Guanosine 5'-[γ -thio]triphosphate induces membrane localization of cytosol-independent phospholipase D activity in a cell-free system from U937 promonocytic leucocytes

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Activation of phospholipase D (PLD) in phagocytic leucocytes requires protein components present in both the plasma membrane and the cytosol, but the catalytic and regulatory factors are not fully defined. We have characterized the effect of guanosine 5'-[γ -thio]triphosphate (GTP[S]) on the subcellular requirements for reconstitution of PLD activity, using a cell-free system from U937 human promonocytic leucocytes. Incubation of permeabilized cells with 100 μ M GTP[S] resulted in a membrane-localized PLD activity which was independent of added cytosol. The PLD activity of membranes from GTP[S]-treated cells was 7-fold greater than the basal activity of control membranes, and could be further augmented by the addition of ATP. This was the first demonstration of a stable agonist-regulated PLD activity in membranes from phagocytic leucocytes which was quantitatively comparable with that seen in a fully reconstituted system. Cytosol from GTP[S]-treated cells had a

decreased capacity to support PLD activation, consistent with GTP[S]-induced depletion of a factor essential for reconstitution of PLD activity. Incubation of isolated membrane and cytosol with GTP[S] also resulted in a cytosol-independent PLD activity in the re-isolated membranes. The effect of GTP[S] could be mimicked by guanosine 5'-[β - γ -imido]triphosphate, but not by aluminium fluoride, consistent with the involvement of a low-molecular-mass GTP-binding protein(s). Incubation of isolated subcellular fractions with GTP[S], followed by removal of unbound nucleotide, suggested that at least one of the GTP-binding proteins involved in the membrane localization of PLD activity was itself present in the membrane fraction. These data were consistent with a model in which activation of GTP-binding protein(s) resulted in the stable assembly of an active PLD signalling complex at the membrane surface.

INTRODUCTION

The activation of phospholipase D (PLD) in phagocytic leucocytes had been linked to three important effector responses of these cells: stimulation of the respiratory burst [1–3], granule secretion [4–6] and phagocytosis [7,8]. The PLD-catalysed production of phosphatidic acid (PA), and its subsequent conversion into diacylglycerol by the enzyme PA phosphohydrolase, provides the major source of diacylglycerols in neutrophils stimulated by the chemotactic peptide, *N*-formylmethionyl-leucyl-phenyl-alanine [9,10]. PA itself possesses bioeffector properties, including activation of protein kinases [11], stimulation of neutrophil superoxide production [2,12,13], and mitogenic activity [14,15].

Although GTP-binding proteins, tyrosine kinases and protein kinase C (PKC) have been shown to stimulate PLD in leucocytes [16–20], the mechanism of lipase activation remains unclear. The primary physiological stimuli which result in the activation of PLD in phagocytic leucocytes are ligands for G-protein-coupled receptors, including *N*-formylated peptides, platelet-activating factor, interleukin-8, complement component C5a and leukotriene B₄ [18,21–24]. Activation of PLD by GTP-binding proteins has also been analysed in permeabilized leucocytes [25–27], and recently, in cell-free assays derived from human neutrophils

[28,29], HL-60 promyelocytic leucocytes [30], and the promonocytic leukaemic cell line, U937 [31]. These studies indicate that non-hydrolysable analogues of GTP rapidly activate a PLD activity which, in the presence of low concentrations of ethanol, can be quantified by the accumulation of the transphosphatidylated product, phosphatidylethanol (PEt). In the HL-60 and U937 cell lines, the stimulation of PLD activity by GTP-binding proteins is potentially augmented by ATP through a tyrosine kinase-based mechanism [25,26,31].

Analysis of PLD activation in cell-free systems derived from various myeloid/phagocytic cell types has demonstrated a requirement for protein components in both the plasma membrane and cytosol [28–33]. However, the subcellular localizations of the relevant catalytic and regulatory components are not fully defined. Recent evidence indicates that low-molecular-mass (LMW) GTP-binding proteins of the Rho and ARF families are involved in the activation of PLD in phagocytic leucocytes [29,32,33].

Permeabilization of phagocytic leucocytes, by either electroporation [25] or streptolysin O [27], results in a time-dependent loss of PLD activity which cannot be subsequently stimulated by guanosine 5'-[γ -thio]triphosphate (GTP[S])/ATP. However, treatment of cells with GTP[S] at the time of permeabilization

Abbreviations used: PLD, phospholipase D; PA, phosphatidic acid; PKC, protein kinase C; PEt, phosphatidylethanol; LMW, low-molecular-mass; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate; PMA, phorbol 12-myristate 13-acetate.

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largely prevents the time-dependent loss of ATP-potentiated PLD activity, suggesting that activation of GTP-binding proteins results in stabilization of a lipase-substrate complex [25,27]. A correlative analysis in U937 cells washed repeatedly following detergent permeabilization supports the hypothesis that GTP[S] causes a membrane stabilization of PLD activity [26].

In the present study, we have analysed the effect of GTP[S] on the subcellular requirements for PLD activity, using a cell-free system derived from U937 cells. The results suggest that activation of a membrane-associated GTP-binding protein leads to the stable membrane localization of a PLD activity which is independent of added cytosol.

METHODS

Cells and labelling of phospholipids

U937 cells (obtained from the American Tissue Type Culture Collection) were maintained in Iscove's medium supplemented with 25 mM Hepes and 10% (v/v) newborn-calf serum at 37 °C in 7.5% CO₂. Cells [(0.5–1.0) × 10⁶] in 500–1000 ml of medium were radioisotopically labelled by incubation with [³H]oleic acid (0.5 µCi/ml) for 18 h in a spinner flask [31].

Permeabilization of U937 cells

Following radiolabelling of cellular phospholipids, cells were washed with Hepes/NaCl buffer [25 mM Hepes (pH 7.4)/125 mM NaCl/0.7 mM MgCl₂/0.5 mM EGTA/10 mM glucose/1 mg/ml BSA [28] and resuspended in 10 ml of the same. Following incubation with 4 mM di-isopropyl fluorophosphate for 25 min at 4 °C, cells were resuspended in Hepes/KCl buffer [25 mM Hepes (pH 7.4)/100 mM KCl/3 mM NaCl/5 mM MgCl₂] supplemented with 2 µM leupeptin/0.5 mM phenyl-methylsulphonyl fluoride/1 mM dithiothreitol/0.5 mg/ml BSA [28]. The level of free Ca²⁺ was adjusted to 1 µM by buffering with 1 mM EGTA. Cells were warmed to 37 °C for 5 min and then permeabilized by the addition of 75 µg/ml β-escin (prepared as a 5 mg/ml stock solution in ethanol). In selected samples, 100 µM GTP[S] or 1 µM sodium orthovanadate + 100 µM H₂O₂ were added concurrently with β-escin. Following a 7 min reaction with 37 °C, cells were placed on ice for 1 min before cell fractionation.

Cellular fractionation and isolation of membrane and cytosol

Subcellular fractions were prepared either from cells newly resuspended in Hepes/KCl buffer or following permeabilization of cells with β-escin. Cell disruption was accomplished by nitrogen cavitation [3.1 MPa (450 lb/in²), 25 min, 4 °C] and plasma-membrane and cytosolic fractions were prepared by a modification of the protocol of Olson et al. [28], as noted in [31]. Cytosol preparations were passed through a 0.22 µm Amicon filter. Membrane and cytosol were utilized within 48 h of isolation. Protein concentrations in the cytosolic and membrane fractions were determined by the method of Bradford [34].

Assay of PLD activity

PLD activity was assayed by the method of Olson et al. [28] with the modifications noted in [31]. Briefly, 25 µl of membrane (75 µg of protein) was incubated with 475 µl of Hepes/KCl buffer (containing 1 µM free Ca²⁺ buffered with 1 mM EGTA) and the appropriate additions (100 µM GTP[S] and/or 1 mM ATP). Ethanol (1.6%) was included in the assay to permit detection of PEt, the metabolically stable transphosphatidylated product, as an index of PLD activity [25,31]. In selected assays, 100 µl of

cytosol (100 µg of protein) was added to 375 µl of Hepes/KCl buffer, prior to initiation of the reaction by the addition of membrane. Following a 30 min incubation at 37 °C, the reaction was stopped by addition of 5 vol. of chloroform/methanol (2:1, v/v). Lipids were extracted, dried and analysed by t.l.c. as described previously [25]. [³H]PEt was quantified by liquid-scintillation spectrophotometry, and counts were normalized for the total amount of ³H-labelled phospholipid in each experiment to correct for variability in cell radiolabelling and permit pooling of data from successive experiments. Basal values of [³H]PEt present in each membrane preparation prior to initiation of the PLD assay were subtracted from all values.

Cell-free assay of membrane-localized PLD activity

Freshly isolated membrane (2.4 mg of protein) and cytosol (3.2 mg of protein) from unstimulated U937 cells were incubated in Hepes/KCl buffer in a total volume of 2.0 ml. Then 100 µM GTP[S] or 1 µM phorbol 12-myristate 13-acetate (PMA)/1 mM MgATP or 10 µM vanadate/100 µM H₂O₂/1 mM MgATP was added, and the level of free Ca²⁺ was buffered at 1 µM with 1 mM EGTA. In selected samples, the free Ca²⁺ concentration was adjusted by varying the addition of CaCl₂ by using a K_D of 10⁻⁷ M for EGTA-Ca²⁺ [35]. Following a 10 min incubation at 37 °C, 4 vol. of ice-cold Hepes/KCl buffer was added and membranes were pelleted by centrifugation at 225000 g for 1 h at 4 °C. Following resuspension of the membrane in Hepes/KCl buffer, the PLD activity of the membrane with or without cytosol, GTP[S], ATP was determined by measuring the accumulation of PEt in the presence of 1.6% ethanol.

Analysis of the subcellular localization of a GTP-binding protein involved in activation of PLD

Membrane (2.4 mg of protein) and cytosolic (3.2 mg of protein) fractions isolated from unstimulated U937 cells were incubated with 100 µM GTP[S] or buffer control (in a total volume of 1.0 ml) for 1 h at 4 °C on a rotating platform. Unbound GTP[S] was removed from the membrane fraction by washing twice with 10 ml of ice-cold Hepes/KCl buffer, followed by resuspension in 700 µl of the same. Unbound GTP[S] was removed from the cytosolic fraction by ultrafiltration twice using a Centricon microconcentrator with a 10 kDa exclusion limit, followed by reconstitution to 1 ml with Hepes/KCl buffer. Previous experiments have shown that this ultrafiltration procedure itself does not alter the capacity of cytosol to support GTP[S]-mediated PLD activity when normalized for the protein concentration.

The PLD activity of the washed membrane and ultrafiltered cytosol was assayed in the presence of the complementary untreated fraction with or without GTP[S]/ATP. The washing and ultrafiltration steps were calculated to dilute the concentration of unbound GTP[S] in the subsequent PLD assay to < 0.5 nM, which was well below the lower limit of 1 µM at which GTP[S] stimulation of PLD can be detected. Membrane and cytosolic fractions which had not been incubated with GTP[S] were mock-washed/ultrafiltered, and PLD activity was similarly determined.

RESULTS

GTP[S] treatment of permeabilized U937 cells results in membrane localization of a PLD activity which is cytosol-independent

In order to analyse the mechanism by which activation of GTP-binding proteins is linked to the stimulation of PLD, we isolated membranes from U937 cells which were treated with GTP[S] in

Table 1 Effect of GTP[S] pretreatment of U937 cells on the PLD activity of isolated plasma membranes alone or with cytosol

U937 cells (3×10^8) in Hepes/KCl buffer were incubated at 37 °C with either 75 μ g/ml β -escin alone (control) or 100 μ M GTP[S] + β -escin for 7 min. Membrane and cytosolic fractions were then prepared as outlined in the Methods section. PLD activity of membranes was assayed under the indicated conditions at 37 °C for 30 min. Free Ca^{2+} was buffered at 1 μ M during the PLD assay. Values represent mean $\pm$ S.E.M. of 5 experiments performed in duplicate. Basal values of [^3H]PEt present in each membrane preparation prior to the PLD assay were subtracted from all values: control 1447 ± 133 , GTP[S] 8751 ± 356 .

Additions to membrane in PLD assay*	PEt (c.p.m./ 10^5 c.p.m. in phospholipid)	
	Control preincubation	GTP[S] preincubation
None	492 $\pm$ 133	3505 $\pm$ 230
GTP[S]	911 $\pm$ 172	4884 $\pm$ 488
ATP	744 $\pm$ 67	6897 $\pm$ 484
GTP[S] + ATP	1773 $\pm$ 264	6685 $\pm$ 629
Cytosol	685 $\pm$ 141	5114 $\pm$ 548
+ GTP[S]	3468 $\pm$ 292	6529 $\pm$ 601
+ ATP	1959 $\pm$ 268	11049 $\pm$ 1103
+ GTP[S]/ATP	7369 $\pm$ 714	11759 $\pm$ 1109

* GTP[S] 100 μ M, MgATP 1 mM.

the presence of the permeabilizing agent, β -escin, or from cells treated with β -escin alone, and then assayed PLD activity in the absence and presence of cytosol. To obtain an accurate determination of the membrane-associated PLD activity, the levels of PEt which accumulated during the initial permeabilization period were subtracted from each of the values obtained from the PLD assay. As shown in Table 1, membranes from GTP[S]-treated cells had an increased level of PLD activity (in the absence of added cytosol) compared with membranes from control cells. The level of PEt accumulation by membranes from GTP[S]-treated cells was 7-fold greater than that of control membranes. This suggested that GTP[S] promoted the membrane localization of a PLD activity which was independent of added cytosol. The elevated PLD activity of membranes isolated from cells pretreated with GTP[S] persisted following repeated washings and storage at 4 °C for 48 h.

Vanadate and PMA potentially activate PLD in U937 cells and other phagocytic leucocytes [10,26,27,36–40]. However, in contrast with the effects of GTP[S], treatment of permeabilized U937 cells with vanadate or of intact cells with PMA did not result in a cytosol-independent PLD activity in subsequently isolated membranes (results not shown). These results suggested that the formation of a stable membrane-localized PLD activity, which can persist following cell disruption and isolation of the membranes, required activation of the relevant GTP-binding protein(s) by poorly hydrolysable GTP analogues.

GTP[S] treatment of membranes isolated from control cells did not result in a level of PLD activity commensurate with that of membranes isolated from cells pretreated with GTP[S] (Table 1). This suggested that a cytosolic component was required for the GTP[S]-mediated induction of a membrane-localized PLD activity. Membranes from control cells (or from cells pretreated with vanadate or PMA) required the presence of cytosol to yield a PLD activity which was stimulated by GTP[S] and potentiated by ATP. The values for PEt accumulation in the control membrane+cytosol system (with or without GTP[S]/ATP) closely parallel those seen in the absence of prior cell perme-

Figure 1 Effect of GTP[S] on the capacity of cytosol to reconstitute PLD activity

U937 cells (3×10^8) were incubated with either 75 μ g/ml β -escin alone (control) or 100 μ M GTP[S] + β -escin for 7 min at 37 °C. Following the incubation, cells were disrupted by nitrogen cavitation, and plasma membrane and cytosolic fractions were prepared by density-gradient ultracentrifugation. Control membrane (75 μ g) was then added to the indicated amounts of cytosol from control ($\square$) or GTP[S]-treated cells ($\blacksquare$) and Hepes/KCl buffer was added to a total volume of 0.5 ml. PEt accumulation stimulated by 100 μ M GTP[S], in a 15 min reaction at 37 °C, was determined as indicated in the Methods section. Data represent means $\pm$ S.D. from one of two similar experiments performed in duplicate.

abilization [31], suggesting that the treatment with β -escin did not significantly alter the mechanism of PLD activation.

Addition of 1 mM ATP to membranes from GTP[S]-treated cells significantly augmented their basal PLD activity (2-fold increase) in the absence of added cytosol (Table 1). In fact, the level of PEt accumulation produced by membranes from GTP[S]-treated cells, assayed in the presence of ATP, was approximately equal to the maximal PEt accumulation observed when control membranes were assayed in the presence of added cytosol, GTP[S] and ATP. This suggested that GTP[S] treatment of permeabilized cells resulted in a quantitatively significant membrane association of a cytosolic factor(s) required for PLD activation. These data further suggested that membranes isolated from GTP[S]-treated cells may be a useful model for studying the co-ordinate regulation of PLD by GTP-binding proteins and ATP.

Re-addition of cytosol from cells treated with GTP[S], to membranes from these same cells, caused an additional 2-fold increase in PLD activity assayed in the presence of ATP (Table 1). This finding suggested that GTP[S] treatment of permeabilized cells did not cause the membrane translocation of the entire pool of the cytosolic factor(s) required for PLD activation and/or that an additional cytosolic factor(s) which was required for maximal lipase activity did not undergo membrane translocation in the presence of GTP[S]. To analyse these possibilities further, we studied whether GTP[S] treatment of permeabilized cells could deplete cytosol of the factor(s) which promote PLD activity, when combined with control membranes. Control membranes were assayed for GTP[S]-stimulated PLD activity in the presence of increasing amounts of cytosol derived from either GTP[S]-treated cells or control cells. At each amount tested, cytosol from GTP[S]-treated cells supported less PLD activity, stimulated either by GTP[S] alone (Figure 1) or GTP[S] + ATP (results not shown). This decreased ability of cytosol from GTP[S]-treated cells to support stimulated PLD activity was

Table 2 Effect of guanine nucleotide analogues on the generation of a membrane-localized PLD activity

Plasma membrane and cytosol from unstimulated U937 cells were incubated in Hepes/KCl buffer with the indicated nucleotides for 10 min at 37 °C. The reaction was terminated with 4 vol. of ice-cold buffer and membranes were pelleted by centrifugation at 225 000 *g* for 1 h at 4 °C. Following resuspension, the PLD activity of the membrane with or without cytosol, GTP[S] and/or ATP was determined by measuring the accumulation of PEt in the presence of 1.6% ethanol and free Ca²⁺ (1 μ M). Basal values of [³H]PEt present in each membrane preparation prior to the PLD assay were subtracted from all values: control 516 $\pm$ 56, GTP[S] 1804 $\pm$ 216, GTP[S]/GDP[S] 984 $\pm$ 83. Data represent means $\pm$ S.E.M. from 5 experiments performed in duplicate.

Additions to membrane in PLD assay*	PEt (c.p.m./10 ⁵ c.p.m. in phospholipid)		
	Control preincubation	GTP[S]† preincubation	GTP[S]/GDP[S] preincubation
None	371 $\pm$ 26	2302 $\pm$ 446	422 $\pm$ 41
GTP[S]	450 $\pm$ 63	4809 $\pm$ 552	1866 $\pm$ 131
ATP	442 $\pm$ 112	5845 $\pm$ 369	3506 $\pm$ 102
GTP[S]/ATP	1520 $\pm$ 350	6906 $\pm$ 458	2637 $\pm$ 122
Cytosol	481 $\pm$ 31	2102 $\pm$ 126	492 $\pm$ 79
+ GTP[S]	2661 $\pm$ 180	6916 $\pm$ 384	3063 $\pm$ 160
+ ATP	834 $\pm$ 27	5750 $\pm$ 228	3587 $\pm$ 109
+ GTP[S]/ATP	4416 $\pm$ 322	11240 $\pm$ 271	6242 $\pm$ 312

* GTP[S] 100 μ M, MgATP 1 mM.

† Concn. of guanine nucleotide analogues in the initial incubation: GTP[S] 100 μ M, GDP[S] 1 mM.

consistent with the hypothesis that GTP[S] treatment of acutely permeabilized cells caused the membrane translocation of a cytosolic factor required for activation of the lipase.

GTP[S]-induced membrane localization of a cytosol-independent PLD activity can be reproduced with isolated subcellular fractions

In order to characterize further the GTP[S]-mediated induction of a membrane-localized PLD activity, membrane and cytosolic fractions were isolated from untreated cells and then recombined *in vitro* in the absence or presence of guanine nucleotide analogues. After a 10 min incubation of the recombined fractions, the membranes were re-separated from the cytosolic fraction, extensively washed, and the PLD activity of the re-isolated membranes was assayed. When membrane and cytosol were incubated in the absence of guanine nucleotides, the re-isolated membrane fraction had a low basal level of PLD activity, which was not significantly augmented by inclusion of GTP[S] in the assay medium (Table 2). However, when cytosol was added to these re-isolated control membranes, a GTP[S]-dependent PLD activity, which was potentiated by ATP, could be reconstituted. Since these results paralleled the stimulation of PLD which occurred with freshly isolated subcellular fractions [31], it suggested that the 10 min incubation of membrane and cytosol, prior to re-isolation of the membranes, did not alter the subcellular distribution of components required for activation of the lipase.

In contrast with the control incubation, co-incubation of isolated plasma membrane and cytosolic fractions in the presence of GTP[S] followed by re-isolation of the membrane, resulted in a membrane-associated PLD activity which was readily assayed in the absence of added cytosol (Table 2). The level of PEt accumulation by membranes which had been preincubated with cytosol in the presence of GTP[S] was 6-fold greater than

Table 3 Effect of aluminium fluoride on membrane localization of PLD activity

Cytosol (3.2 mg) and membrane (2.4 mg) fractions from unstimulated U937 cells were incubated in Hepes/KCl buffer, either alone (control), or in the presence of 20 mM NaF + 10 μ M AlCl₃ + 10 μ M GDP, or 100 μ M GTP[S]. Following a 10 min incubation at 37 °C, 4 vol. of ice-cold buffer were added and membranes were pelleted by centrifugation. Membranes were washed and resuspended in Hepes/KCl buffer and PLD activity of membranes with or without ATP, GTP[S] or cytosol was determined in the presence of 1 μ M free Ca²⁺ and 1.6% ethanol, by analysing the accumulation of [³H]PEt, as noted in the Methods section. Basal values of [³H]PEt present in each membrane preparation prior to the PLD assay were subtracted from all values: control 482 $\pm$ 52, aluminium fluoride 470 $\pm$ 60, GTP[S] 1752 $\pm$ 305. Data represent means $\pm$ S.E.M. from three experiments performed in duplicate.

Additions to membrane in PLD assay*	PEt (c.p.m./10 ⁵ c.p.m. in phospholipid)		
	Control preincubation	Aluminium fluoride preincubation	GTP[S] preincubation
None	337 $\pm$ 37	305 $\pm$ 35	2176 $\pm$ 376
ATP	527 $\pm$ 79	557 $\pm$ 68	4897 $\pm$ 551
Cytosol + GTP[S] + ATP	5038 $\pm$ 611	5404 $\pm$ 511	7829 $\pm$ 373

* GTP[S] 100 μ M, MgATP 1 mM.

membranes preincubated with cytosol in the absence of GTP[S]. Inclusion of MgATP in the assay medium significantly augmented PLD activity in these GTP[S]/cytosol-pretreated membranes, but had no effect on membranes which were incubated with cytosol in the absence of GTP[S]. When the GTP[S]-treated membranes were assayed in the presence of MgATP, the level of PEt accumulation was 17-fold greater than that observed with control membranes. The stimulus dependence of the membrane-localized PLD activity closely paralleled the activation of PLD by GTP[S]/ATP in permeabilized U937 cells [26] and in the complete cell-free system [31]. This suggested that the membrane-localized PLD activity was likely to represent the same enzymic activity observed under these other experimental conditions.

The ability of GTP[S] to induce this membrane-localized PLD activity was inhibited when guanosine 5'-[β -thio]diphosphate (GDP[S]) was included in the pre-incubation with GTP[S] and cytosol (Table 2). A 10-fold molar excess of GDP[S] during this pre-incubation decreased the basal PEt accumulation assayed in the re-isolated membranes by approx. 80% and the ATP-potentiated PEt accumulation by 40%. The inhibitory effect of GDP[S] was consistent with the hypothesis that a GTP-binding protein mediates the membrane localization of PLD activity induced by GTP[S]. Furthermore, incubation of membrane and cytosolic fractions in the presence of guanosine 5'-[β -imido]-triphosphate (p[NH]ppG), another poorly hydrolysable GTP analogue, also resulted in increased PLD activity in the re-isolated membranes [PEt (c.p.m.): control 371 $\pm$ 26; p[NH]ppG 708 $\pm$ 73, *n* = 3].

The effects of GTP[S] and p[NH]ppG could be mediated by either a heterotrimeric G-protein or a LMW GTP-binding protein. In order to gain further insight into the nature of the relevant GTP-binding protein, we studied the effects of aluminium fluoride on reconstitution of PLD activity, as this compound has been reported to activate heterotrimeric, but not LMW, GTP-binding proteins [41]. Incubation of membrane and cytosol with aluminium fluoride (20 mM NaF + 10 μ M AlCl₃ + 10 μ M GDP) for 10 min at 37 °C did not result in an increase in the basal or stimulated PLD activity of re-isolated membranes (Table 3).

Another parameter used to differentiate the classes of GTP-binding proteins involved in a particular biological response is the Mg^{2+} -dependence of the phenomenon, as LMW GTP-binding proteins exhibit a much lower Mg^{2+} requirement than heterotrimeric G-proteins [29,42,43]. To study the Mg^{2+} -dependence of the GTP[S] effect on membrane PLD activity, Mg^{2+} -free buffer was utilized for cell disruption, washing of the plasma membrane fraction, and for incubation of membrane + cytosol with or without GTP[S]. Despite the use of Mg^{2+} -free buffer, membranes derived from the GTP[S] incubation possessed an elevated level of PLD activity [PEt (c.p.m.): control 332 ± 77 ; GTP[S] 2906 ± 221 ; mean $\pm$ S.D., $n = 2$]. The lack of effect of aluminium fluoride, and the low Mg^{2+} requirement for GTP[S] induction of membrane PLD activity, were both consistent with the involvement of a LMW GTP-binding protein.

PMA and vanadate are potent activators of PLD in intact and acutely permeabilized U937 cells [26,36,37]. We additionally tested whether pre-incubation of isolated membranes and cytosol with either PMA (plus ATP) or vanadate (plus ATP) might induce the membrane localization of PLD activity. Co-incubation of membrane and cytosolic fractions with either 1 μ M PMA or 10 μ M vanadate (+ 100 μ M H_2O_2 to generate vanadyl peroxides) did not increase the basal PLD activity associated with the re-isolated membranes. The basal levels of PEt accumulation (3H /c.p.m./ 10^5 c.p.m. of total phospholipid; mean $\pm$ S.E.M., $n = 3$ experiments) in these re-isolated membranes were 327 ± 25 in control membranes, 277 ± 77 in PMA-pretreated membranes and 344 ± 58 in the vanadate-pretreated membranes. The inability of PMA or vanadate to promote membrane localization of PLD activity when added to isolated subcellular fractions paralleled their similar lack of effect on this parameter when incubated with intact or permeabilized cells. These results reinforced the hypothesis that activation of GTP-binding proteins was required for the induction of a stable membrane-localized PLD activity.

Effect of Ca^{2+} on membrane localization of PLD activity in the absence and presence of GTP-binding protein activation

Increased levels of the intracellular free Ca^{2+} promote the membrane association of several cytosolic proteins, including the 85 kDa phospholipase A_2 and certain isoforms of PKC [44–46]. We have therefore investigated the effects of varying the level of Ca^{2+} (either alone or in conjunction with GTP[S]) during the preincubation of membranes and cytosol, prior to re-isolation of the membranes and assay of PLD activity (Table 4). When present in the preincubation medium at 1 μ M, Ca^{2+} did not promote the formation of a membrane-localized PLD activity in the absence of GTP[S]. The basal level of PEt accumulation by re-isolated membranes, as well as that assayed in the presence of GTP[S], ATP and cytosol, did not differ significantly regardless of whether the membranes had been pre-incubated with free $Ca^{2+} < 1$ nM or free $Ca^{2+} = 1$ μ M. In contrast, GTP[S] promoted the membrane localization of PLD activity during preincubation of the membranes and cytosol even when free Ca^{2+} was < 1 nM. In this case, the basal level of membrane-associated PLD activity was approx. 2.5 times that seen in the absence of GTP[S] and was increased 3.5-fold by the addition of ATP.

Even though Ca^{2+} ions were not required for the GTP[S]-induced membrane localization of PLD activity, the presence of 0.1–1 μ M Ca^{2+} , along with GTP[S], during preincubation of membranes and cytosol did potentiate the basal and stimulated PLD activity associated with the re-isolated membrane (Table 4). PEt accumulation by re-isolated membranes was increased when the preincubation [Ca^{2+}] was raised from < 1 nM to 100 nM (the latter value approximating to the level of free intracellular Ca^{2+}

Table 4 Effect of Ca^{2+} on membrane-associated PLD activity in the absence and presence of GTP[S]

Membrane and cytosol from unstimulated U937 cells were incubated in Hepes/KCl buffer for 10 min at 37°C. Each reaction contained 1 mM EGTA and either no added $CaCl_2$ (free $Ca^{2+} < 1$ nM) or sufficient $CaCl_2$ to yield the indicated level of free Ca^{2+} . Following the reaction, membranes were isolated and PLD activity of the membrane with or without the indicated additions was determined. The level of free Ca^{2+} was maintained at 1 μ M during the PLD assay. Results are means $\pm$ S.D. of two experiments performed in duplicate. Basal amounts of [3H]PEt present in each membrane preparation prior to the PLD assay were subtracted from all values: $Ca^{2+} (< 1$ nM) 467 ± 78 , Ca^{2+} (1 μ M) 547 ± 109 , Ca^{2+} (1 nM) + GTP[S], 1223 ± 203 , Ca^{2+} (0.1 μ M) + GTP[S], 1578 ± 316 , Ca^{2+} (1 μ M) + GTP[S], 1416 ± 278 .

(a) No GTP[S] during preincubation		PEt (c.p.m./ 10^5 c.p.m. in phospholipid)	
Additions to membrane in PLD assay*		< 1 nM Ca^{2+}	1 μ M Ca^{2+}
None		497 $\pm$ 59	498 $\pm$ 10
GTP[S]		1378 $\pm$ 138	621 $\pm$ 78
ATP		994 $\pm$ 191	934 $\pm$ 146
GTP[S], ATP		1454 $\pm$ 102	1764 $\pm$ 350
Cytosol, GTP[S], ATP		5646 $\pm$ 328	5302 $\pm$ 577

(b) GTP[S] during preincubation		PEt (c.p.m./ 10^5 c.p.m. in phospholipid)		
Additions to membrane in PLD assay*		< 1 nM Ca^{2+}	100 nM Ca^{2+}	1 μ M Ca^{2+}
None		1233 $\pm$ 178	1636 $\pm$ 253	3995 $\pm$ 660
GTP[S]		1621 $\pm$ 172	2082 $\pm$ 158	6154 $\pm$ 1526
ATP		4398 $\pm$ 233	5490 $\pm$ 102	7830 $\pm$ 1195
GTP[S], ATP		4668 $\pm$ 108	5760 $\pm$ 278	9462 $\pm$ 1319
Cytosol, GTP[S], ATP		6900 $\pm$ 267	7726 $\pm$ 300	14554 $\pm$ 1833

* GTP[S] 100 μ M, MgATP 1 mM.

in resting leucocytes [47,48]). Raising the preincubation [Ca^{2+}] to 1 μ M (a concentration observed in leucocytes stimulated by Ca^{2+} -mobilizing agonists, [47,48]), resulted in a further increase in the GTP[S]-dependent localization of PLD activity with the re-isolated membranes. This influence of Ca^{2+} on the induction of a cytosol-independent membrane-localized PLD activity paralleled its effects on GTP-binding protein-dependent PLD activity observed in the complete reconstituted system [31]. In both cases, Ca^{2+} was not required for activation of PLD by GTP-binding proteins, but it did increase the magnitude of stimulated PLD activity.

A membrane-associated GTP-binding protein is involved in the activation of PLD by GTP[S] and its potentiation by ATP

The above experiments suggested that stable activation of GTP-binding proteins altered the subcellular loci of the components required for reconstitution of PLD activity. To characterize this phenomenon further, experiments were designed to investigate the subcellular localization of the relevant GTP-binding protein(s). Membrane or cytosolic fractions were separately incubated with 100 μ M GTP[S] for 1 h at 4°C to promote nucleotide binding to GTP-binding proteins present in these subcellular fractions. Unbound GTP[S] was removed either by washing the membranes twice, followed by resuspension in nucleotide-free buffer, or by repeated ultrafiltration of cytosol. Each of these treated fractions was then combined with the complementary untreated fraction (cytosol or membrane), and

Table 5 Effect of GTP[S] pretreatment of membrane or cytosol on reconstitution of PLD activity

Membrane or cytosolic fractions were separately incubated with 100 μ M GTP[S] for 1 h at 4 °C. Unbound GTP[S] was removed by repeated washing of the membrane or ultrafiltration of cytosol as described in the Methods section. PLD activity of the respective fractions was assayed in the presence of the complementary untreated fraction with or without GTP[S]/ATP. The level of free Ca^{2+} was maintained at 1 μ M during the PLD assay. PLD activity of untreated membrane and cytosol was also determined. Basal amounts of [^3H]PEt present in each membrane preparation prior to the PLD assay were subtracted from all values: control 163 ± 25 , GTP[S] preincubation of membranes 299 ± 56 , GTP[S] preincubation of cytosol 131 ± 15 . Results are means $\pm$ S.E.M. of three experiments performed in duplicate.

Additions to PLD assay*	PET (c.p.m./10 ⁵ c.p.m. in phospholipid)		
	None	Membrane	Cytosol
None	235 $\pm$ 33	442 $\pm$ 60	202 $\pm$ 54
GTP[S]	2055 $\pm$ 38	2171 $\pm$ 75	1902 $\pm$ 115
ATP	550 $\pm$ 51	1423 $\pm$ 136	434 $\pm$ 78
GTP[S] + ATP	4919 $\pm$ 339	6179 $\pm$ 923	4871 $\pm$ 135

* GTP[S] 100 μ M, MgATP 1 mM.

the basal and stimulated levels of PLD activity were determined (Table 5). Treatment of the plasma-membrane fraction with GTP[S], followed by washing and recombination with untreated cytosol, resulted in a 2-fold increase in PLD activity compared with that observed in assays wherein neither subcellular fraction was pretreated. In contrast, pretreatment of cytosol with GTP[S], followed by repeated ultrafiltration and recombination with untreated membranes, did not result in levels of PET accumulation which were significantly different from those observed with control membranes and cytosol. Addition of 100 μ M GTP[S] and fresh cytosol to the reconstituted assay mixtures resulted in similar levels of PET accumulation, even when the membranes had been pretreated with GTP[S]. This latter observation suggested that the extensive washing procedure may have resulted in significant dissociation of GTP[S] from the relevant GTP-binding protein(s). Alternatively, this may indicate that additional GTP-binding proteins in the cytosolic fraction were recruited to PLD signalling complexes in the presence, but not in the absence, of the membrane fraction. ATP significantly augmented the level of PET accumulation in assays containing GTP[S]-pretreated membranes, but had a considerably smaller effect in assays containing control components or GTP[S]-treated cytosol. Mock-washing/ultrafiltration of membranes or cytosol which had not been incubated with GTP[S] did not alter the basal or stimulated levels of the PLD activity (results not shown). Preliminary experiments indicated that preincubation of membrane or cytosol with GTP[S] at 25 °C for 15 min (rather than at 4 °C for 1 h) yielded similar results, with increased basal and stimulated PLD activity in samples in which the membrane fraction had been pretreated with GTP[S].

DISCUSSION

GTP-binding proteins are important components of PLD activation in phagocytic leukocytes. In intact phagocytes, PLD is rapidly activated by diverse agonists [*N*-formylmethionyl-leucyl-phenylalanine, interleukin-8, platelet-activating factor, C5a] which occupy 7-transmembrane domain receptors coupled to heterotrimeric G-proteins [9,10,18–20]. In permeabilized phagocytes and cell-free systems derived from phagocytes, phospholipase D is readily activated by GTP[S] and other poorly

hydrolysable GTP analogues [25–33, 40]. In the present study, we have utilized membranes and cytosol isolated from the U937 human promonocyte cell line to analyse the effects of GTP[S] on the subcellular components required for reconstitution of PLD activity. All previous reports using reconstituted cell-free systems derived from phagocytic leukocytes have indicated that both membrane and cytosolic fractions must be present for the measurement of significant PLD activity [28–33]. Our present results demonstrate that activation of GTP-binding proteins, either in permeabilized cells prior to fractionation or in reconstituted mixtures of isolated membranes and cytosol, induces a stable membrane-associated PLD activity which can be readily assayed in the absence of cytosol. This membrane-associated PLD activity which results from GTP-binding protein stimulation is quantitatively similar to that attained in the fully reconstituted system [28,31].

The membrane-localized PLD activity shares many characteristics with the GTP[S]-dependent PLD activity observed in permeabilized U937 cells [26] or in the complete cell-free system (comprising membranes and cytosol) derived from these cells [31]. These features include: (1) marked potentiation by ATP; (2) similar efficacy profiles for activation by various guanine nucleotide analogues; and (3) potentiation by micromolar levels of free Ca^{2+} in the absence of an absolute requirement for Ca^{2+} . These similar characteristics suggest that the membrane-localized PLD activity is likely to represent the same GTP[S]-stimulated enzymic activity observed in permeabilized cells and in the complete cell-free system. Thus membranes from GTP[S]-treated cells may serve as a useful model system for further molecular characterization of PLD activity in phagocytic leukocytes, and for comparison with membrane PLD activities from other tissues/cell types.

Subcellular-fraction-specific incubation with GTP[S] suggested that at least one of the GTP-binding protein(s) responsible for PLD activation is membrane-associated (Table 5). Our results are concordant with those of Bowman et al. [29], who have recently characterized GTP-binding protein-dependent activation of PLD in a cell-free system derived from human neutrophils. In their study, GTP[S] pre-binding experiments confirmed that the activating GTP-binding protein was localized in the plasma membrane fraction. Furthermore, based on aluminium fluoride insensitivity, Mg^{2+} requirement for GTP[S] binding, and modulation of PLD activity by *Rho* GDP-dissociation inhibitor and *smg*-GDP dissociation stimulator, these authors have proposed that a LMW GTP-binding protein of the *Rho* family mediates GTP[S]-dependent activation of PLD. Similarly, in our studies, aluminium fluoride could not substitute for GTP[S] in inducing a membrane-localized PLD activity. Because aluminium fluoride preferentially activates heterotrimeric G-proteins [41], the lack of effect of this compound on reconstitution of membrane PLD activity is consistent with the involvement of a LMW GTP-binding protein. The ability of GTP[S] to induce the membrane-localized PLD activity even in the presence of low Mg^{2+} (< 30 μ M) is also consistent with involvement of a LMW GTP-binding protein [42,43].

We observed that GTP[S] supported further PLD activity when added to mixtures containing GTP[S]-pretreated membranes and cytosol (Table 5). This suggests the additional participation of soluble GTP-binding proteins in the activation of PLD. Two groups have recently demonstrated that soluble LMW GTP-binding proteins belonging to the family of ADP-ribosylation factors (ARF) proteins can stimulate PLD activity present in permeabilized HL-60 cells [32] or HL-60 membranes [33]. While inactive, GDP-liganded ARF proteins are primarily localized in the cytoplasmic fraction from unstimulated cells,

they can associate with membranes when in the GTP-liganded state [49]. Significantly, efficient GDP/GTP exchange by ARF proteins requires the presence of membranes or exogenous phospholipids. The inability of GTP[S]-pretreated cytosol to support PLD activity when subsequently recombined with membranes (Table 5) is consistent with these latter characteristics of ARF proteins, since the absence of membranes during the pretreatment would prevent the efficient exchange of GDP for GTP[S] by cytosolic ARFs.

Thus data presented in this study and in previous studies indicates that PLD activities in phagocytic leucocytes may be regulated by LMW GTP-binding proteins located in both membranous and cytosolic compartments. Regardless of the subcellular loci of PLD and the relevant GTP-binding proteins in resting leucocytes, it is likely that the induction of catalytically active PLD involves the assembly of a lipase-substrate GTP-binding protein complex at membrane surfaces. Our data indicate that one or more cytosolic factors are stably recruited to such signalling complexes upon ligation of GTP[S] by the relevant GTP-binding regulatory protein(s). Even after multiple washings of membranes containing the putative complexes, significant PLD activity can be measured in the absence of additional cytosol and GTP[S]. The apparent stability of these complexes may prove useful in future studies aimed at identification of the resident signalling proteins.

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