

ARF1-regulated phospholipase D in human neutrophils is enhanced by PMA and MgATP

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Abstract Human neutrophil PLD activity stimulated with GTP- γ -S was reconstituted with recombinant ARF1 in cytosol-depleted cells. PMA-pretreatment of intact cells greatly enhanced the subsequent reconstitution of the ARF1-regulated PLD activity. This enhancement was only observed provided that the intact cells were pretreated with PMA, suggesting the stable recruitment of a cytosolic component, presumably protein kinase C, to the membranes. rARF1-reconstituted PLD activity was not dependent on MgATP, but could be considerably enhanced by MgATP. Maximal effects of MgATP were seen at 1 mM. This enhancement by MgATP could not be attributed to protein kinase C. Neomycin was found to inhibit ARF1-regulated PLD activity suggesting the requirement for polyphosphoinositides. We conclude: (i) that many of the observed effects of PMA may be dependent on the presence of the small GTP-binding protein, ARF, and (ii) polyphosphoinositides are required for ARF1-stimulated PLD activity.

Key words: Phospholipase D; Neutrophil; ADP ribosylating factor; PMA; MgATP

1. Introduction

Phospholipase D (PLD) hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. In intact cells, this enzyme activity is stimulated by a number of cell-surface receptors including G-protein-coupled receptors as well as receptors that use tyrosine kinase as their signaling mechanism [1,2]. In addition to receptor-directed agonists, both PMA, a protein kinase C (PKC) activator, and Ca^{2+} ionophores activate PLD activity in many cell types [1] including neutrophils [2–4]. Further evidence in support of PKC comes from studies where down regulation of PKC abolished agonist-stimulated PLD activity [5] whilst overexpression of PKC α or β 1 isoforms upregulates both PMA-activated as well as receptor-activated PLD activity [6–8]. More recently it has been reported that direct addition of PKC isoforms β or α stimulated PLD activity in membranes [9].

From studies in permeabilized cells and other cell-free systems, it has emerged that PLD activity can be regulated by guanine nucleotides indicating a role for a putative GTP-binding protein [10–12]. Several studies have indicated that maximal PLD activity required the interplay of PKC and a GTP-binding protein [11,12]. In neutrophils and the related cell-line HL60, many studies have indicated that there is a requirement for both cytosol and membranes to observe GTP- γ -S-regulated PLD activity [13–15]. In HL60 cells, the cytosolic factor has now been identified as two highly-related small GTP binding proteins of the ARF family, ARF1 and ARF3 [16,17].

We show that ARF1 regulates PLD in human neutrophils and this activity can be modulated by PMA pretreatment and by MgATP. When PMA is used to recruit PKC to membranes

prior to cytosol-depletion, ARF1-reconstituted PLD activity is enhanced. MgATP also increased ARF1-reconstituted PLD activity and this effect was attributed to the increased availability of polyphosphoinositides. We conclude (i) that many of the observed effects of PMA may be dependent on the presence of the small GTP-binding protein, ARF and (ii) polyphosphoinositides are required for ARF1-stimulated PLD activity.

2. Materials and methods

2.1. Materials

All reagents were obtained as previously specified [12]. Recombinant ARF1 (rARF1) was prepared as described previously [17].

2.2. Preparation of human neutrophils

Human neutrophils were prepared by established procedures from 50 ml of anti-coagulated blood obtained from healthy volunteers [18]. In brief, after sedimentation of red cells with an equal volume of 2% dextran in phosphate buffered saline (0.9%) (pH 7.2), the leucocytes were layered onto Ficoll-hypaque and centrifuged at 2,000 rpm for 20 min to separate the neutrophils from other white cells. The contaminating red cells were removed by hypotonic lysis. Typical yield of neutrophils from 50 ml of blood was $5\text{--}8 \times 10^7$ cells.

2.3. Labelling of neutrophils with [^3H]alkyl-lyso-PC

Neutrophils ($5\text{--}8 \times 10^7$ cells) were washed twice in HEPES buffer (20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM MgCl_2 , 1 mM CaCl_2 and 0.1 mg/ml BSA, pH 7.2) and finally resuspended in 2 ml. The cells were incubated at 37°C with [^3H]alkyl-lyso-PC (10 μCi) for 30 min. Lyso-PC is incorporated into the cells and rapidly acylated to PC. The majority of the label is found in PC (Fig. 3A). The labelled cells were sedimented by centrifugation to remove unincorporated label and the cells washed with PIPES buffer (20 mM PIPES, 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, and 0.1 mg/ml BSA, pH 6.8).

2.4. Assay for GTP- γ -S-dependent PLD activity in permeabilized cells

The labelled cells were suspended in PIPES buffer and aliquots (25 μl) transferred to tubes containing 0.4 i.u./ml streptolysin O, MgATP (1 mM), MgCl_2 (2 mM), EtOH (1%). The Ca^{2+} concentration was maintained with Ca^{2+} . EGTA buffers as described previously [19]. GTP- γ -S (10 μM) was also present where indicated. These cells are referred to as 'acutely permeabilized'.

After incubation for 30 min at 37°C, the samples were quenched with 500 μl chloroform/methanol (1:1, v/v). After phase separation with 250 μl of water, the chloroform phase was recovered and a mixture of PA and PEt [12] was added for localization of the lipids with brief staining

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Abbreviations: PLD, phospholipase D; PI 3-kinase, phosphoinositide 3-kinase; PA, phosphatidic acid; PEt, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; ARF, ADP-ribosylating factor; G-proteins, GTP-binding proteins; fMetLeuPhe, *N*-formyl-methionyl-leucyl-phenylalanine; GTP- γ -S, guanosine 5' [γ -thio]triphosphate; PLC, inositol lipid-specific phospholipase; PI, phosphatidylinositol; PC, phosphatidylcholine.

with I_2 vapour. Radiolabelled PA, PEt and PC were separated by tlc in a solvent system using $CHCl_3/MeOH/acetic\ acid/H_2O$ (75:45:3:1, by volume) on oxalate-treated plates as described previously [12]. The tlc plates were either analysed by two methods. The spots identified as PA, PEt and PC were scraped and counted by liquid scintillation. In some experiments, the tlc plate was put into contact with a phosphor imaging plate and the results scanned on a Fuji Bioimage analyser.

2.5. Reconstitution of ARF-regulated PLD in cytosol-depleted cells

The labelled cells were suspended in 4.5 ml of PIPES buffer and permeabilized with streptolysin O (0.4 IU/ml) for 10 min at 37°C [17]. The cells were then diluted with 40 ml of ice-cold PIPES buffer and centrifuged to remove the leaked cytosol. These cells are referred to as 'cytosol-depleted' cells. PLD activation was initiated by adding 25 μ l of cytosol-depleted cells to tubes containing rARF1 (40 μ M), GTP- γ -S (10 μ M), Ca^{2+} (1 μ M, buffered with 3 mM EGTA), ethanol (1%), $MgCl_2$ (2 mM) and MgATP (1 mM) as indicated in a final volume of 100 μ l. Assays were processed as for the 'acutely permeabilized' cells.

2.6. Metabolic inhibition of cells prior to permeabilization

In those experiments where intracellular ATP levels were depleted prior to permeabilization, glucose was omitted from the permeabilization buffer. The cells were metabolically inhibited by incubation with 5.6 mM deoxyglucose and 5 μ M antimycin A for 5 min at 37°C before the addition of streptolysin O.

2.7. Presentation of results

All determinations were performed in duplicate and did not vary by more than 5%. The data shown are from representative experiments. All experiments were repeated under identical conditions and most observations were verified on 3–4 occasions with slight modifications in the protocol.

3. Results

3.1. rARF1 reconstitutes PLD activity in cytosol-depleted neutrophils

PLD catalyses the hydrolytic cleavage of the terminal phosphodiester bond of PC with the formation of PA and the water-soluble headgroup, choline. In the experiments reported here, the PC pool is specifically labelled by incubating human neutrophils with lyso-PC. This is readily taken up by cells and acylated to PC (see Fig. 3A). A property of PLD is the unique ability to transfer the phosphatidyl portion of the PC molecule to appropriate nucleophiles such as ethanol. In this case, the product that is formed is phosphatidylethanol (PEt). Due to its stability and also because it is an unambiguous product of PLD activity, production of PEt was monitored in the experiments described below.

Fig. 1A illustrates that addition of GTP- γ -S simultaneously with the permeabilizing agent, streptolysin O (SLO), to human neutrophils results in the activation of PLD. (These cells are referred to as acutely-permeabilized). Transphosphatidylolation, in the presence of 1% ethanol, is always incomplete and therefore PEt is always accompanied by a corresponding increase in PA (Fig. 1B, see also Fig. 3A). Activation of PLD by GTP- γ -S is impaired when the cells are permeabilized first to deplete the endogenous cytosolic proteins (Fig. 1A,B). Some GTP- γ -S-regulated PLD activity is always retained and this is due to the presence of membrane-associated ARF proteins (data not shown). Incubation of the cytosol-depleted cells with rARF1 more than fully restores the ability of GTP- γ -S to activate the endogenous PLD (Fig. 1A,B).

Addition of rARF1 alone has little effect on PLD activity in cytosol-depleted neutrophils but in the presence of GTP- γ -S, rARF1 stimulates PLD activity in a concentration-dependent

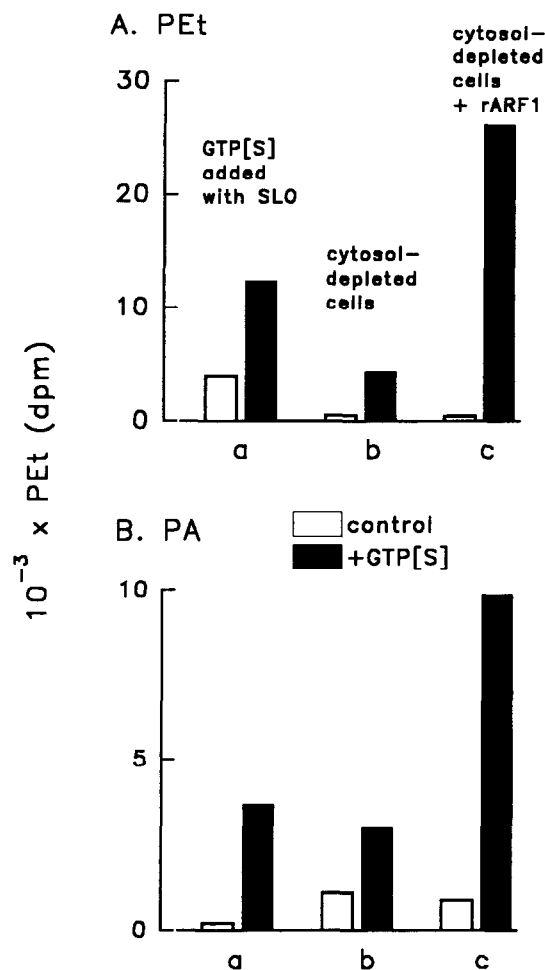


Fig. 1. rARF1 can restore GTP- γ -S-stimulated PLD activity in cytosol-depleted human neutrophils (A) PEt and (B) PA. (a) Neutrophils were permeabilized with streptolysin O $\pm$ 10 μ M GTP- γ -S. (b) Neutrophils were permeabilized with streptolysin O for 10 min to deplete the cytosolic proteins, washed and then incubated $\pm$ 10 μ M GTP- γ -S. (c) Cytosol-depleted cells as in (b) were incubated with rARF1 (4 nmol/assay; 40 μ M) $\pm$ 10 μ M GTP- γ -S. The PLD activity was assayed for 30 min in the presence of 1 mM MgATP, 1 μ M Ca^{2+} and 1% ethanol (assay volume, 100 μ l).

manner (Fig. 2A). Near maximal activation is observed at 5 nmol of rARF1, i.e. 50 μ M. rARF1 used in these experiments is not myristoylated and therefore it is required at 100 times greater concentration compared to myristoylated ARF1 [17]. To test the requirement for Ca^{2+} , neutrophils were reconstituted with rARF1 either in the presence of Ca^{2+} buffered at pCa 8 (10 nM) or pCa 6 (1 μ M) (Fig. 2B). In the absence of GTP- γ -S, Ca^{2+} alone at either concentration does not influence PLD activity. GTP- γ -S induced a robust activation of PLD at either Ca^{2+} concentration; the response was always slightly greater at 1 μ M compared to 10 nM.

3.2. PMA pretreatment enhances rARF1-reconstituted PLD activity

The phorbol ester, PMA has been shown to stimulate PLD activity in many cell-types including human neutrophils [1,2,4]. We therefore examined the influence of PMA on rARF1-reconstituted PLD activities. The intact cells were pretreated with

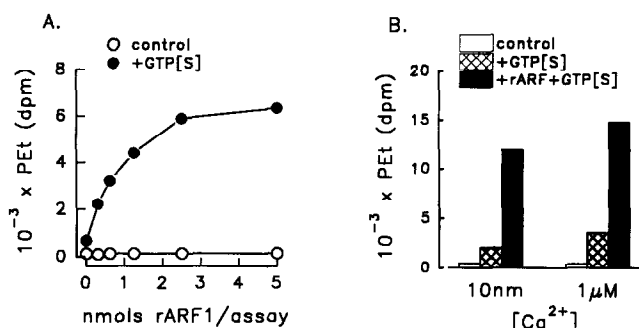


Fig. 2 rARF1-stimulated PLD activity (A) concentration dependence of rARF1 and (B) dependence on Ca²⁺. (A) Cytosol-depleted neutrophils were assayed for PLD activity for 30min in the presence of 1 mM MgATP, 1 μM Ca²⁺ and 1% ethanol in the presence or absence of GTP-γ-S (10 μM). (B) Legend as in A except that rARF1 was 4nmol/assay and Ca²⁺ was present at 10 nM or 1 μM Ca²⁺.

100 nM PMA for 5 min and then permeabilized for 10 min to remove the endogenous proteins. The control cells and PMA-pretreated cells were then reconstituted with rARF1. In the control cells, increases in PA and PEt are seen in the presence of GTP-γ-S and rARF1 only (Fig. 3A). Pretreatment with PMA doubles the rARF1 reconstitution of GTP-γ-S-stimulated PLD activity (Fig. 3A,B). It should be noted that PMA pretreatment alone raised both basal and GTP-γ-S-stimulated PLD activity. These increases are probably dependent on the residual endogenous ARF proteins. In addition to the rise in PA and PEt, it was also noted that there was a substantial increment in radioactivity in the region where neutral lipids run (Fig. 3A). Preliminary data indicate that these changes are in mono- and diglycerides as well as an unidentified lipid. This increase in the neutral lipids were particularly observed in PMA-pretreated cells suggesting that PMA stimulated an additional lipase activity, possibly a PC-PLC.

Enhancement of rARF1-reconstituted PLD activity by PMA was only observed when the cells were treated with PMA prior to permeabilization (Fig. 3B). Direct addition of PMA during the assay had no effect. This implies that PMA allows the recruitment of a factor from the cytosol during the pretreatment of the intact cells. The factor must be stably-associated with the cell membranes, since the cells are permeabilized with SLO for 10min and subsequently washed.

3.3. Effects of MgATP on rARF1-regulated PLD activity

It has been previously demonstrated that MgATP will enhance GTP-γ-S-stimulated PLD activity in permeabilized HL60 cells [12, 20]. This enhancement by MgATP was attributed to the activation of G-protein-mediated PLC and hence to the activation of PKC [12,19]. It was therefore of interest to examine whether reconstitution of PLD activity in cytosol-depleted neutrophils with rARF1 was also affected by MgATP. In cytosol-depleted HL60 cells, GTP-γ-S-mediated activation of PLC is impaired due to loss of another cytosolic protein, phosphatidylinositol transfer protein [21]. Fig. 4A demonstrates that rARF1-reconstituted PLD activity is substantial in the absence of MgATP. However, in the presence of MgATP, it more than doubles.

The concentration of MgATP required for maximal potentiation is 1 mM, with half maximal at 100 μM. This requirement

for MgATP excludes protein kinases since they have a half maximal requirement in the μM range [22]. Inositol lipid kinases, however, show such a MgATP dependence [19]. In order to investigate whether the requirement for MgATP reflects the involvement of polyphosphoinositides, we examined the effect of neomycin. Neomycin inhibited both the MgATP-dependent and independent components of PLD activity. The MgATP-dependent component was inhibited by 80% whilst the MgATP-independent component by 60% (Fig. 4B).

It has been demonstrated that wortmannin specifically inhibits phosphoinositide 3-kinase (PI 3-kinase) in neutrophils, thus preventing the fMetLeuPhe-mediated production of PIP₃ [23,24]. We have examined the effect of wortmannin on rARF1-regulated PLD and found that wortmannin up to 1 μM was without effect. In the absence of wortmannin, PEt production due to rARF1 and GTP-γ-S was 8,240 dpm and it was 8,908 dpm in the presence of 1 μM wortmannin.

4. Discussion

Phospholipase D activation by cell-surface receptors is a well-documented response observed in many cell-types but its function is not yet established [1]. The mechanism of its regu-

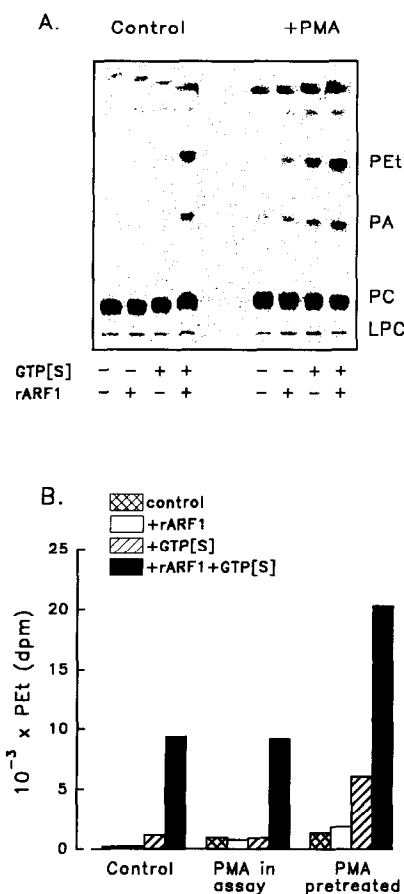


Fig. 3 PMA pretreatment potentiates rARF1-regulated PLD activity. Human neutrophils were pretreated with PMA (100 nM) for 5 min prior to permeabilization with streptolysin O for 10 min. The cells were washed and incubated with 10 μM GTP-γ-S, 4 nmol rARF1, 1 μM Ca²⁺, 1 mM MgATP and 1% ethanol for 30 min to assay for PLD activity. 100 nM PMA was added into the assay where indicated. Analysis of radioactivity (A) by Bioimager and (B) by scintillation counting.

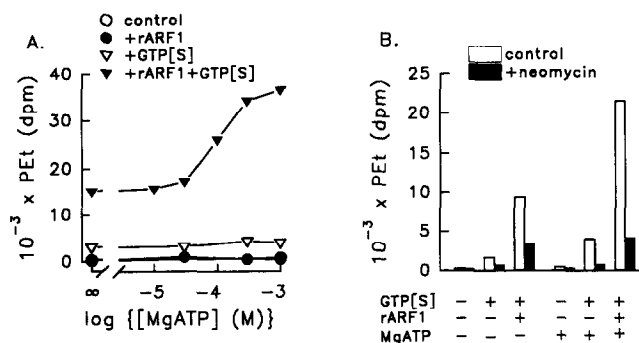


Fig. 4 (A) MgATP potentiates ARF-regulated PLD activity. Neutrophils were metabolically inhibited prior to permeabilization to reduce intracellular levels of MgATP. Cytosol-depleted neutrophils were assayed for PLD activity for 30 min in the presence at $1 \mu\text{M}$ Ca^{2+} and 1% ethanol. rARF1 (4 nmol), GTP- γ -S (10 μM) and indicated concentrations of MgATP were presented where indicated. (B) Neomycin inhibits rARF1-regulated PLD activity in the presence or absence of MgATP. Legend as in A except that the concentration of neomycin and MgATP were both at 1 mM.

lation has been intensively studied, and evidence for both GTP-binding proteins and PKC has been reported [1]. ARF has been recently identified as the GTP-binding protein responsible for regulating PLD in undifferentiated HL60 cells, a promyelocytic cell-line [16,17]. In this study we report the requirement for ARF as an activator of PLD in human neutrophils and show that the ARF1-dependent PLD activity is greatly potentiated by PMA, an activator of PKC and by MgATP.

Many of the characteristics of rARF1-reconstituted PLD activity in the cytosol-depleted human neutrophils examined here resemble those observed in HL60's, a related cell line, when endogenous cytosol was present [12,20]. For example, synergistic activation of PLD with PMA and GTP- γ -S and enhancement by MgATP are observed under both situations. In studies in HL60's where GTP- γ -S was added during permeabilization, PLD activity was found to be greatly enhanced as the concentration of Ca^{2+} was increased from nanomolar to micromolar [12,20]. It was therefore surprising to note that rARF1-reconstituted PLD activity (Fig. 2B) was not dependent on Ca^{2+} to the same degree. This may reflect cell-specific differences.

PKCs are predominantly cytosolic in neutrophils and their activation with PMA involves the increased association of the enzymes with membranes [25]. 9 isoforms of PKCs have been identified; and classed as conventional PKCs (cPKC) which are Ca^{2+} -dependent or novel PKCs (nPKC) which are Ca^{2+} -independent [26]. Neutrophils possess both cPKCs and nPKCs [27]. In neutrophils the predominant isoform that translocates to membranes in response to PMA is the cPKC isoform β [25,27]. Since PMA pretreatment is necessary to observe the increase in the rARF1-regulated PLD activity, the most likely candidate responsible for this effect is the translocated PKC β . The target for the PKC is not known but could conceivably be PLD itself.

The observation that GTP- γ -S-regulated PLD activity is enhanced by MgATP has previously been interpreted to be due to the activation of PLC and hence PKC [12]. This interpretation is no longer tenable for two reasons. In cytosol-depleted cells used in this study, [1] an effect of PMA is only observed

provided that the PKCs are recruited to the membrane prior to permeabilization. [2] PLC activation is greatly reduced due to loss of a cytosolic protein, phosphatidylinositol transfer protein [21].

It has recently been reported that ARF can only activate a solubilized PLD in the presence of $\text{PI}(4,5)\text{P}_2$ [16]. We have previously shown that in the absence of MgATP, the polyphosphoinositide levels drop significantly and re-addition of MgATP at millimolar concentrations restores these levels [28]. The enhancement with MgATP when ARF-regulated PLD is examined in its natural environment may reflect the increased availability of PIP_2 . The observation that neomycin, which binds to polyphosphoinositides selectively [29] inhibited ARF1-regulated PLD activity specifically provides direct proof that the polyphosphoinositides play a role not only *in vitro* [16] but also when the substrate, PC, is presented in its natural environment.

The fungal metabolite wortmannin is a potent inhibitor of PI 3-kinase in neutrophils [23,24] as well as fMetLeuPhe-mediated PLD activation in intact neutrophils [30–32]. PMA or A23187 stimulation of PLD is unaffected by wortmannin, suggesting that direct inhibition of the enzyme is unlikely, and that events upstream of PLD activation are the target for wortmannin inhibition [33]. Since wortmannin is without effect on rARF1-regulated PLD, it follows that it is not PIP_3 that enhances rARF1-reconstituted PLD activity in the presence of MgATP.

A major goal is to identify by which mechanism receptor activation leads to the activation of PLD in neutrophils. We would assume that there is an ARF exchange factor that is responsible for exchanging GDP for GTP on ARF. A possible signal that may be responsible for ARF activation could be derived from the PI 3-kinase pathway. The fMetLeuPhe receptor activates PI 3-kinase through $\beta\gamma$ -subunits and wortmannin has been shown to be a direct inhibitor of this activity [23,24,34]. fMetLeuPhe-mediated PLD activation in intact neutrophils is also potently inhibited by wortmannin [30–32]. Since we show that ARF1-regulated PLD activity is not inhibited directly by wortmannin, this would support the concept that the PLD pathway is downstream to the PI 3-kinase pathway.

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