could stimulate different PLD isoenzymes, they often appear to act synergistically rather than additively, implying a convergence of regulatory mechanisms on a single isoenzyme. In particular, previous studies in this laboratory have shown that GTP[S] and either phorbol esters or Ca^{2+} exert synergistic effects on the PLD activities of rabbit platelet membranes [7] and permeabilized human platelets [21,22].

In platelets, the dominant signal-transduction pathway has long been thought to be the receptor- and G-protein-mediated activation of phosphoinositide-specific phospholipase C, leading to the formation of diacylglycerol and activation of PKC, and to the mobilization of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate [23]. In this system, phosphatidate is formed largely by the action of diacylglycerol kinase [24]. However, the activation of PLD by thrombin has been demonstrated in intact human platelets [22,25,26], and the evidence available now indicates that 10–20% of the phosphatidate accumulating in response to this stimulus derives from the action of PLD [22,26].

Abbreviations used: PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; DMSO, dimethyl sulphoxide; PET, phosphatidylethanol; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β -imidazole]triphosphate; p[NH]ppA, adenosine 5'-[β -imidazole]triphosphate; NDPK, nucleoside diphosphate kinase; CK, creatine kinase; PCr, phosphocreatine; DNFB, 2,4-dinitrofluorobenzene; G_p , GTP-binding protein that activates PLD.

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In permeabilized platelets, close correlations have been noted between GTP[S]-induced secretion and PLD activity, particularly in the absence of Ca^{2+} , when there is no significant activation of phospholipase C [21,22]. These observations have suggested that phosphatidate formed by PLD could have a role as a signal for exocytosis from platelets. If so, the unidentified GTP-binding protein that activates PLD (G_D) could be identical with the protein (G_p) postulated by Gomperts and colleagues [27] as a mediator of exocytosis in various cell types. Similar conclusions were reached recently from experiments with permeabilized HL-60 cells [28]. Studies with a variety of permeabilized cells [29–31], and with permeabilized platelets [32], have indicated that several nucleoside triphosphates in addition to GTP or GTP[S] can support or potentiate secretion. The mechanisms of action of these compounds are not clear. We have now investigated the effects of a wide range of nucleoside triphosphates on the PLD activity of rabbit platelet membranes and have obtained evidence that G_D can be activated by these compounds, and also by phosphocreatine (PCr), through the intermediary actions of nucleoside diphosphate kinase (NDPK) and creatine kinase (CK).

EXPERIMENTAL

Materials

ATP, XTP, UTP, CTP, GDP, IDP, dTDP, PCr, CK (rabbit muscle, 250 units/mg of protein) and 2,4-dinitrofluorobenzene (DNFB) were obtained from Sigma (St. Louis, MO, U.S.A.), and adenosine 5'-[γ -thio]triphosphate (ATP[S]), guanosine and adenosine 5'-[β -imido]triphosphates (p[NH]ppG and p[NH]ppA), ITP, ADP and UDP were from Boehringer Mannheim Canada Ltd. (Laval, Que., Canada). Aqueous counting scintillant (ACS), [γ - ^{32}P]ATP (6000 Ci/mmol) and [$8\text{-}^{14}\text{C}$]GTP (52 mCi/mmol) were supplied by Amersham (Oakville, ON, Canada) and [$8\text{-}^{14}\text{C}$]ATP (50 mCi/mmol) by ICN Biomedicals Canada Ltd. (Mississauga, ON, Canada). Cellulose powder for t.l.c. (MN 300 HR) was purchased from Brinkmann Instruments (Rexdale, ON, Canada). Ro 31-8220 was a gift from Roche Products Ltd. (Welwyn Garden City, Herts., U.K.). All other materials were from sources listed previously [7].

Labelling of rabbit platelets and preparation of membranes for measurements of PLD activity

Platelet phospholipids were labelled with [^3H]glycerol, and the PLD activities of isolated membranes were assayed by measurement of the formation of [^3H]phosphatidylethanol ([^3H]PEt) in incubations that included 300 mM ethanol [7]. These procedures were carried out as described in [7], with minor modifications. Platelets were suspended in plasma at 5×10^9 /ml, incubated for 2 h at 37 °C with [^3H]glycerol at 33–50 $\mu\text{Ci}/\text{ml}$ and then washed by centrifugation and resuspension in Ca^{2+} -free Tyrode's solution (pH 6.5) containing BSA (3.5 mg/ml), apyrase (30 $\mu\text{g}/\text{ml}$), 2.6 mM EGTA and 10 mM Pipes. The platelets were finally resuspended at 5×10^9 /ml in the same medium without BSA or EGTA and incubated for 2 min at 37 °C with 1 μM PMA or vehicle [dimethyl sulfoxide (DMSO); final concn. 0.2%, v/v]. After addition of 5 mM EDTA, the platelet suspensions were then lysed by freezing and thawing twice. Membranes were isolated by centrifugation at 30000 g_{av} for 40 min at 4 °C, homogenized once in hypertonic buffer (1 M KCl/5 mM EDTA/25 mM Hepes, pH 7.4) and once in hypotonic buffer (the same without KCl), before final resuspension at about 1 mg of protein/ml in 100 mM KCl/2.5 mM EGTA/25 mM Hepes,

pH 7.4 (buffer A). The membrane preparations contained $(1.0\text{--}1.5) \times 10^6$ d.p.m. of ^3H -labelled phospholipid/mg of protein. In some experiments, membranes were incubated for 15 min at 25 °C in hypotonic buffer containing 100 μM DNFB or vehicle (DMSO; final concn. 0.1%, v/v), before extraction with hypertonic and hypotonic buffers.

Incubations and measurement of [^3H]PEt formation

All incubations were carried out in triplicate. Incubation mixtures (0.5 ml) comprised 0.2 ml of membrane suspension, 0.2 ml of buffer A containing sufficient MgCl_2 , CaCl_2 and KOH to give the required concentrations of $\text{Mg}^{2+}_{\text{free}}$ and $\text{Ca}^{2+}_{\text{free}}$ at pH 7.4, and 0.1 ml of buffer A containing other additions. In most experiments, CaCl_2 was omitted and the final MgCl_2 concentration was 11.4 mM, giving 10 mM $\text{Mg}^{2+}_{\text{free}}$. Concentrations of $\text{Ca}^{2+}_{\text{free}}$ and $\text{Mg}^{2+}_{\text{free}}$ were calculated in accordance with [33]. Apart from unincubated controls, all incubations were for 10 min at 37 °C and included 300 mM ethanol. Incubations were terminated by addition of 0.5 ml of buffer A and 3.75 ml of chloroform/methanol (1:2, v/v), followed 10 min later by 1.5 ml of water and 1 ml of chloroform to separate organic and aqueous phases. The [^3H]PEt extracted in the organic phase was mixed with carrier PEt and phosphatidic acid, purified by t.l.c. and counted for radioactivity, as described in [7]. The ^3H associated with carrier PEt after t.l.c. of extracts of unincubated membranes was subtracted from all values obtained in incubations with ethanol. Results from triplicate samples were expressed as means $\pm$ S.E. of the difference and normalized with respect to membrane protein, determined by the Lowry method. The significance of changes was evaluated by two-sided unpaired or paired *t* tests, as appropriate.

Metabolism of [γ - ^{32}P]ATP by platelet membranes

The formation of [^{32}P]GTP after incubation of platelet membranes with [γ - ^{32}P]ATP was determined. Experience showed that it was essential to remove labelled impurities from commercial [γ - ^{32}P]ATP. Approx. 30 μCi of [γ - ^{32}P]ATP was purified by t.l.c. on a cellulose plate with isobutyric acid/1.0 M NH_3 /0.1 M EDTA (125:75:2, by vol.) as the solvent; the labelled ATP (located by autoradiography) was eluted from the cellulose with buffer A. Samples of membrane suspension were then incubated with 10 μM ATP (plus 0.5 μCi of [γ - ^{32}P]ATP) and the additions described above (final volume 0.5 ml, including ethanol). Incubations were terminated by addition of 0.25 ml of 30% (w/v) trichloroacetic acid together with 0.05 μCi of [^{14}C]ATP and 0.05 μCi of [^{14}C]GTP, added to monitor the recovery of the ^{32}P -labelled compounds. After centrifugation and extraction of trichloroacetic acid from the supernatant with 3 vol. of water-saturated diethyl ether, 20 μl fractions were mixed with carrier nucleoside triphosphates and analysed by t.l.c. in two dimensions on cellulose plates. The first-dimension solvent was butan-1-ol/acetone/acetic acid/14.8 M NH_3 /water (90:30:20:1:60, by vol.) and the second was that used to purify [γ - ^{32}P]ATP. To obtain a complete separation of GTP and UTP, it was necessary to run plates at least twice in the first solvent. Cellulose containing ATP, GTP and UTP was scraped into scintillation vials containing 0.5 ml of water and 8 ml of ACS, sonicated and counted for both ^{32}P and ^{14}C radioactivity, with corrections for cross-over. Values for [^{32}P]ATP and [^{32}P]GTP were adjusted for the recovery of the corresponding ^{14}C -labelled compounds; the recovery of [^{32}P]UTP was assumed to be the same as that of GTP. The ^{32}P -labelled compounds measured were expressed as molar concentrations, even when partly membrane-bound. Some

incubation mixtures (final vol. 1 ml) were filtered before addition of trichloroacetic acid. Use of both an upper $3\ \mu\text{m}$ (SS) and a lower $0.22\ \mu\text{m}$ (GS) Millipore filter permitted removal of membranes from 0.5–1 ml of incubation mixture in less than 1 min. The metabolism of GTP in incubation mixtures was also studied by using $[^{14}\text{C}]\text{GTP}$ and similar methods for separation of the products to those described for $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, except that the recoveries of $[^{14}\text{C}]\text{GTP}$ and $[^{14}\text{C}]\text{GDP}$ were monitored by addition of $5\ \mu\text{mol}$ of the unlabelled compounds and spectrophotometric determination of the amounts eluted from t.l.c. plates with water.

Enzyme assays

The NDPK activities of platelet membrane and supernatant fractions were determined as described in [34], with dTDP ($0.4\ \text{mM}$) and ATP ($2\ \text{mM}$) as substrates. CK activities were assayed with PCr ($30\ \text{mM}$) and ADP ($2\ \text{mM}$) as substrates [35]. Both assays were carried out at $30\ ^\circ\text{C}$; 1 unit of enzyme activity is defined as the formation of $1\ \mu\text{mol}$ of product/min.

RESULTS

Effects of Mg^{2+} and Ca^{2+} on PLD activity

In previous work [7], PLD activity was determined in the presence of physiological concentrations of bivalent cations, that is $0.5\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$ and $0\text{--}1\ \mu\text{M}\ \text{Ca}^{2+}_{\text{free}}$. The results showed that

Figure 1 Effects of Mg^{2+} and GTP[S] on the PLD activities of membranes from control and PMA-treated platelets

Membranes were isolated from platelets that had been labelled with $[^3\text{H}]\text{glycerol}$ and had been incubated either without PMA (○, ●) or with PMA (□, ■), as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at $37\ ^\circ\text{C}$ without GTP[S] (○, □) or with $10\ \mu\text{M}$ GTP[S] (●, ■) in the presence of the concentrations of $\text{Mg}^{2+}_{\text{free}}$ indicated and $300\ \text{mM}$ ethanol. Membrane $[^3\text{H}]\text{PEt}$ was then determined and appropriate blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment.

Figure 2 Comparison of the effects of GTP[S], GTP, ATP[S] and ATP on the PLD activity of membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with $[^3\text{H}]\text{glycerol}$ and incubated with PMA, as described in the Experimental section. Samples of membrane suspensions were then incubated for 10 min at $37\ ^\circ\text{C}$ with either no addition (○) or the indicated concentrations of GTP[S] (●), GTP (▲), ATP[S] (▼) or ATP (■), all in the presence of $10\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$ and $300\ \text{mM}$ ethanol. Membrane $[^3\text{H}]\text{PEt}$ was determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment.

under these conditions Ca^{2+} enhanced the stimulatory effect of GTP[S]. In the present study, the concentration of $\text{Mg}^{2+}_{\text{free}}$ was varied to determine the optimal conditions for activation of the enzyme. Virtually no activity was observed at any Mg^{2+} concentration in control membranes (Figure 1). However, incubation of the intact platelets with PMA before membrane isolation or of the isolated membranes with GTP[S] led to substantial $[^3\text{H}]\text{PEt}$ formation, which was optimal with $10\ \text{mM}\ \text{Mg}^{2+}$. Together, these treatments were powerfully synergistic, and under these conditions, maximal PLD activity was again observed with $10\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$ (Figure 1). Half-maximal activation required $1.6\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$. Although addition of $1\ \mu\text{M}\ \text{Ca}^{2+}_{\text{free}}$ enhanced enzyme activity with $0.5\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$ [7], this had little or no effect in percentage terms in the presence of $10\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$.

Effects of nucleoside triphosphates and diphosphates on membrane PLD activities

The effects of these compounds were studied in the presence of $10\ \text{mM}\ \text{Mg}^{2+}$, using membranes from PMA-treated platelets. Under these optimal conditions, accurate results were obtained using smaller amounts of membrane than in previous work [7]. With $10\ \text{mM}\ \text{Mg}^{2+}_{\text{free}}$, the maximum effects of GTP[S] and GTP, amounting to approx. 5-fold increases in PLD activity, were observed with $1\ \mu\text{M}$ of the former compound and $100\ \mu\text{M}$ of the latter (Figure 2) and were not significantly different ($P > 0.05$, $n = 5$). Half-maximal effects were seen with $0.05\ \mu\text{M}$

Figure 3 Effects of nucleoside triphosphates and diphosphates on the PLD activity of membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at 37 °C with and without 100 μM GTP, and with the indicated concentrations of ITP (●), XTP (▲), UTP (■), IDP (○), ADP (▽) or UDP (□), all in the presence of 10 mM $\text{Mg}^{2+}_{\text{free}}$ and 300 mM ethanol. Membrane [^3H]PEt was determined and blanks were subtracted. The increases in [^3H]PEt formation caused by the compounds studied were then expressed as percentages of the increase observed with 100 μM GTP. Values are means $\pm$ S.E. of the quotient from triplicate measurements in a single experiment.

GTP[S] and 3 μM GTP. To our surprise, ATP[S] and ATP were also stimulatory. Whereas ATP[S] showed a progressively increasing effect over the concentration range 1–1000 μM , ATP had a biphasic action with a maximum at 10 μM (Figure 2). ATP[S] stimulated PLD activity by 4.1 ± 0.1 -fold at 1 mM and ATP by 2.2 ± 0.1 -fold at 10 μM (mean values $\pm$ S.E.M., $n = 5$ and 7 respectively). p[NH]ppG stimulated PLD activity to about the same extent and over the same concentration range as GTP (results not shown). Commercial p[NH]ppA was weakly stimulatory, but this effect was diminished after purification of the compound by t.l.c. (by the method described for [γ - ^{32}P]ATP; see the Experimental section) and probably resulted from contamination with p[NH]ppG. The actions of other nucleoside triphosphates were also studied; ITP, XTP and UTP all had powerful stimulatory effects on PLD activity with maximum effects that were comparable with (ITP and XTP) or slightly weaker (UTP) than that of GTP (Figure 3). With these compounds, maximal increases in PLD activity were observed at about 1 mM and half-maximal effects with 10–20 μM concentrations. At 1 mM, CTP had an effect comparable with that of XTP (Figure 4). Nucleoside diphosphates had either much weaker effects on PLD, causing up to 2-fold increases in activity at 1 mM (ADP and IDP), or had no effect at all (UDP) (Figure 3). GDP

Figure 4 Effects of UDP on the activation of PLD by nucleoside triphosphates in membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at 37 °C with the indicated concentrations of UDP and either no nucleoside triphosphate or derivative (○), 10 μM GTP[S] (●), 100 μM ITP (▲), 100 μM XTP (▽) or 100 μM CTP (■), all in the presence of 10 mM $\text{Mg}^{2+}_{\text{free}}$ and 300 mM ethanol. [^3H]PEt formation was then determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment.

was also weakly stimulatory, causing $21 \pm 4\%$ of the maximum effect of GTP at 100 μM (mean $\pm$ S.E.M., $n = 3$).

The activation of PLD by ATP[S] and by the wide range of nucleoside triphosphates studied suggested that the formation of GTP[S] or GTP by the action of NDPK on endogenous GDP might mediate the effects of these compounds. This conclusion was supported by the ability of UDP, which can inhibit NDPK [36], to decrease the stimulatory effects of 100 μM ATP[S] (Table 1) and of 100 μM XTP or CTP (Figure 4). In addition, 1 mM UDP essentially blocked the stimulation of PLD activity by ATP (Figure 5). UDP was less effective as an inhibitor of the action of ITP and, significantly, was without effect on the activation of PLD by GTP[S] or GTP (Table 1, Figure 4). This indicates that the latter compounds did not exert their effects through NDPK and that the actions of ATP[S] and ATP were not attributable to contamination with small amounts of guanine nucleotides. It should be noted that exogenous GDP (1 μM) did not potentiate the effects of 10 or 100 μM ATP[S] (results not shown). The action of ATP[S] was also inhibited by the G-protein antagonist guanosine 5'-[β -thio]diphosphate (GDP[S]) (Table 1), which is consistent with evidence that NDPK can catalyse the synthesis of GTP[S] from ATP[S] and endogenous GDP [37–39]. Direct assay of NDPK in rabbit platelet membranes demonstrated the presence of enzyme activity amounting to 0.132 ± 0.003 unit/mg of protein (mean $\pm$ S.E.M., $n = 3$). In view of the rigorous washing conditions used to prepare the membranes, it is likely that this NDPK is firmly bound to membranes, rather than loosely

Table 1 Effects of UDP and of GDP[S] on the activation of PLD by PCr and CK in membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at 37 °C with the indicated additions at the following concentrations: UDP, 1 mM; GDP[S], 1 mM; GTP[S], 0.1 μM ; GTP, 1 μM ; ATP[S], 100 μM ; PCr, 1 mM; CK, 10 units/ml. All incubation mixtures also contained 10 mM $\text{Mg}^{2+}_{\text{free}}$ and 300 mM ethanol. Membrane [^3H]PEt was then determined and appropriate blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate determinations in the same experiment. Significant inhibition is shown (* $P < 0.01$, unpaired t tests).

Additions		$10^{-3} \times [^3\text{H}]\text{PEt}$ formed (d.p.m./mg of protein)
Activator	Inhibitor	
None	None	6.4 ± 0.1
	UDP	6.4 ± 0.1
	GDP[S]	8.5 ± 0.1
GTP[S]	None	22.6 ± 0.2
	UDP	21.5 ± 0.4
GTP	None	14.8 ± 0.4
	UDP	16.6 ± 0.1
ATP[S]	None	19.5 ± 0.3
	UDP	$13.8 \pm 0.1^*$
	GDP[S]	$13.5 \pm 0.1^*$
PCr	None	16.9 ± 0.7
	UDP	$8.9 \pm 0.2^*$
	GDP[S]	$10.8 \pm 0.2^*$
PCr + CK	None	18.5 ± 1.0
	UDP	$12.0 \pm 0.7^*$
	GDP[S]	$11.9 \pm 0.3^*$

adsorbed. However, rabbit platelet cytosol contained a much higher NDPK activity (2.1 units/mg of protein).

Comparison of the time course of activation of PLD by 10 μM ATP with the effects of a range of concentrations of GTP showed that addition of 0.4 μM GTP was required to reproduce the effect of the ATP (Figure 6a). However, measurements of the [^{32}P]GTP formed on incubation of the same platelet membranes with 10 μM [γ - ^{32}P]ATP showed that the concentration of [^{32}P]GTP present increased to only 0.2 μM during the first 2 min and that this concentration was maintained for a further 3 min (Figure 6b). At the same time, the [^{32}P]ATP concentration decreased by over 40%. Almost identical results were obtained in two additional experiments, in one of which incubations were continued for 10 min, at which time both the [^{32}P]ATP and [^{32}P]GTP had decreased by over 90%. Addition of 1 mM UDP almost abolished the stimulation of PLD by 10 μM ATP (Figure 6a) and at the same time prevented any measurable formation of [^{32}P]GTP (Figure 6b). The actions of UDP were accompanied by an accelerated removal of [^{32}P]ATP and the formation of roughly corresponding amounts of [^{32}P]UTP (Figure 6b). These results establish that the membrane NDPK forms small but significant amounts of GTP after addition of ATP. Although the above quantitative comparison could be interpreted as indicating that GTP formed by NDPK is more effective as an activator of PLD than is exogenous GTP, it is necessary to allow for the breakdown of the latter. This was determined by incubating platelet membranes with 0.4 μM [^{14}C]GTP; approximately half was degraded to [^{14}C]GDP after 2 min at 37 °C (results not shown). We conclude that the apparent discrepancy between the effects on PLD of exogenous GTP and of GTP generated by NDPK in the presence of ATP is probably attributable to the more rapid

Figure 5 Effects of staurosporine and of UDP on the activation of PLD by ATP in membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at 37 °C with the indicated concentrations of ATP and no other addition (●), 2 μM staurosporine (■) or 1 mM UDP (▲). All incubation mixtures contained 10 mM $\text{Mg}^{2+}_{\text{free}}$, 300 mM ethanol and 0.2% DMSO, the solvent for staurosporine. Membrane [^3H]PEt was then determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment.

disappearance of the former in the absence of regeneration by NDPK. The above results do not indicate whether NDPK acts on membrane-bound GDP or on GDP that has been released from the membranes. We therefore determined the percentage of the [^{32}P]GTP formed in the presence of [γ - ^{32}P]ATP that is found in filtrates of samples incubated for 2 min; values of 55% and 61% were obtained in two separate experiments, suggesting that NDPK acts, at least in part, on soluble GDP.

Inhibitory effects of high ATP concentrations

Addition of 1 mM ATP decreased the stimulation of PLD caused by 10 μM GTP by 38% (mean, $n = 2$). This observation, combined with the distinctive biphasic action of ATP, suggested that high concentrations of this compound could inhibit PLD activity by a mechanism not available to the other nucleoside triphosphates. In support of this view, we found that staurosporine, an inhibitor of PKC and other protein kinases, could diminish or abolish the inhibitory action of ATP, thereby markedly increasing the stimulation of PLD by high ATP concentrations (Figure 5). On average, 2 μM staurosporine increased the activation of PLD by 1 mM ATP from 1.7 ± 0.1 -fold to 3.2 ± 0.3 -fold (means $\pm$ S.E.M., $n = 5$). At 0.1 μM , staurosporine was ineffective. In contrast, 0.1–2 μM staurosporine caused weak (about 20%) inhibitions of membrane PLD activity measured in the absence of additions, in the presence of low ATP concentrations (Figure 5) or in the presence of 0.1–100 μM

Figure 6 Relationship between the stimulation of PLD by ATP and ATP metabolism in membranes from PMA-treated platelets; effects of UDP

Membranes were isolated from platelets that had been labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. (a) Samples of membrane suspension were incubated at 37 °C for the periods indicated with no nucleotide derivative (Δ), 10 μM ATP ($\circ$), 1 mM UDP ($\blacktriangle$) or 10 μM ATP and 1 mM UDP ($\bullet$), all in the presence of 10 mM Mg^{2+} free and 300 mM ethanol. [^3H]PEt was determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements. For comparison, other membrane samples were incubated for the same periods with 0.2 μM GTP (-----), 0.4 μM GTP (---) or 1 μM GTP (—) and the increases in [^3H]PEt determined. (b) Samples of membrane suspension were incubated under the same experimental conditions as in (a) but with 10 μM [γ - ^{32}P]ATP ($\circ$, $\square$) or 10 μM [γ - ^{32}P]ATP and 1 mM UDP ($\bullet$, $\blacksquare$, $\blacktriangledown$). The concentrations of [^{32}P]ATP ($\circ$, $\bullet$), [^{32}P]GTP ($\square$, $\blacksquare$) and [^{32}P]UTP ($\blacktriangledown$) present after incubation for the indicated periods are shown; values are means $\pm$ S.E.M. from three determinations.

GTP[S] or GTP (results not shown). Addition of 10 μM Ro 31-8220, a compound that is chemically related to staurosporine but a more specific inhibitor of PKC [40], diminished the PLD activity of platelet membranes in the presence of both low and high ATP concentrations (by 35% with 10 μM ATP and 19% with 1 mM ATP). These results suggest that, although membrane-bound PKC may enhance PLD activity, a different protein kinase or perhaps another staurosporine-binding protein must also regulate the enzyme.

Effects of PCr and CK on membrane PLD activity

In some systems, addition of PCr with CK has been shown to decrease the activation of G-proteins by ATP[S], presumably by removing GDP [38,39]. However, addition of 1 mM PCr and 10 units of rabbit muscle CK/ml did not prevent the activation of platelet membrane PLD by ATP[S], and alone stimulated the PLD activity of membranes from PMA-treated platelets by 3.0 ± 0.1 -fold (mean $\pm$ S.E.M., $n = 5$), an effect of the same magnitude as that observed with 1 mM ATP[S] and significantly larger than that seen with 10 μM ATP ($P < 0.05$, unpaired t test). Investigation showed that whereas CK had no effect by itself, PCr alone could stimulate membrane PLD activity. At concentrations ≥ 1 mM, PCr was equally effective in the absence or presence of CK. However, the effects of lower concentrations of PCr (0.04–0.4 mM) were potentiated by exogenous CK (Figure 7). This was explained by the presence of CK in rabbit platelet membranes (0.42 ± 0.04 unit/mg of protein; mean $\pm$ S.E.M., $n = 4$). Again, a much higher enzyme activity was found in the platelet supernatant fraction (5.1 units/mg of protein; mean, $n = 2$). Treatment of platelet membranes with the CK inhibitor DNFB (50–100 μM) markedly decreased the stimulation of PLD

Figure 7 Effects of PCr and CK on the PLD activity of membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of membrane suspension were then incubated for 10 min at 37 °C with the indicated concentrations of PCr in the absence ($\circ$) or presence ($\bullet$) of 10 units of CK/ml, all with 10 mM Mg^{2+} free and 300 mM ethanol in the medium. Membrane [^3H]PEt was determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment.

Table 2 Effects of DNFB on the activation of PLD by PCr in membranes from PMA-treated platelets

Membranes were isolated from platelets that had been both labelled with [^3H]glycerol and incubated with PMA. Before washing as described in the Experimental section, membranes were first incubated for 15 min at 25 °C with either 0.1% DMSO (control membranes) or 100 μM DNFB and 0.1% DMSO (DNFB-treated membranes). When ready for assay of PLD, these membrane preparations had CK activities of 0.42 and 0.07 unit/mg of protein respectively. Samples of each membrane suspension were incubated for 10 min at 37 °C with the indicated additions at the following concentrations: CK, 10 units/ml; PCr, 1 mM; GTP, 100 μM . All incubation mixtures also contained 10 mM Mg^{2+} free and 300 mM ethanol. Membrane [^3H]PEt was then determined and appropriate blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in a single experiment. Significant inhibition is shown (* $P < 0.001$; unpaired t test).

Additions to PLD assay	[^3H]PEt formed (d.p.m./mg of protein)	
	Control membranes	DNFB-treated membranes
None	5.0 ± 0.2	5.0 ± 0.1
CK	4.9 ± 0.2	5.2 ± 0.1
PCr	14.4 ± 0.1	$7.9 \pm 0.1^*$
PCr + CK	18.9 ± 0.8	19.8 ± 0.5
GTP	29.7 ± 0.2	30.1 ± 0.6

by PCr alone, but had no effect on the activation of PLD by PCr and exogenous CK, or by GTP (Table 2). To determine more precisely the mechanism of activation of PLD by PCr and CK, we studied the actions of 1 mM UDP and of 1 mM GDP[S] on this effect. Both compounds were inhibitory (Table 1); however,

Figure 8 Effects of membrane concentration on the stimulation of PLD activity by nucleoside triphosphates and by PCr

Membranes were isolated from platelets that had been labelled with [^3H]glycerol and incubated with PMA, as described in the Experimental section. Samples of undiluted membrane suspension and of suspension diluted 2-, 4- or 8-fold with buffer A were incubated for 10 min at 37 °C with the additions listed below, all in the presence of 10 mM $\text{Mg}^{2+}_{\text{free}}$ and 300 mM ethanol. After incubation of the samples, membrane [^3H]PEt was determined and blanks were subtracted; values are means $\pm$ S.E. of the difference from triplicate measurements in each experiment. (a) In this experiment, samples with undiluted suspension contained 0.21 mg of membrane protein. The additions were: none ($\circ$); 0.1 μM GTP[S] ($\bullet$); 0.4 μM GTP ($\triangle$); 10 μM ATP (∇); 1 mM PCr ($\blacksquare$). (b) In this case, samples with undiluted suspension contained 0.31 mg of membrane protein. The additions were: none ($\circ$); 1 mM ATP[S] ($\diamond$); 0.1 mM ATP[S] ($\diamond$); 4 μM GTP ($\blacktriangle$); 40 μM ATP ($\blacktriangledown$).

UDP was less effective with PCr, and particularly with PCr and CK, than with exogenous ATP. Since UDP did not inhibit the effect of 1 μM GTP (Table 1) and neither UDP or GDP[S] inhibited platelet membrane or rabbit skeletal-muscle CK, even when the ADP concentration in the assay was lowered to 20 μM , we conclude that PCr stimulated PLD mainly through the sequential actions of CK, NDPK and a GTP-binding protein (G_D) by utilizing membrane-associated ADP and GDP, and to a lesser extent through CK and G_D alone.

Effects of membrane concentration on the actions of nucleoside triphosphates and PCr

To determine whether NDPK or CK can act directly on membrane-bound GDP or can, in some way, channel GTP to G_D , we studied the effects of dilution of platelet membrane suspension on the responsiveness of membrane PLD to activating compounds. In principle, effects that do not depend on the release of diffusible intermediates into the incubation medium should be unaffected by dilution of the membranes. As expected, neither the control nor the GTP[S]-stimulated PLD activities of platelet membranes were affected by dilution (Figure 8a). Although the action of 4 μM GTP (Figure 8b) was similarly unaffected, that of 0.4 μM GTP (Figure 8a) was enhanced by

dilution of the membranes. The latter observation can readily be explained by the rapid breakdown of low concentrations of GTP, described above. In contrast with the effects of the above compounds, the stimulation of PLD activity by 1 mM PCr (Figure 8a) or 40 μM ATP (Figure 8b) was markedly attenuated by dilution of the membranes. The action of 10 μM ATP was less affected by dilution (Figure 8a), probably as a result of decreased ATP breakdown. These results strongly suggest that most or all of the effects of PCr and ATP are mediated by diffusible intermediates. ATP[S], on the other hand, appeared to differ in its effects from the above compounds (Figure 8b). With 1 mM ATP[S], no effect of dilution was seen, and with 0.1 mM ATP[S], an 8-fold dilution of the membranes decreased the activation of PLD by only one-half. This suggests that ATP[S] and NDPK may act in part without releasing free GTP[S] into the incubation medium.

DISCUSSION

Our results show that ATP[S] and a wide variety of nucleoside triphosphates, including ATP, XTP and CTP, can activate PLD in rabbit platelet membranes. Several lines of evidence indicate that these effects are mediated by NDPK. First, this enzyme can transfer the thiophosphate group of nucleoside 5'-[γ -thio]triphosphates to nucleoside diphosphates [37] in a reaction that is already known to occur in human platelet membranes, in which GTP[S] can be generated from ATP[S] and endogenous GDP [38]. The other nucleoside triphosphates that were effective are also substrates of NDPK [41]. Second, the effects of these compounds on PLD activity were decreased or blocked by a high concentration of UDP, which can form an abortive complex with the enzyme or serve as a competitive substrate [41] and has long been used to inhibit GTP formation from endogenous GDP [36]. Third, incubation of [γ - ^{32}P]ATP with platelet membranes led to the formation of [^{32}P]GTP in amounts which, when allowance was made for GTP breakdown, were compatible with the observed increase in PLD activity. Fourth, we confirmed that NDPK is present in rabbit platelet membranes, using an assay that is specific for this enzyme [34].

Although the earliest studies on platelet NDPK were motivated by a hypothesis that the enzyme might be a receptor for extracellular ADP [42], over the last 10 years evidence from a variety of cell systems, including platelets, has suggested that NDPK may be functionally linked to GTP-binding proteins, and may play a role in signal transduction by transferring high-energy phosphate to GDP bound to or closely associated with these proteins [43,44]. Such a role for NDPK has been proposed in relation to G_s [36,38,45], G_i [46], G_k [47,48], the GTP-binding proteins that activate NADPH oxidase in HL-60 cells [39] and various other low- M_r GTP-binding proteins [49]. However, it has always been difficult to understand how NDPK could utilize GDP that was still bound to GTP-binding proteins, and studies purporting to demonstrate this reaction with purified proteins [49–51] are now recognized to have been flawed [52–54]. Nevertheless, the fact that close physical and functional associations of GTP-binding proteins with NDPK have so frequently been observed suggests that, even if NDPK cannot directly transfer phosphate to bound GDP, mechanisms may exist for the channelling of GTP from NDPK to G-proteins. Recent support for this view is provided by observations showing that GTP[S] formed after addition of ATP[S] to HL-60 cell membranes inhibits ligand binding by receptors much more effectively than does exogenous GTP[S] [55,56]. It is also relevant that in some instances receptor activation appears to enhance membrane NDPK activity [38,46,57]. Thus, in platelet membranes, prosta-

glandin E_1 potentiated the formation of GTP[S] after addition of ATP[S] [38] and adrenaline appeared to enhance the conversion of endogenous GDP into GTP [46]. In a study with *Dictyostelium* membranes, the receptor-stimulated increase in NDPK activity could not be accounted for by an enhanced release of GDP [57]. Despite these intriguing observations, the importance of membrane-bound NDPK in intact cells remains unclear, since in this situation the supply of GTP is less likely to be a rate-limiting factor in G-protein activation. A role for NDPK in the local regulation of the GTP/GDP ratio has not, however, been excluded [43,44].

Our studies add the unidentified GTP-binding protein that regulates PLD (G_D) to those that can be functionally coupled with NDPK. However, in this instance, no convincing evidence was obtained that GTP formed by NDPK is used in preference to exogenous GTP; the apparent difference (Figure 6) could be accounted for by the more rapid degradation of the latter GTP, which cannot be regenerated. To determine whether direct phosphate transfer from NDPK to G_D , or channelling of guanine nucleotide, can occur in platelet membranes, we have applied a dilution test that does not appear to have been used previously. Thus, in principle, actions of NDPK that do not involve a freely diffusible intermediate should be unaffected by membrane dilution, and vice versa. The results showed that, although the stimulation of PLD by GTP[S] and GTP did not decrease after membrane dilution, the effects of ATP were largely abolished. With ATP[S], more equivocal results were obtained, but at least part of the effect of 0.1 mM ATP[S] could be attributed to the formation of a diffusible intermediate, presumably GTP[S] [38]. Although the remaining component of the action of ATP[S] could reflect direct thiophosphate transfer or channelling of GTP[S], as proposed by others [43,44,56], it is also possible that the almost irreversible high-affinity binding of GTP[S] to G-proteins in the presence of Mg^{2+} gives the action of ATP[S] its distinctive properties [54].

In contrast with the findings of others studying ligand binding to HL-60 cell membranes [55,56], exogenous GDP did not potentiate the action of ATP[S] in the platelet system, suggesting that the amount of endogenous membrane-bound GDP was more than sufficient for NDPK activity. However, attempts to scavenge this endogenous GDP with PCr and CK did not diminish the activation of PLD by ATP[S], and stimulated PLD activity in the absence of other additions. This result contrasts with those obtained in other studies on the effects of PCr and CK on the activation of G-proteins in human platelet membranes, which have generally shown an inhibition of the action of ATP[S] attributable to the removal of GDP [38,46]. That this was not observed is probably explained by the ability of PCr and CK to generate GTP in amounts sufficient to activate PLD as effectively as the smaller amounts of GTP[S] formed on addition of ATP[S]. PCr alone was also stimulatory; this is explained by the presence of CK in rabbit (though not human) platelets [58]. Our experiments show that a small fraction of this CK was firmly membrane-bound, like NDPK. The inhibitory effects of GDP[S] and UDP indicate that PCr and CK can activate G_D and that much of this effect is exerted through the intermediary action of NDPK. We suggest that CK, NDPK and G_D can be coupled in series in rabbit platelet membranes and that endogenous ADP plays a role similar to that of GDP. The isoenzymes of CK and NDPK present in rabbit platelets have not been identified, but it is likely that their relative activities with ADP and GDP as substrates [41,59] favour this indirect mechanism of generating GTP on addition of PCr. It is, however, necessary to explain why PCr and CK can stimulate PLD more effectively than exogenous ATP. Although there is evidence that CK associated with membranes

plays an important role in the channelling of high-energy phosphate to ATP-dependent ion pumps, notably the Na^+/K^+ -ATPase of the cardiac sarcolemma [60] and the Ca^{2+} -ATPase of muscle sarcoplasmic reticulum [61], channelling of ATP and GTP formed by CK in rabbit platelet membranes is unlikely, because dilution of these membranes almost abolished the activation of PLD by PCr. Most probably, 1 mM PCr drives the formation of more GTP than does 10 μ M ATP by maintaining all the available adenine and guanine nucleotides as the triphosphates, whereas this concentration of exogenous ATP is rapidly degraded.

The decreased activation of PLD at high (mM) ATP concentrations presumably reflects an inhibition of the action of GTP formed by NDPK, since a similar inhibitory effect was seen when ATP was added with exogenous GTP. This phenomenon could be of physiological interest, since reversal of this inhibition could be required before PLD activity can be expressed in intact platelets. The mechanism responsible for the inhibition of PLD by ATP is still uncertain. However, the ability of micromolar concentrations of staurosporine, but not of a more selective PKC inhibitor, Ro 31-8220 [40], to overcome this inhibition suggests that a protein kinase other than PKC could be involved. This could imply a physiological mechanism in which PLD is activated directly or indirectly by protein dephosphorylation. In this context, treatment of intact platelets with 10 μ M staurosporine increased the PLD activity in isolated platelet membranes [7]. However, alternative explanations of the action of staurosporine are possible. Recently, the stimulation of PLD in neutrophils by high concentrations of this compound has been attributed to direct activation of a pertussis-toxin-sensitive G-protein [62]. Although the activation of rabbit platelet membrane PLD by GTP is not inhibited by pertussis toxin (J. S. Sherwood and R. J. Haslam, unpublished work), the possibility that staurosporine acts on a platelet protein other than a protein kinase cannot yet be excluded.

As yet, there is no good evidence for a direct activation of platelet membrane PLD by a receptor-coupled G-protein. Although thrombin stimulates PLD in intact human platelets [22,25,26], this stimulus has little or no effect on the PLD activity of isolated rabbit platelet membranes, whether or not GTP or GTP[S] is present [7]. This contrasts with the phosphoinositide-specific phospholipase C activity of similarly prepared membranes, which is increased by thrombin in the presence of GTP [63]. A further significant difference lies in the ability of GTP to activate platelet membrane PLD to the same maximum extent as GTP[S], whereas, in the absence of a receptor agonist, activation of phospholipase C by GTP amounted to only 20% of the effect of GTP[S] [63]. These considerations suggest that two different GTP-binding proteins are involved and that, under the conditions of our experiments, the species that activates PLD may release GDP more readily and/or have a lower GTPase activity.

Since activation of PLD by GTP[S] may generate signals important for secretion from permeabilized platelets [21,22] and HL-60 cells [28], it is of interest that some of the nucleoside triphosphates that stimulated platelet membrane PLD activity have been reported to support or potentiate exocytosis from various permeabilized cells. Thus CTP or UTP can replace ATP in supporting the Ca^{2+} -dependent secretion of 5-hydroxytryptamine and lysosomal enzymes from electroporated permeabilized platelets [32]. In contrast, XTP or ITP (but not CTP or UTP) can potentiate secretion of β -glucuronidase from streptolysin-O-permeabilized HL-60 cells, without activating phospholipase C [31]; the former compounds also stimulated PLD activity [28]. In permeabilized rat mast cells, ITP and XTP were even more effective in supporting exocytosis than was GTP or ATP [29], whereas in permeabilized rabbit neutrophils, UTP, XTP and

CTP could all support secretion of β -glucuronidase, but less efficiently than GTP [30]. Although the differences between these cells require explanation, perhaps in terms of variations in the nucleotide affinities of the NDPK isoenzymes present [41] or of other differences in nucleotide metabolism, these results are compatible with the hypothesis that NDPK and G_D mediate the secretion from permeabilized cells caused by these compounds. It follows that G_E , the GTP-binding protein thought to play a specific role in exocytosis from these cells [27], may be identical with G_D . Our results have an additional implication. Thus, the dependence of secretion from some permeabilized cells on ATP or ATP[S] (e.g. [64]) has been interpreted as indicating that protein phosphorylation or thiophosphorylation plays a role in the exocytotic process. This view should be reconsidered, since these compounds may exert some of their effects as a result of the formation of GTP or GTP[S] by NDPK.

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