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Synergistic activation of phospholipase D by protein kinase C- and G-protein-mediated pathways in streptolysin O-permeabilized HL60 cells

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Stimulation of phospholipase D (PLD) by cell surface receptors has been observed in many cell types. We have investigated the mechanism of activation of this enzyme in undifferentiated HL60 cells. GTP analogues and Ca^{2+} (buffered in the nanomolar to micromolar range) were introduced into HL60 cells in the presence of the permeabilizing agent, streptolysin O. We report that guanosine 5'-[γ-thio]triphosphate (GTP[S]) is a potent activator of phospholipase D when Ca^{2+} is available at micromolar levels. Phorbol 12-myristate 13-acetate or Ca^{2+} alone can also stimulate PLD, but to a limited extent. The activation of PLD by GTP[S] can be partially dissociated from GTP[S]-stimulated phosphoinositide-specific phospholipase C, suggesting that a G-protein may be directly involved in regulating PLD. However, maximal activation of PLD only occurs under conditions that are permissive to phospholipase C stimulation. We conclude that PLD activation is under dual control, i.e. protein kinase C- as well as G-protein-mediated regulation. Synergistic activation occurs when both pathways are simultaneously stimulated. We conclude that full activation of PLD requires protein kinase C, increased Ca^{2+} and a GTP-binding protein. Evidence for cytosolic components that may also be involved in obtaining full activation of PLD is also presented.

INTRODUCTION

The process of signal transduction in a wide variety of cells involves the production of second messenger molecules derived from membrane phospholipids. Thus far three phospholipases C, A_2 and D, have been shown to be stimulated on cell surface activation by specific receptors. These three phospholipases are often activated by the same agonist. It is now firmly established that the phosphoinositide signalling pathway is coupled to some receptors by G-proteins [1], but the mechanism of activation of phospholipases A_2 and D by receptors remains unclear.

Activation of phospholipase D (PLD) by receptor-mediated stimulation in intact cells has been demonstrated in a number of cell types. Examples include fMetLeuPhe-mediated stimulation of neutrophils and HL60 cells [2,3], purinergic and bradykinin activation of endothelial cells [4], muscarinic stimulation of 1321N1 astrocytoma cells [5] and canine brain [6], gonadotropin-releasing hormone activation of ovarian granulosa cells [7], vasopressin-stimulation of hepatocytes [8] and fibroblasts [9], and platelet-derived growth factor stimulation of NIH-3T3 cells [10]. All of these agonists also stimulate phosphoinositide-specific phospholipase C (PLC) [11] and hence generation of diacylglycerol (DG), leading to protein kinase C activation. Since the phorbol ester phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, has also been found to be a stimulator of PLD in most of these cell types [5,7,9,10,12,13], it is possible that PLD activation may be a secondary consequence of PLC activation. Overexpression of protein kinase C β1 leads to enhanced PLD activity in fibroblasts, further strengthening the view that PLD activation may be under the control of protein kinase C-mediated phosphorylation [14].

An alternative route to PLD activation has been proposed based on the observations that GTP[S] can activate PLD activity in liver and platelet membranes [8,15,16], in permeabilized endothelial cells [17] and in HL60 homogenates [18,19]. In neutrophils, fMetLeuPhe induces the activation of PLD which cannot be prevented with protein kinase C inhibitors [20]. This supports the suggestion that regulation of PLD by receptors may be under alternative control mechanism, i.e. G-proteins.

In the present study we have investigated the regulatory pathways that can lead to activation of PLD in streptolysin O-permeabilized HL60 cells. Our findings strongly suggest that PLD activation is under dual control, i.e. protein kinase C- as well as G-protein-mediated regulation. Moreover, a marked synergy is observed when both pathways are activated simultaneously.

MATERIALS AND METHODS

Materials

Culture media and supplements were purchased from Flow Laboratories. Fetal calf serum was obtained from Imperial Laboratories. ATP, UTP, GTP, GTP[S], guanosine 5'-[βγ-imido]triphosphate (Gpp[NH]p), guanosine 5'-[βγ-methylene]triphosphate (Gpp[CH_2]p), guanosine 5'-[β-thio]-diphosphate (GDP[S]), GDP and fatty-acid-free BSA were all obtained from Boehringer–Mannheim. Streptolysin O was obtained from Wellcome Diagnostics, Dartford, Kent, U.K., and [^{3}H]alkyl-lyso-PtdCho (92 Ci/mmol) was purchased from Amersham. All other reagents, including peanut PLD, phosphatidylcholine (PtdCho), phosphatidic acid (PtdOH), PMA, neomycin, 2-deoxyglucose and antimycin, were purchased from Sigma. Phosphatidylethanol (PtdEt) was obtained from Avanti Lipids. Staurosporine was obtained from Calbiochem.

Abbreviations used: DG, diacylglycerol; GTP[S], guanosine 5'-[γ-thio]triphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate; Gpp[NH]p, guanosine 5'-[βγ-imido]triphosphate; Gpp[CH_2]p, guanosine 5'-[βγ-methylene]triphosphate; PMA, phorbol 12-myristate 13-acetate; PLD, phospholipase D; PLC, phosphoinositide-specific phospholipase C; PtdOH, phosphatidate; PtdEt, phosphatidylethanol; PtdCho, phosphatidylcholine.

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Preparation of PtdOH and PtdEt standards for t.l.c.

A mixture of PtdOH plus PtdEt was prepared from PtdCho digestion with peanut PLD in the presence of ethanol (2%). PtdCho (4 mg) was incubated with 1000 units of peanut PLD in the presence of Ca^{2+} (10 mM) in acetate buffer (0.2 M, pH 5.6) in the presence of ether overnight at room temperature. At the end of the incubation, the ether was evaporated and the lipids extracted with 3.75 ml of chloroform/methanol (1:2, v/v), followed by addition of 1.25 ml of chloroform and 1.25 ml of water. After phase separation, the chloroform phase was recovered. The sample was run on t.l.c. and two spots corresponding to PtdEt and PtdOH were observed. Authentic PtdEt and PtdOH were run in parallel for confirmation.

Cell labelling

HL60 cells were grown in liquid suspension in RPMI 1640 as reported previously [21]. Undifferentiated cells were used in all experiments. Before labelling with [^{3}H]alkyl-lyso-PtdCho, HL60 cells were washed three times in buffer comprising 20 mM-Hepes, 137 mM-NaCl, 2.7 mM-KCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5.6 mM-glucose and 1 mg of fatty acid-free BSA/ml (pH 7.2). Cells (1 ml) were resuspended in the same medium at 5×10^7 cells/ml and incubated at 37 °C for 30 min in the presence of 5 μCi of [^{3}H]alkyl-lyso-PtdCho/ml [3]. Prior to permeabilization, the cells were washed twice in permeabilization buffer (pH 6.8) comprising 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Pipes, 5.6 mM-glucose and 1% BSA and then resuspended in the same medium. In experiments where the cells were metabolically inhibited to decrease endogenous levels of ATP, glucose was omitted from the buffer.

Permeabilization and measurement of PLD activation

Streptolysin O was used to permeabilize the cells as previously described [21]. Permeabilization and PLD activation were initiated simultaneously by adding aliquots of the labelled cell suspension (100 μl) to tubes containing 100 μl of the permeabilization buffer with the indicated additions (i.e. Ca^{2+} buffers, ethanol, streptolysin O, MgATP, GTP analogues etc.). Ca^{2+} was buffered with 3 mM-EGTA as described previously [21]. The final concentrations of the individual components were: streptolysin O, 0.4 i.u./ml; $MgCl_2$, 1 mM; Ca^{2+}, buffered with 3 mM-EGTA to give the indicated concentrations; MgATP, 1 mM; ethanol, 2%. Radioactivity present in PtdCho was assessed in all experiments and was in the range $(2–4) \times 10^5$ d.p.m. per incubation. The incubations were carried out at 37 °C for 15 min and the reactions were terminated by addition of 1.5 ml of ice-cold chloroform/methanol (1:2, v/v) and vortex-mixing. A mixture of unlabelled PtdOH plus PtdEt was added at this stage for localization of the reaction products by t.l.c. Phases were separated by sequential addition of 0.2 ml of water, 0.5 ml of chloroform and 0.5 ml of 2 M-KCl as described previously [2].

The lower, lipid-containing phase was collected and dried under vacuum and then redissolved in 50 μl of chloroform. Samples were spotted on Whatman LK6TLC silica plates previously impregnated with a 1% potassium oxalate solution, dried at room temperature and activated for 30 min at 110 °C. The plates were developed in chloroform/methanol/acetic acid/water (75:45:3:1, by vol.), dried at room temperature and the lipid spots were visualized with iodine vapours. R_F values for the lipids were: sphingomyelin, 0.08; PtdCho, 0.135; PtdOH, 0.445; phosphatidylethanolamine, 0.49; PtdEt, 0.67. The spots corresponding to PtdOH and PtdEt standards were marked, and after iodine sublimation at room temperature they were excised. The silica was put into scintillation vials, and lipids were extracted with 0.25 μl of methanol and counted for radioactivity after addition of 4 ml of scintillation liquid.

Metabolic inhibition of cells prior to permeabilization

In the experiments where intracellular levels of ATP were to be decreased before permeabilization, glucose was excluded from the permeabilization buffer and the cells were metabolically inhibited. This was achieved by adding 5.6 mM-deoxyglucose and 5 μM-antimycin A to the intact cells for 5 min prior to permeabilization. Under these conditions, intact HL60 cells or neutrophils, when challenged with an agonist, are unable to respond functionally, i.e. secretion is inhibited [22].

Ability of the cells to stimulate PLD in response to GTP[S] is lost if the cells are permeabilized first and GTP[S] is added after a delay of 0.5–15 min

HL60 cells were permeabilized with 0.8 i.u. of streptolysin/ml at pCa 7 (buffered with 100 μM-EGTA). These cells are subsequently referred to as pre-permeabilized cells. In some incubations GTP[S] or pCa 5 was also added with streptolysin O during the pre-permeabilization. At the indicated times, 100 μl aliquots were transferred to tubes containing GTP[S], MgATP and 2% ethanol at pCa 5 to determine PtdEt formation. After incubating the samples for 15 min, the samples were quenched and processed as described above for determining PtdEt formation.

Expression of data

All results presented are from individual experiments repeated on at least three occasions with similar results. All determinations were carried out either in duplicate or in triplicate. For the data in the Tables, the results are presented as averages $\pm$ S.E.M. For the Figures, the error bars were often smaller than the symbols and have been excluded for clarity.

RESULTS

Characteristics of GTP[S]-stimulated PLD activity in permeabilized cells

Addition of GTP[S] to HL60 cells (prelabelled with [^{3}H]alkyl-lyso-PtdCho) in the presence of the permeabilizing agent streptolysin O led to a rapid and prolonged generation of labelled PtdOH (Fig. 1*a*). The source of the labelled PtdOH could only be PtdCho, as the cells were prelabelled with [^{3}H]alkyl-lyso-PtdCho and this is rapidly converted to PtdCho by the cells [3]. To confirm that PtdOH formation was predominantly via a PLD route, we utilized the ability of PLD to catalyse a transphosphatidylation to ethanol, generating the unique phospholipid PtdEt [23]. In the presence of 2% ethanol the increase in labelled PtdOH was abrogated at the expense of labelled PtdEt formation (Fig. 1*b*).

The initial rates of formation of PtdOH and PtdEt were linear and similar in both the absence and the presence of ethanol. In the absence of ethanol the level of PtdOH declined after 30 min in this experiment, an indication that PtdOH is further metabolized. The rates of PtdOH production and disappearance, but not those of PtdEt, were variable in different preparations. We attribute this variability to the presence of phosphatases that can degrade the PtdOH. In the presence of ethanol, PtdEt formation reached a plateau by 30 min and then remained stable for up to 60 min, the longest period examined. As PtdEt is less labile than PtdOH, subsequent experiments were done in the presence of ethanol and the resultant formation of PtdEt was measured.

GTP and the GTP analogues were tested for their ability to stimulate PLD activity in permeabilized cells (Fig. 2). PtdEt

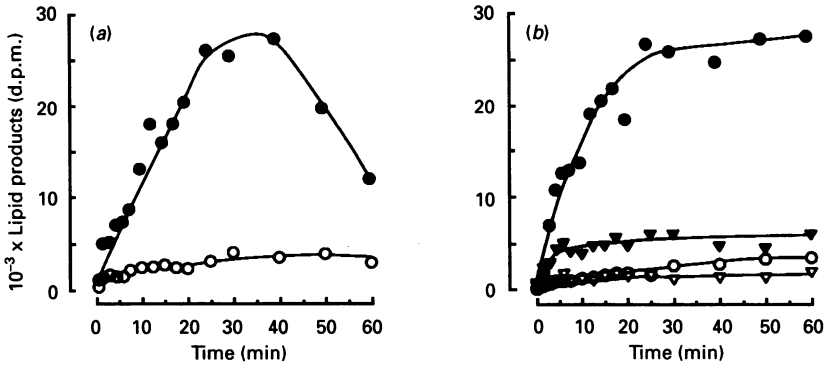

Fig. 1. Time course of (*a*) PtdOH and (*b*) PtdOH and PtdEt formation in HL60 cells in the absence and presence respectively of ethanol

PtdOH and PtdEt formation was stimulated by 30 μM-GTP[S] plus pCa 5, or pCa 5 alone, in HL60 cells permeabilized with streptolysin O. (*a*) Incubations in the absence of ethanol; ○, pCa 5; ●, pCa 5 plus GTP[S]. (*b*) Incubations in the presence of 2% ethanol; ○, ●, PtdEt; ▽, ▼, PtdOH; ○, ▽, pCa 5; ●, ▼, pCa 5 plus GTP[S].

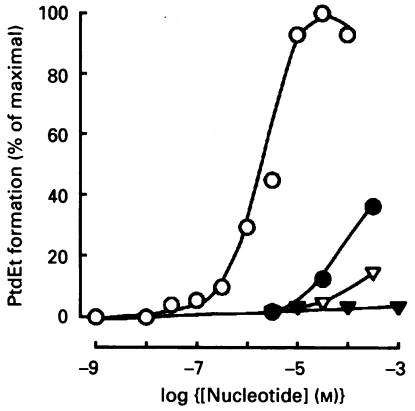

Fig. 2. Concentration-dependence of PtdEt formation stimulated with GTP and GTP analogues in permeabilized HL60 cells

The permeabilized cells were incubated for 15 min with pCa 5 plus the indicated nucleotide at 37 °C, and the generation of PtdEt was determined. The maximal response was observed with 30 μM-GTP[S] plus pCa 5, and this was 21880 d.p.m. The response at pCa 5 was 3100 d.p.m. This response at pCa 5 was set at 0% and the response with GTP[S] plus Ca^{2+} was set at 100%. Results for GTP and the analogues were then expressed as a percentage of this maximal response. ○, GTP[S]; ●, Gpp[NH]p; ▽, Gpp[CH_2]p; ▼, GTP.

Table 1. Effect of GDP[S] and GDP on PLD activation stimulated by GTP[S]

HL60 cells were permeabilized in the presence of pCa 5 or pCa 7 and the indicated additions of GTP[S], GDP and GDP[S]. Results are expressed as means ± S.E.M. ($n = 3$).

	[^{3}H]PtdPt (d.p.m.)	
	Control	GTP[S] (10 μM)
pCa 7	295±82	–
pCa 5	5222±311	19231±703
pCa 5+GDP (1 mM)	4866±1105	5167±452
pCa 5+GDP[S] (1 mM)	2544±180	12989±345

formation was maximal at 10 μM-GTP[S]. The response to the other GTP analogues, Gpp[NH]p and Gpp[CH_2]p, is expressed as a percentage of the maximal response observed with 10 μM-GTP[S] for comparison. Gpp[NH]p and Gpp[CH_2]p also stimulated PtdEt formation, but to a lesser extent. The relative potency for the three analogues was GTP[S] > Gpp[NH]p > Gpp[CH_2]p; this is similar to that for the activation of other G-protein-regulated events, including adenylate cyclase [24], cyclic GMP-phosphodiesterase [25], PLC [21] and phospholipase A_2 [26], and for exocytosis [27]. GTP up to 1 mM had no effect (Fig. 2).

In order to establish that the effect of GTP[S] was mediated via a G-protein, use was made of GDP and GDP[S] as potential inhibitors of G-proteins. Table 1 illustrates that the response to GTP[S] could be inhibited totally by GDP and partially by GDP[S]. It was observed that GDP[S] (but not GDP) consistently partially inhibited the activation of PLD by Ca^{2+} (pCa 5) alone.

In the experiments described so far, the Ca^{2+} concentration was maintained at pCa 5. To examine the Ca^{2+} requirement for PLD activation, the cells were permeabilized in the presence of Ca^{2+} buffered in the range pCa 8 to pCa 5 (Fig. 3*a*). Stimulation of PLD by GTP[S] was greatest when Ca^{2+} was available at pCa 5. At pCa 7, stimulation of PLD by GTP[S] was small but clearly detectable (Fig. 3*b*). Ca^{2+} alone, when present above 1 μM, also stimulated PtdEt formation to a limited extent (Fig. 3*a*).

Inter-relationship between activation of PLC and PLD

We have previously demonstrated that addition of GTP[S] to permeabilized HL60 cells also leads to the activation of PLC [21]. {There is no stimulation of phospholipase A_2 by GTP[S] in undifferentiated HL60 cells (J. Stutchfield & S. Cockcroft, unpublished work), unlike in differentiated HL60 cells [26].} It is therefore possible that the ability of GTP[S] to stimulate PLD is a consequence of PLC activation. The two well-defined second messengers formed by the PLC pathway are inositol trisphosphate ($InsP_3$), which mobilizes intracellular Ca^{2+}, and DG, which mediates protein kinase C activation. Since the experiments were performed in the presence of Ca^{2+} buffered with 3 mM-EGTA, any mobilization of Ca^{2+} by $InsP_3$ would be inconsequential. However, activation of protein kinase C by DG and hence protein phosphorylation can still occur, since the experiments presented so far were all conducted in the presence of MgATP (1 mM).

Effect of PMA. To examine the effect of protein kinase C activation, PMA was used. The effect of PMA was examined in intact cells as well as in permeabilized cells. In intact cells PMA stimulated PLD with a very slow time course (Fig. 4). Moreover, the extent of the response was small even after 1 h in comparison with the response observed with GTP[S] in permeabilized cells (compare Figs. 1 and 4). In order to examine the effect of PMA in permeabilized cells, intact cells were pre-treated for 10 min at 37 °C with 100 nM-PMA to allow a prior activation of protein

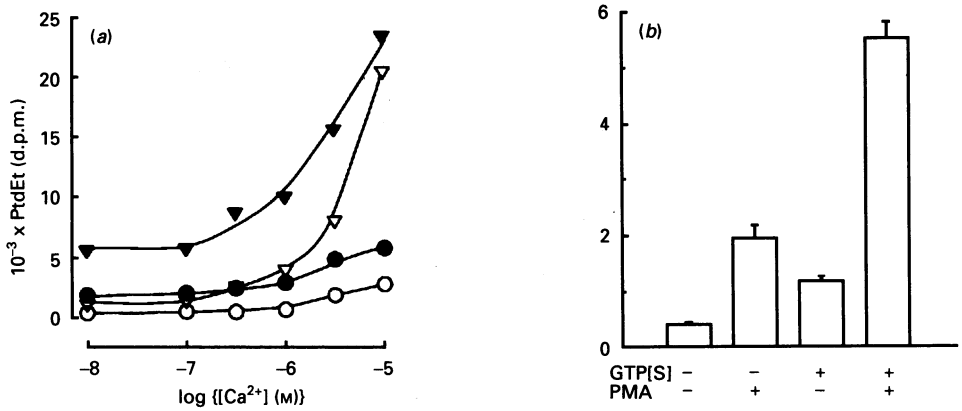

Fig. 3. Synergistic activation of PLD by GTP[S] and PMA

(*a*) PMA reduces the Ca^{2+} requirement for PLD activation by GTP[S]. Intact HL60 cells were pre-treated with 100 nM-PMA for 10 min where indicated, and then permeabilized in the presence of Ca^{2+} buffered in the pCa range 8–5 in the presence and the absence of 30 μM-GTP[S]. ○, Control; ●, 100 nM-PMA; ▽, 30 μM-GTP[S]; ▼, PMA+GTP[S]. (*b*) Synergistic activation of PLD with 30 μM-GTP[S] and 100 nM-PMA at pCa 7.

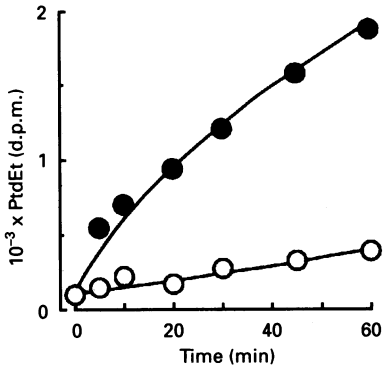

Fig. 4. Time course of PtdEt formation stimulated by PMA (100 nM) in intact HL60 cells

Labelled HL60 cells were suspended in buffer comprising 20 mM-Hepes, 137 mM-NaCl, 2.7 mM-KCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5.6 mM-glucose and 1 mg of fatty-acid-free BSA/ml. PMA (100 nM) was added to the cells and the time course of PtdEt formation was assessed at the indicated times. ○, No addition; ●, 100 nM-PMA.

Table 2. Effect of staurosporine on PMA- and GTP[S]-induced PtdEt formation in permeabilized HL60 cells

Intact HL60 cells were pre-incubated with 50 nM-staurosporine for 10 min. PMA was then added to the intact cells for a further 10 min. After this 20 min period, the cells were permeabilized in the presence of pCa 5 in either the presence or the absence of 1 μM-GTP[S]. After incubation for 10 min, the samples were quenched and assayed for PtdEt formation. Results are expressed as means ± S.E.M. (n = 3).

	[^{3}H]PtdEt (d.p.m.)	
	Control	+Staurosporine (50 nM)
No additions	2805±416	2458±456
PMA (100 nM)	4117±427	2905±232
GTP[S] (1 μM)	4968±121	5192±548

kinase C. Ethanol was not added during the pre-treatment stage, and hence no PtdEt formation could occur. PtdEt production was subsequently measured after permeabilization in the presence of Ca^{2+} buffered in the range pCa 8 to pCa 5. In the permeabilized cells PMA stimulated PtdEt formation at all concentrations of Ca^{2+} (Figs. 3*a* and 3*b*). The response stimulated by PMA should be compared to the response obtained with GTP[S]. GTP[S] was far more potent in stimulating PtdEt formation than was PMA, particularly at pCa 5. This result suggests that stimulation by GTP[S] cannot be solely due to protein kinase C activation as a result of DG formed from PLC.

If the effects of PMA and GTP[S] were mediated by separate mechanisms, then it would be interesting to examine whether activation of protein kinase C by PMA prior to permeabilization influenced GTP[S]-stimulated PtdEt formation. The intact cells were treated with PMA for 10 min followed by determination of GTP[S]-stimulated PtdEt formation at different Ca^{2+} concentrations. GTP[S] was a poor activator of PtdEt formation at low Ca^{2+} concentrations (Figs. 3*a* and 3*b*). The effect of PMA pre-treatment was to stimulate PtdEt formation at substantially lower Ca^{2+} concentrations such that GTP[S] stimulated a considerable response even at pCa 7 (Figs. 3*a* and 3*b*). The results obtained so far indicate that PMA cannot substitute for GTP[S]. The effect of PMA is to sensitize the system such that PtdEt formation is observed at substantially lower concentrations of Ca^{2+}.

The effect of staurosporine, a non-specific inhibitor of protein kinase C, was examined to assess whether the response to PMA and GTP[S] could be selectively blocked. If the dominant effect of GTP[S] in stimulating PtdEt formation was directly via a G-protein, then it would be predicted that staurosporine should only inhibit the PMA-induced PtdEt formation, but not that induced by GTP[S]. Addition of 50 nM-staurosporine to intact cells for 10 min blocked the response to PMA measured in permeabilized cells (Table 2). In contrast, staurosporine pre-treatment had no effect on the GTP[S]-stimulated response. Staurosporine could not be used at higher concentrations because it stimulates production of PtdEt by itself (results not shown).

Effect of MgATP. The above experiments suggest that the effect of GTP[S] is not likely to be due to the secondary activation of protein kinase C, but to an activation of a G-protein. It is, however, possible that PLC activation is still required before PLD activation can occur. In order to examine more closely the dependence of PLD activation on a prior activation of PLC, we have compared the MgATP requirement for these two phospholipases (Figs. 5*a* and 5*b*). PtdCho is an abundant lipid [2] and therefore the substrate for PLD will not be limiting in the

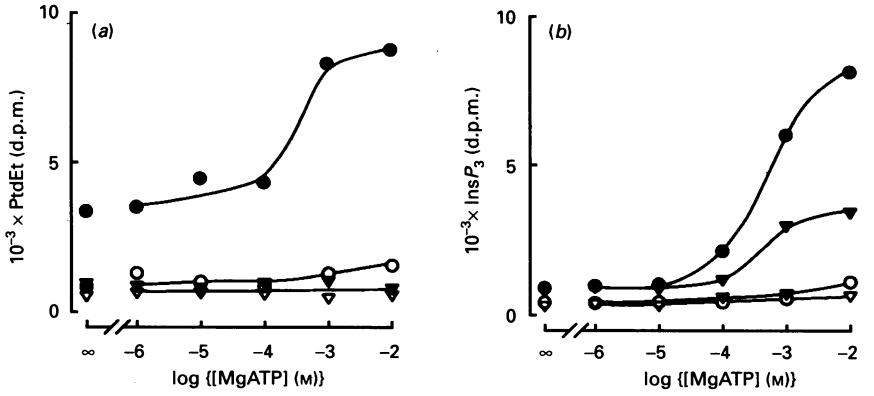

Fig. 5. Comparison of MgATP-dependence of (*a*) PtdEt and (*b*) $InsP_3$ formation in HL60 cells permeabilized with streptolysin O

The cells were metabolically inhibited prior to permeabilization to reduce the intracellular concentration of ATP. (*a*) PLD activation was assessed in cells pre-labelled with lyso-PtdCho, and (*b*) PLC was assessed in [^{3}H]inositol-labelled cells upon stimulation with GTP[S]. Assays for the two phospholipases were carried out at pCa 7 and pCa 5 at various concentrations of MgATP: ○, pCa 5; ●, pCa 5+GTP[S]; ▽, pCa 7; ▼, pCa 7+GTP[S]. Data for PLC activation are redrawn from a previous study for comparison [21].

short term. In contrast, Ptd$InsP_2$ is a minor lipid and therefore the substrate for PLC will be limiting in the course of the experiment [21,28]. The resting Ptd$InsP_2$ level only accounts for a small proportion of the total substrate that is hydrolysed by PLC, and therefore the Ptd$InsP_2$ has to be replenished from PtdIns by phosphorylation with MgATP. To minimize the contribution made by the presence of pre-formed Ptd$InsP_2$, the cells were also metabolically inhibited prior to permeabilization. Metabolic inhibition decreases the ATP levels such that, under these conditions, PLC-mediated hydrolysis of Ptd$InsP_2$ is minimal but not completely abolished (Fig. 5*b*; see also [28]).

Figs. 5(*a*) and 5(*b*) provide a comparison of the permissive effect of MgATP on the GTP[S]-induced activation of the two phospholipases, D and C respectively, at two Ca^{2+} concentrations. At pCa 5, a substantial stimulation of PLD occurred with GTP[S] in the absence of any added MgATP. Under the same conditions the stimulation of PLC was minimal. As the concentration of MgATP was increased to above 100 μM, product formation by both phospholipases was enhanced. This result suggests that PLC activation could be a contributory factor in the enhancement of PLD activity in the presence of MgATP.

The Ca^{2+}-dependence of the two phospholipases was used as a means to establish this interdependence. Activation of the two phospholipases was assessed at two Ca^{2+} concentrations, pCa 7 and pCa 5. At pCa 5, activation of both phospholipases was stimulated by GTP[S], but at pCa 7 only PLC activation could be observed (compare Figs. 5*a* and 5*b*). This clearly establishes that activation of PLC does not lead to PLD activation *per se*, but that the presence of Ca^{2+} in the micromolar levels is mandatory.

Despite metabolic inhibition and no added MgATP, there was a small but measurable formation of $InsP_3$ with GTP[S] at pCa 5 (Fig. 5*b*). Thus, to establish whether the GTP[S]-mediated activation of PLD in the absence of MgATP could be related to protein kinase C activation due to residual endogenous ATP, we compared the effects of GTP[S] and PMA under identical experimental conditions. The cells were metabolically inhibited and then stimulated with either GTP[S] or PMA, both added at the time of permeabilization. Table 3 illustrates that minimal stimulation of PLD activity was observed with PMA. In contrast, GTP[S] was still able to stimulate a substantial production of PtdEt. This would exclude a role for protein kinase C in the activation obtained with GTP[S] in the absence of MgATP.

Table 3. Effects of UTP, neomycin and PMA on GTP[S]-stimulated PLD activation in the presence and the absence of MgATP

HL60 cells were permeabilized in the presence of pCa 5 and the indicated additions in either the presence or the absence of 1 mM-MgATP. The cells were metabolically inhibited prior to permeabilization. Results are expressed as means ± S.E.M. (n = 3). n.d., not determined.

	[^{3}H]PtdEt (d.p.m.)	
	pCa 5	pCa 5+MgATP
Control	698±23	2127±8
GTP[S] (30 μM)	6593±452	22091±3535
UTP (1 mM)	1427±57	n.d.
UTP+GTP[S]	7088±853	n.d.
Neomycin (1 mM)	650±86	727±17
Neomycin+GTP[S]	3415±127	4905±400
PMA (100 nM)	1193±193	n.d.
PMA+GTP[S]	8812±462	n.d.

Mechanism of MgATP in enhancing GTP[S]-mediated PLD activation

Although a substantial stimulation of PLD by GTP[S] was observed in the absence of MgATP, provision of MgATP increased PLD activation substantially. Two separate roles of ATP have to be considered. (1) One role for ATP is as a ligand acting at the cell surface receptor. We have previously demonstrated the presence of such a receptor in undifferentiated HL60 cells [29]. (2) Another role for MgATP is intracellular. One possibility is that MgATP is permissive to GTP[S]-mediated activation of PLC, and hence protein kinase C-mediated phosphorylation of PLD or associated activating factor(s).

To examine the first possibility we studied the effect of another nucleotide, UTP, which is also an agonist for the HL60 cell ATP receptor [29]. We have compared the effects of UTP with those of ATP. Table 3 illustrates that UTP cannot substitute for ATP. In a separate experiment, MgUTP was used and this also cannot substitute for MgATP (results not shown). Another observation that eliminates an ATP receptor effect is the observation that ATP is effective at enhancing PLD activation in the millimolar

range (Fig. 5*a*). Its receptor-mediated effects are apparent in the 1–100 μM range [29].

The second possibility, i.e. that the effect of ATP is exerted intracellularly, is probably more likely. The enhancing effect of MgATP on GTP[S]-stimulated PLD may be due to PLC activation. Neomycin was used to investigate this possibility. One of the many effects of neomycin is to inhibit PLC activation by substrate sequestration [30,31]. Here it is demonstrated that neomycin lowered PLD activation when measured in the presence of MgATP, but had a less dramatic effect in the absence of MgATP (Table 3). The concentration of neomycin used in these experiments is more than sufficient to totally inhibit PLC activation [32].

If the primary effect of MgATP is to promote protein phosphorylation by protein kinase C, then it should be possible to decrease the ATP enhancement of PLD activation by pre-treating the cells with PMA prior to permeabilization. The results of such an experiment are presented in Table 4. Pre-treatment of HL60 cells with PMA was carried out for 10 min, and PLD activation by GTP[S] was assessed after permeabilization in the presence or absence of MgATP. Pre-treatment with PMA did not decrease the MgATP requirement for full activation of PLD.

Table 4. PMA pre-treatment does not decrease the MgATP enhancement of PLD activation by GTP[S]

HL60 cells were pre-treated with PMA (100 nM) for 10 min followed by permeabilization in the presence of pCa 5. GTP[S] (30 μM) and MgATP (1 mM) were present where indicated. Results are expressed as means ± S.E.M. (n = 3).

		[^{3}H]PtdEt (d.p.m.)	
	MgATP	Control	PMA pre-treatment
Control	–	1160±98	1507±242
GTP[S]	–	11896±1033	16309±4346
Control	+	2787±252	5741±586
GTP[S]	+	31868±3280	37340±3200

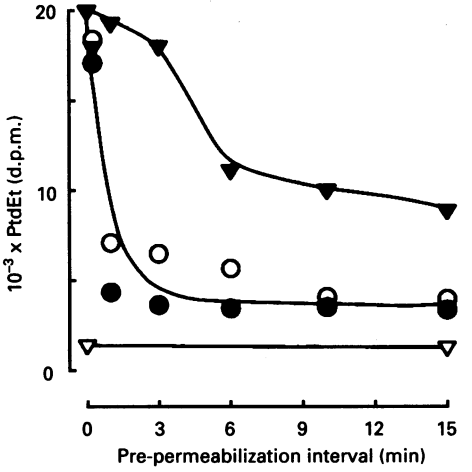

Fig. 6. Decay of PLD activation when GTP[S] plus pCa 5 were added subsequent to permeabilization: prevention by the presence of GTP[S] but not Ca^{2+} (pCa 5) during pre-permeabilization

HL60 cells were pre-permeabilized with streptolysin O at pCa 7 (○, ▽), pCa 5 (●), or pCa 7 plus 100 μM-GTP[S] (▼). At the end of the pre-permeabilization interval, the cells were incubated for a further 15 min to assess for PLD activation. The pre-permeabilized cells were incubated with either GTP[S] (50 μM) plus pCa 5 (○, ●, ▼), or at pCa 7 (▽) in the presence of 2% ethanol and 1 mM-MgATP.

Progressive loss of PLD activation on addition of GTP[S] after initiation of permeabilization

In the experiments described so far, GTP[S] was added to the cells at the time of permeabilization. In two recent studies it was reported that both cytosol and membranes are required for optimal GTP[S]-stimulated PLD activity in phagocytic cells [19,33]. In order to establish whether cytosolic components are indeed necessary for activating PLD in permeabilized cells, we took advantage of the fact that there is a time-dependent leakage of proteins, including lactate dehydrogenase and PLC, from streptolysin O-permeabilized HL60 cells [21,34]. Release of lactate dehydrogenase is essentially complete by 5 min [21] and that of PLC isoenzyme(s) by 20 min [34]. The cells were permeabilized with streptolysin O at pCa 7 in the absence of MgATP, and after a specified permeabilization interval the cells were transferred to tubes containing GTP[S], MgATP, ethanol and Ca^{2+} buffered at pCa 5 to assay for PtdEt production. Activation of PLD by GTP[S] plus Ca^{2+} declined as the pre-permeabilization interval was increased from 0 to 3 min (Fig. 6). A residual response, however, could still be observed even when addition of GTP[S] plus Ca^{2+} was delayed for up to 15 min, the longest time examined.

GTP[S] alleviates the decline in stimulated PLD activity that results from prolonged cell permeabilization

The loss of responsiveness to GTP[S] plus Ca^{2+} was further investigated by examining the ability of Ca^{2+} (pCa 5) or GTP[S] to prevent it. During the pre-permeabilization interval, Ca^{2+} or GTP[S] was also present. The rationale was that Ca^{2+} or GTP[S] may recruit a factor from the cytosol and this allows PLD to function. The cells were pre-permeabilized either at pCa 7 plus GTP[S] or at pCa 5. MgATP was not added during this stage. Under these conditions, activation of PLD was minimal. (During the pre-permeabilization stage no PtdEt formation would occur, since ethanol was excluded at this stage.) At specified intervals, the pre-permeabilized cells were assessed for PLD activation by transferring the cells to tubes containing pCa 5 plus GTP[S], MgATP and ethanol. It was found that GTP[S] but not Ca^{2+} had a protective effect (Fig. 6). In a separate experiment, PMA pretreatment had no effect on the decay of responsiveness (results not shown). The loss of GTP[S]-stimulated PtdEt production was minimal during the first 3 min of pre-permeabilization when GTP[S] was present during the pre-permeabilization stage. After this time a decline in responsiveness was apparent; nonetheless, the residual response remaining even after 15 min of pre-permeabilization was substantial, accounting for 50% of the original response (Fig. 6).

DISCUSSION

In this paper we demonstrate that PLD can be activated by non-hydrolysable analogues of GTP. Since the addition of guanine nucleotides to permeabilized HL60 cells also leads to stimulation of PLC, it was of importance to establish whether PLD activation by GTP analogues is downstream from PLC stimulation, or whether independent pathways for PLD stimulation exist. The results presented here indicate that two separate elements of PLD regulation are observed: a possible direct G-protein-mediated activation and a protein kinase C-mediated activation. Synergistic activation of PLD occurs when both pathways are stimulated simultaneously.

The main evidence supporting the conclusion that GTP[S] or protein kinase C may directly regulate PLD can be summarized as follows. (a) In metabolically inhibited cells, conditions under which PLC activation is virtually abolished, it is still possible to stimulate PLD activation with GTP[S]. This response is approx. 30% of the maximal response observed with GTP[S] in the presence of MgATP. Direct activation of PLD by a G-protein probably accounts for this component. (2) Evidence for protein kinase C-mediated activation of PLD is clearly provided by the observation that PMA is able to stimulate PLD activation in intact cells and in permeabilized cells, but only in the presence of MgATP. The response observed with PMA represents about 20% of the maximal response.

Although dual regulation of PLD activity occurs, these two pathways synergize to provide the full activation of PLD. GTP[S], in the presence of MgATP, will activate the PLC pathway and hence protein kinase C. In addition, GTP[S] can also interact with the putative G-protein regulating PLD. The simultaneous activation of these two components leads to a response which is greater than the response seen if either pathway was activated alone. The results with neomycin and MgATP clearly support the contribution made by the PLC pathway.

Neomycin was found to partially inhibit GTP[S]-stimulated PtdEt formation in the absence of MgATP. Since the response in the absence of MgATP can be directly attributed to activation via a G-protein, this result might suggest that PLD is inhibited directly by neomycin. Liscovitch *et al.* [35] have recently provided evidence for such a possibility for the synaptic membrane PLD.

Activation of the PLD pathway is highly Ca^{2+}-dependent. This dependence on Ca^{2+} clearly establishes this pathway as being distal to PLC activation in intact cells. A rise in cytosolic Ca^{2+} would have to precede PLD activation, at least in intact cells. One can therefore predict that agonists that are stimulators of the PLD pathway are likely to be activators of the PLC pathway as well. If one examines the agonists that trigger the PLD pathway, these same agonists are also activators of the PLC pathway [36]. Muscarinic cholinergic receptors present a good example of this inter-relationship. The muscarinic receptor consists of five subtypes, of which m1, m3 and m5 (but not m2 and m4) mediate a strong stimulation of PLC. It appears that a strong activation of PLD is observed with m1 and m3, but not with m2 or m4 [37,38]. Although PLD activation by GTP[S] is highly Ca^{2+}-dependent, it is interesting to note that PMA pretreatment reveals a PLD activity that is stimulated by GTP[S] and is independent of Ca^{2+}. Thus one interpretation is that there are two PLD enzymes. One is activated by Ca^{2+} and stimulated by GTP[S]; the other is insensitive to Ca^{2+}, requires protein kinase C-mediated modification and can then be activated by G-protein.

From the results presented here, we suggest that PLD activation can occur either as a consequence of second messengers originating from PLC activation or directly via a G-protein. The individual contributions of these two routes approximate to 20% and 30% respectively when compared with the maximal response observed when both pathways are stimulated simultaneously.

PLD activity (assayed with an exogenous substrate in the presence of detergent) has generally been found to be associated with the membrane fraction in mammalian cells [39–41], as well as the cytosol [42]. GTP[S]-stimulated PLD activity has been demonstrated in membrane fractions in platelets [16], NIH 3T3 cells [43], liver cells [15] and cerebral cortex [44], and the conditions of the assay were such as to eliminate any contribution made by the PLC pathway. We would suggest that the response observed in membrane preparations only represents a small proportion of the total response, and this component is represented by the residual response that is observed in our experiments after the extensive loss of cytosolic components during the pre-permeabilization (see Fig. 6). In a recent study of platelet membranes [16], PMA was also shown to enhance GTP[S]-stimulated PLD activity, and it was concluded that the full activation of platelet PLD may require the interplay of protein kinase C, increased Ca^{2+} and a GTP-binding protein. It is our hypothesis that the membrane system does not contain all the required components and that cytosolic factor(s) are recruited in a G-protein-dependent manner; these factors then play a role in obtaining the full PLD response.

The physiological significance of PLD activation in cells is not apparent, but its role is likely to be related to providing an intracellular signal for cell activation. This signal is most likely to be PtdOH. In human neutrophils the mass of PtdOH increases to a maximal value of 10.7 nmol/10^8 cells within 20 s from a basal level of 1.7 nmol/10^8 cells [2,45]. If PtdCho is the main source of PtdOH, then this amounts to a net hydrolysis of 2.8% of the total PtdCho pool. The time course for PtdOH formation coincides with the time course of secretion from intact human neutrophils stimulated by fMetLeuPhe [2]. Abrogation of PtdOH production in intact cells by alcohols leads to inhibition of regulated secretory events in many cell types, e.g. secretion from mast cells [46,47], platelets [48], neutrophils [49] and differentiated HL60 cells (J. Stutchfield & S. Cockcroft, unpublished work). However, generation of endogenous PtdOH by addition of exogenous PLD stimulates physiological responses in many cell types, including insulin release from islets [50] and aldosterone secretion from adrenal glomerulosa cells [51]. We thus conclude that PtdOH plays a key role as a second messenger, and the clarification of this function would aid in our understanding of cellular activation.

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