Aluminum fluoride inhibits phospholipase D activation by a GTP-binding protein-independent mechanism

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Abstract Aluminum fluoride (AlF_4^-) inhibited guanine nucleotide-activated phospholipase D (PLD) in rat submandibular gland cell-free lysates in a concentration-dependent response. This effect was consistent in permeabilized cells with endogenous phospholipid PLD substrates. Inhibition was not caused by either fluoride or aluminum alone and was reversed by aluminum chelation. Inhibition of PLD by aluminum fluoride was not mediated by cAMP, phosphatases 1, 2A or 2B, or phosphatidate phosphohydrolase. AlF_4^- had a similar inhibitory effect on rArf-stimulated PLD, but did not block the translocation of Arf from cytosol to membranes, indicating a post-GTP-binding-protein site of action. Oleate-sensitive PLD, which is not guanine nucleotide-dependent, was also inhibited by AlF_4^-, supporting a G protein-independent mechanism of action. A submandibular Golgi-enriched membrane preparation had high PLD activity which was also potently inhibited by AlF_4^-, leading to speculation that the known fluoride inhibition of Golgi vesicle transport may be PLD-mediated. It is proposed that aluminum fluoride inhibits different forms of PLD by a mechanism that is independent of GTP-binding proteins and that acts via a membrane-associated target which may be the enzyme itself.

Key words: Phospholipase D; Aluminum fluoride; GTP-binding protein; Salivary gland

1. Introduction

Phospholipase D (PLD) is increasingly recognized as an important signal-transducing enzyme involved in the regulation of several cellular processes [1,2]. The major substrate for the enzyme is membrane phosphatidylcholine (PC). The hydrolysis product, phosphatidic acid (PA), may itself act as a signaling molecule, and is also the precursor of additional regulatory molecules including diacylglycerol, lyso-PA and arachidonic acid (AA) [1–3]. For example, we previously showed that in submandibular cells, PA is a prime source of AA [4], a multipotent regulator of protein synthesis, calcium flux and mucin secretion in the model [5]. Two forms of phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent PLD, designated PLD1 and PLD2, have been cloned from mammalian tissues [6,7]. PLD1 is regulated by the small GTPases, Arf and RhoA and by the enzyme protein kinase C (PKC). PLD2 has a high constitutive activity and may be regulated by unknown inhibitory factors [1–3]. An additional form of fatty acid (oleate)-sensitive PLD has been demonstrated in brain [8] and other tissues. Most cellular studies so far reported on the enzyme have dealt with the guanine nucleotide/Arf-sensitive PLD1.

A number of agonists that activate PLD are known to act on membrane receptors coupled to heterotrimeric GTP-binding proteins [9]. In an earlier study we found that muscarinic stimulation of submandibular cells activated PLD, partially by acting through the receptor-coupled Gq protein of the PIP_2/phospholipase C (PLC) signal transduction pathway, with the downstream mediation of PKC [4]. We later demonstrated that, in the same model, a Gq-independent pathway, involving the sequential actions of an unidentified heterotrimeric G protein and Arf, also led to PLD activation [10]. In the same study, it was shown that AlF_4^- too activated PLD in intact cells, consistent with its effect on other intact cell models [11,12] and with its known capacity to activate heterotrimeric G proteins. In this effect, AlF_4^- mimics the γ-phosphate group of GTP to stabilize the Gα subunit of the regulatory protein in the transitional state [13,14]. AlF_4^- reacts only with heterotrimeric G proteins in this way, and not with small GTP-binding species such as Arf [15].

Although AlF_4^- activates PLD in intact cells, it has failed to activate the enzyme in cell-free extracts of HL60 cells and neutrophils [16,17]. This prompted us to examine the effects of this probe on PLD in lysates of submandibular acinar cells and in permeabilized cells, in an attempt to gain a better understanding of PLD regulation in this model. We report here an inhibitory effect of aluminum fluoride on PLD in submandibular extracts by a mechanism that is independent of its stimulation of heterotrimeric GTP-binding proteins.

2. Materials and methods

2.1. Materials

Dipalmitoyl-phosphatidylcholine (PC), dioleoyl-phosphatidylethanolamine (PE), and phosphatidylethanol (PEth) were purchased from Avanti Polar Lipids, Alabaster, AL, USA. PIP_2 purified from bovine brain was obtained from Sigma-Aldrich, Oakville, Ont., Canada. Dipalmitoyl-[2-palmitoyl-9,10-^{3}H]phosphatidylcholine and dipalmitoylphosphatidyl-[methyl-^{3}H]choline were from Dupont NEN (Mandel Scientific, Guelph, Ont., Canada). [5,6,8,9,11,12,14,15-^{3}H]AA was a product of Amersham Pharmacia, Baie d'Urfe, Que., Canada. GTPγS and Complete Mini Protease inhibitor tablets were from Boehringer Mannheim, Laval, Que., Canada. Whatman Silica Gel 60 TLC plates were purchased from Fisher Scientific, Winnipeg, Man., Canada. Ultrapure sodium fluoride and all the other reagents were from Sigma-Aldrich.

2.2. Preparation of rat submandibular acinar cells

Submandibular acinar cells from male Sprague-Dawley rats (200–250 g) were prepared by collagenase dissociation and maintained in short-term culture in a modified Hanks' balanced salt solution as described previously [4].

2.3. Preparation of submandibular membrane and cytosolic fractions

Fresh isolated rat submandibular acinar cells were resuspended in

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buffer A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM DTT, pH 7.0). One tablet of Complete Mini protease inhibitor cocktail (Boehringer Mannheim) was added per 10 ml buffer A. The cells were disrupted by homogenization plus brief sonication. Unbroken cells and nuclei were removed by centrifugation at $500\times g$ for 10 min. Total membrane and cytosol were obtained by centrifugation at $100\,000\times g$ for 90 min. The supernatant (cytosol) was removed. The pellet (membrane) was resuspended in buffer A with the same concentration of the protease inhibitors. Membrane and cytosol fractions were aliquoted and stored at −80°C.

2.4. PLD assays

Cell-free PLD assays were carried out using phospholipid vesicles (PE/PIP_2/PC, in a molar ratio of 16:1.4:1) prepared according to Brown et al. [16], and containing dipalmitoyl-[2-palmitoyl-9,10-^{3}H]PC (0.075 μCi per 10 μl lipid vesicle). PLD activity was assayed by quantifying radiolabeled PEth, produced in the transphosphatidylation reaction and isolated by thin layer chromatography [4]. To start the reaction, 10 μl lipid vesicles and 1% ethanol were added to the assay system (50 mM HEPES, 80 mM KCl, 3.0 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 1 mM $CaCl_2$, pH 7.0) which contained 50–100 μg membrane and/or cytosol fraction. In most experiments, 10 μM GTPγS was used to stimulate PLD activity. The assays were conducted at 37°C for 20 min in a total assay volume of 60 μl. The reaction was stopped, PEth was extracted and quantified as described previously [10]. PLD assays in cells permeabilized by 50 μg/ml digitonin and in intact cells were conducted by labeling the cellular phospholipids with [^{3}H]AA and measuring radiolabeled PEth formation [10].

Oleate-dependent PLD activity was assayed as described by Massenburgh et al. [8]. 10 μl phosphatidylcholine vesicles containing 10 nmol of dipalmitoyl-PC and 0.3 μCi of dipalmitoyl-[2-palmitoyl-9,10-^{3}H]PC were incubated for 30 min in a reaction system that included Na-HEPES (50 mM, pH 7.2), 4 mM sodium oleate, 1 mM EGTA, 1 mM $MgCl_2$, 50–100 μg submandibular total cell lysate and 1% ethanol. Generated PEth, reflecting PLD activity, was measured as above.

2.5. Purification of recombinant myristoylated Arf from Escherichia coli

Recombinant myristoylated Arf1 protein was purified from bacteria according to an established protocol [18]. The *E. coli* strain which co-expressed the Arf1 gene and the yeast *N*-myristoyltransferase gene (NMT1) was kindly provided by Dr. R. Bhullar, University of Manitoba.

2.6. Determination of Arf translocation by Western blotting

Arf translocation was determined in a permeabilized cell system by an immunoblotting technique as described previously [10]. A polyclonal anti-Arf antibody was kindly provided by Dr. R. Bhullar, University of Manitoba.

2.7. Preparation of Golgi-enriched membrane fractions

Golgi-enriched membrane fractions were prepared from the post-nuclear supernatant of cell extracts by a modification of the two-step sucrose gradient centrifugation procedure of Balch et al. [19]. After centrifugation at $70\,000\times g$ (av) in a hanging bucket rotor for 4 h, material at the 0.8/1.2 M sucrose interface (Golgi-enriched membranes) was collected by long-tip pipette. Membranes were stored at −70°C until use. Protein content was determined by the Bradford method [20] with the Bio-Rad dye reagent, and 5 μg membrane protein used for each PLD assay (see Section 2.4). The enrichment of the membrane preparations was assessed by assay of the Golgi marker enzyme, galactosyltransferase [21].

3. Results

3.1. Activation of PLD by AlF_4^- in intact submandibular cells

Consistent with our previous observation [10], AlF_4^-, a widely used activator of heterotrimeric GTP-binding proteins, stimulated PLD to around four-fold control levels in intact rat submandibular acinar cells. This effect was dependent on aluminum ion concentration, with a maximal response at 3 mM NaF/2 μM $AlCl_3$ (Fig. 1A). Since fluoride can etch aluminum ions from lab glassware to form AlF_4^- [22], all experiments were carried out in plastic tubes and with ultrapure NaF to rule out uncontrolled aluminum contamination. NaF alone did not stimulate PLD. The stimulatory effect of AlF_4^- on PLD was blocked by the aluminum ion chelator deferoxamine (data not shown). From our previous studies on this model, it is assumed that activation of PLD by aluminum fluoride in intact cells results from a stimulatory effect on a heterotrimeric GTP-binding protein [4,10].

3.2. Inhibition of GTPγS-stimulated PLD by AlF_4^- in a cell-free assay system

In total submandibular lysates (membranes plus cytosol),

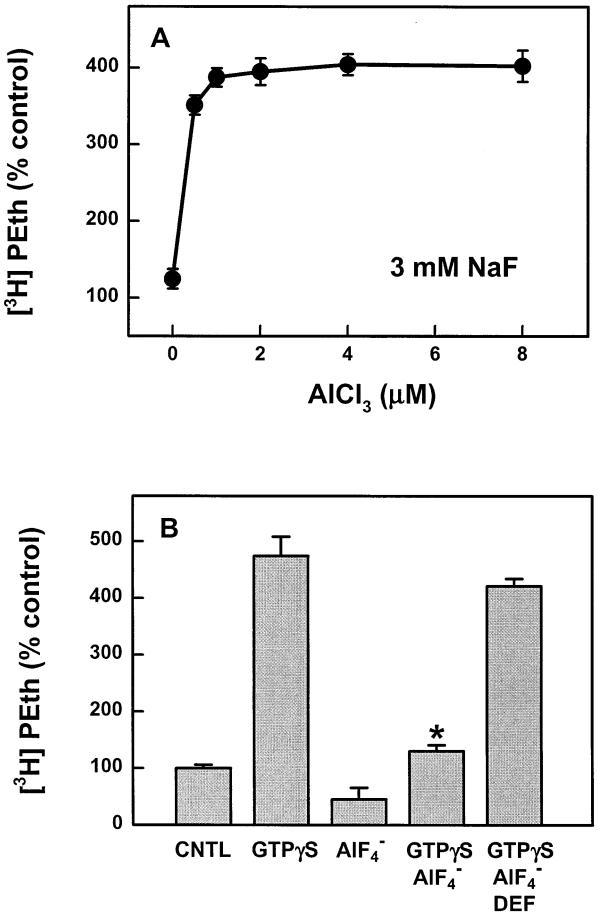

Fig. 1. Aluminum fluoride activates PLD in intact cells but inhibits PLD in cell extracts. A: Aluminum dependence of AlF_4^- stimulation of PLD in intact submandibular acinar cells. Cells were radiolabeled with [^{3}H]AA and then treated with 3 mM NaF plus the indicated concentrations of $AlCl_3$ in the presence of 1% ethanol as substrate for the transphosphatidylation reaction. PLD activity was assayed as described in Section 2. Values are means ± S.D., $n=3$. B: Inhibition of GTPγS-stimulated PLD activity by AlF_4^- in submandibular cell extracts. Cell extracts (54 μg membrane protein plus 30 μg cytosol protein) were preincubated with or without AlF_4^- (3 mM NaF, 20 μM $AlCl_3$) for 5 min before the addition of 10 μM GTPγS. In one group, 100 μM deferoxamine (DEF) was preincubated with cell extracts for 5 min before the addition of AlF_4^-. Values are means ± S.D., $n=3$. The average GTPγS response represents the formation of 17.4 pmol PEth in 30 min. $^*P<0.01$ compared with the response to GTPγS.

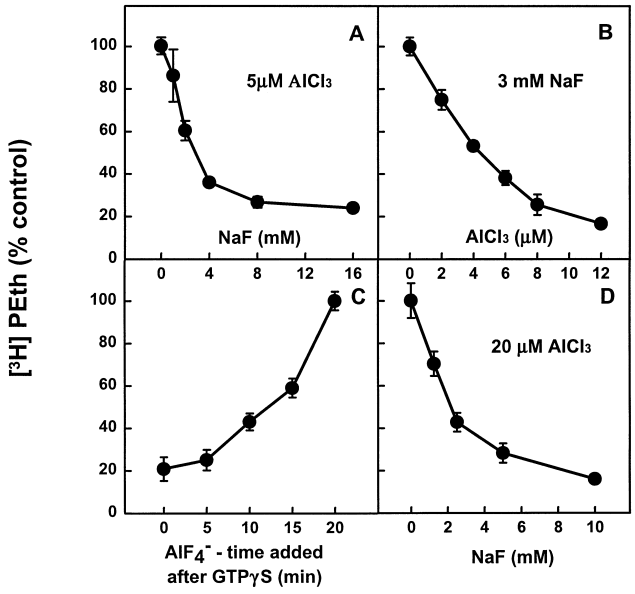

Fig. 2. Concentration/time dependence of AlF_4^- inhibition on GTPγS-stimulated PLD activity in cell extracts or permeabilized cells. A: Submandibular cell extracts were preincubated with the indicated concentrations of NaF together with a fixed concentration of 5 μM $AlCl_3$ to form the active AlF_4^-. B: Cell extracts were preincubated with indicated concentrations of $AlCl_3$ together with a fixed concentration of 3 mM NaF. In both A and B, 10 μM GTPγS was used to stimulate PLD activity after AlF_4^- preincubation for 5 min. Values are means ± S.D., $n=3$. C: Time response of AlF_4^- inhibition on GTPγS-stimulated PLD activity. AlF_4^- (3 mM NaF+ 20 μM $AlCl_3$) was added to the cell-free assay system at different time points during the 20 min reaction time course *after* the reaction had been started by 10 μM GTPγS. Values are means ± S.D., $n=3$. D: AlF_4^- effect on GTPγS (100 μM)-stimulated PLD activity in permeabilized submandibular cells. Cells prelabeled with [^{3}H]AA were permeabilized by 50 μg/ml digitonin in the presence of indicated concentrations of NaF and 20 μM $AlCl_3$. Values are means ± S.D., $n=3$.

10 μM GTPγS stimulated PLD activity to about five-fold basal level (Fig. 1B). As in other experimental models [15,16], AlF_4^- did not activate PLD in this cell-free system. Unexpectedly, we found that AlF_4^- potently inhibited GTPγS-stimulated PLD activity. Inhibition was prevented by the aluminum chelator deferoxamine (Fig. 1B). In concentration-effect studies, either Al^{3+} or F^- was used at a fixed dose, while the other was varied. At 5 μM $AlCl_3$, NaF inhibited GTPγS-activated PLD in a concentration-dependent response (Fig. 2A). Similarly, with NaF held at 3 mM, inhibition was dependent on Al^{3+} concentration over the range 0–12 μM $AlCl_3$ (Fig. 2B). NaF alone had no effect on PLD activity. AlF_4^- inhibited PLD not only when it was preincubated with cell lysates before GTPγS stimulation, but also lowered enzyme activity in a time-dependent way when it was added to the assay system after GTPγS stimulation (Fig. 2C). The GTP analog GTPγS is unhydrolyzable, and not readily displaced from a G protein. Thus, the capacity of AlF_4^- to inhibit PLD after GTPγS stimulation suggests that this effect may be unrelated to its property of activating heterotrimeric G proteins.

3.3. Inhibition of GTPγS-dependent PLD by AlF_4^- in permeabilized cells

The inhibition of PLD by aluminum fluoride was confirmed in permeabilized cells in a concentration-response effect (Fig. 2D). GTPγS enhanced PLD to approximately 5.5-fold basal levels. An optimal combination of 20 μM $AlCl_3$/10 mM NaF inhibited GTPγS-induced PLD activity by 80%. In this assay system, the PLD substrates are prelabeled endogenous membrane phospholipids, suggesting that the AlF_4^- inhibition of PLD observed in the cell-free assay was not an artifact related to the use of exogenous phospholipid vesicles as substrate.

3.4. Investigation of potential mechanisms of AlF_4^- inhibition of PLD

The effects of known aluminum fluoride-sensitive pathways on PLD activation were investigated. Stimulation of the heterotrimeric Gs protein, leading to cAMP elevation, is one of the classic effects of AlF_4^- [22], and the elevation of intracellular cAMP has an inhibitory effect on fMetLeuPhe-mediated PLD activation in neutrophils [23]. We previously reported cross-talk regulation between cAMP and phospholipid signaling pathways in submandibular cells [24]. However, in the present study, three activators of the cAMP pathway, cholera toxin, forskolin, and *db*cAMP, failed to show any significant effects on either basal or GTPγS-stimulated PLD activity (data not shown).

Fluoride has been reported to inhibit different classes of protein phosphatases [25]. The alteration of phosphorylation status of regulatory proteins or of PLD itself may potentially affect PLD activation. Okadaic acid is a known inhibitor of

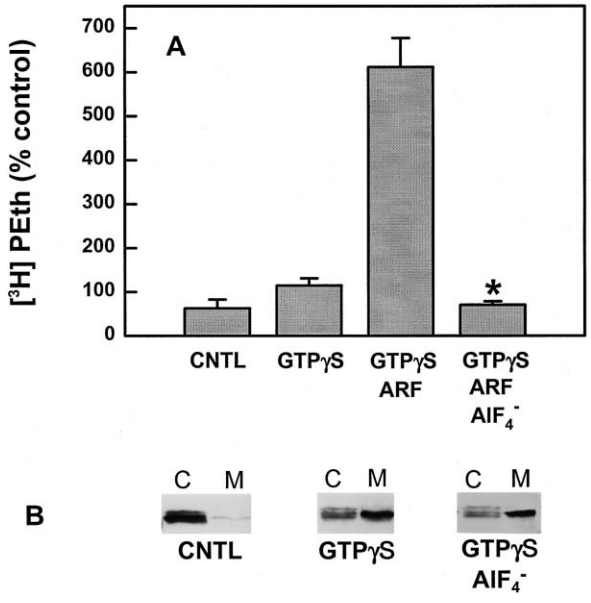

Fig. 3. AlF_4^- effect on rArf-stimulated PLD activity and Arf translocation. A: [^{3}H]AA-labeled cells were permeabilized with 50 μg/ml digitonin, followed by several washes to deplete the cytosolic components. Cytosol-depleted cells were preincubated with or without AlF_4^- for 5 min, then 10 μM GTPγS plus or minus 1.8 μM recombinant myristoylated Arf were added for a 20 min assay period. Values are means of duplicate assays in a representative experiment, bars show the variations from the mean. The Arf response represents the formation of 35 pmol PEth. *$P<0.01$ compared with the response to Arf. B: Lack of effect of aluminum fluoride on the translocation of Arf from cytosol to membranes. Cells were permeabilized by 50 μg/ml digitonin in the presence or absence of AlF_4^- (10 mM NaF+20 μM $AlCl_3$) and 10 μM GTPγS for 20 min. Proteins in the membrane and cytosol fractions were separated by SDS-PAGE and Western blotting was performed with anti-Arf polyclonal antibody as described in Section 2.

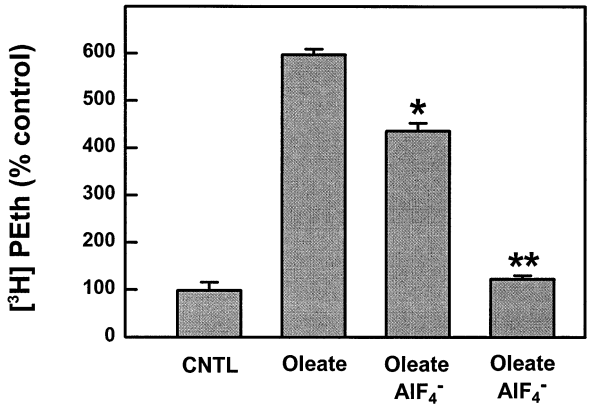

Fig. 4. AlF_4^- effect on an oleate-sensitive form of PLD. Cell membranes (60 μg protein) were preincubated with or without AlF_4^- for 5 min (10 mM NaF plus 2* or 20** μM $AlCl_3$). 10 nmol dipalmitoyl-PC and 1% ethanol were added to the buffer system which contained 4 mM sodium oleate. PEth was measured as before. Values are means ± S.D., $n = 3$. The average activity without inhibitor represents 16.1 pmol PEth formation. $^*P < 0.05$, $^{**}P < 0.01$ compared with oleate-sensitive PLD activity.

protein phosphatases 1, 2A and 2B [26]. In the concentration range 0.1 nM–10 μM, okadaic had no effect on either basal or GTPγS-stimulated PLD activity (data not shown).

Fluoride is also known as an inhibitor of phosphatidate phosphohydrolase (PAP) [27], an enzyme that converts the PC hydrolysis product of PLD action, PA, to diacylglycerol (DAG). We previously demonstrated that the PAP inhibitor propranolol (100 μM) lowered DAG and elevated PA in submandibular cells after carbachol stimulation of PLD, raising the possibility of product inhibitory feedback of the enzyme by elevated PA [4]. In the present study, however, we found that inhibition of PAP by propranolol had no effect on PLD activity, suggesting that the AlF_4^- inhibitory effect was independent of its possible action on PAP (results not shown).

In a previous study, we found that Arf/GTPγS stimulation of PLD involved the translocation of Arf from cytosol to membranes [10]. In the present study, rArf stimulation of PLD in submandibular membranes was also inhibited by AlF_4^- (Fig. 3A). However, Western blotting experiments with an anti-Arf antibody showed that AlF_4^- treatment did not prevent GTPγS-induced Arf translocation from cytosol to membranes (Fig. 3B), suggesting a post-translocation site of inhibitory action.

3.5. Effect of AlF_4^- on oleate-sensitive PLD

Fatty acid (oleate)-sensitive PLD, which is not guanine nucleotide-dependent, was also detected in submandibular membranes. Stimulation with oleate in membrane preparations elevated PLD to approximately six-fold control values (Fig. 4). Again, AlF_4^- inhibited PLD in a concentration-dependent way (Fig. 4). This effect in a GTP-binding protein-independent pathway supports the proposal that GTP-binding proteins are not involved in the aluminum fluoride inhibition of PLD.

3.6. Effect of AlF_4^- on PLD in Golgi-enriched membranes

Golgi-enriched membranes, which are essentially devoid of endoplasmic reticulum, endosomes and multivesicular vesicles [29], have been used in several cell types to study PLD activation and function [28,29]. In the present study, enrichment of the preparations from submandibular cells was confirmed by assay for galactosyltransferase, a Golgi enzyme marker. Enzyme activity in extracts of the 0.8/1.2 M sucrose interface was 19-fold that of total cell membranes (data not shown).

Golgi-enriched membranes from submandibular cells had a high basal PLD activity, which was approximately 10-fold the value of that for total cell membranes. Treatment with GTPγS increased basal levels by a factor of 2.4 (Fig. 5). AlF_4^- potently inhibited Golgi PLD in both basal and guanine nucleotide-stimulated preparations to almost negligible activities (Fig. 5).

4. Discussion

Aluminum fluoride is an activator of heterotrimeric G proteins in receptor-coupled signaling pathways. Fluoride alone is often found to have the same effect, attributed to its capacity to etch aluminum from laboratory glassware to form the active AlF_4^- species [22]. In intact cells, including neutrophils [11,12], Cos-7 cells [30] and submandibular acinar cells ([10], present study), fluoride or AlF_4^- activates PLD, presumably via a heterotrimeric GTP-binding protein. Recent evidence suggests that the G protein, G_{13}, couples to PLD [30]. We previously showed that in submandibular acinar cells, a heterotrimeric G protein and the small GTPase, Arf, may be sequentially involved in muscarinic receptor-coupled PLD activation in the model [10].

Aluminum fluoride is unable to activate PLD in cell extracts or in assays with purified components ([16,17], J. Exton, personal communication). However, AlF_4^- does activate another phospholipase, PLC, in intact cells, permeabilized cells and cell extracts [31,32], by its action on the PLC-coupled Gq regulatory protein. Thus, the inhibitory effect on PLD found in the present study is not universal for phospholipases and is not the result of some difference between AlF_4^- effects in cell-free versus intact cell conditions. Our findings also confirmed that AlF_4^- does not inhibit the enzyme via a Gs-coupled cAMP elevation in a crosstalk regulatory effect.

Several lines of evidence also suggest that the AlF_4^- inhibitory effect is not dependent on its well-known activation of heterotrimeric GTP-binding proteins. Guanine nucleotide-sensitive PLD could be inhibited by AlF_4^- added after GTPγS, a

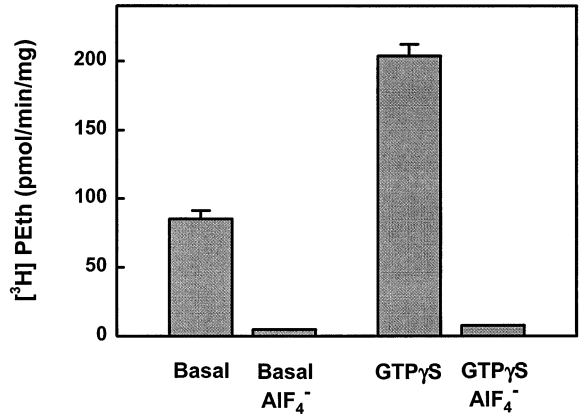

Fig. 5. Effect of AlF_4^- on basal and GTPγS-stimulated PLD in a Golgi membrane-enriched preparation from rat submandibular cells. Membranes (5 μg/assay) were incubated with or without AlF_4^- (10 mM NaF+20 μM $AlCl_3$) for 5 min and then in the presence or absence of 10 μM GTPγS for 20 min. PLD was assayed as before. Values are means ± S.D., $n = 3$.

non-hydrolyzable GTP analog which binds GTP-binding proteins with a negligible off-rate [14], and is not, therefore, easily replaced by GDP-AlF_4^-. In addition, oleate-sensitive PLD, which is not regulated by guanine nucleotide-binding proteins [8,33], is also inhibited by AlF_4^- so that there may be a common regulatory mechanism for different forms of PLD. Furthermore, AlF_4^- also inhibited rArf-stimulated PLD activity but did not affect the translocation of Arf from the cytosol to membranes. This indicates that the site of action of AlF_4^- in PLD inhibition is located at a post-Arf-translocation locus. Since AlF_4^- inhibited PLD in the membrane fraction alone, a non-GTP-binding membrane-associated target is proposed. This could be a PLD regulatory protein, or the enzyme itself.

PLD has been proposed to regulate intracellular vesicle transport [34,35], especially that of COP1-coated vesicles across Golgi stacks [29,39], and abundant PLD activity has been identified in Golgi-enriched membranes [28]. Intra-Golgi transport in a cell-free system is inhibited by AlF_4^- which appears to act through an AlF_4^--sensitive factor on the Golgi membrane [36–38]. In the present study, we found that PLD activity was high in Golgi-enriched membranes from submandibular cells and that this activity was potently inhibited by AlF_4^- in both the basal and GTPγS-stimulated condition. It is therefore possible that AlF_4^- blocks vesicle transport in the Golgi by inhibition of PLD. If so, aluminum fluoride should prove a useful probe in future studies on the role and mechanism of action of PLD in intracellular vesicle transport.

In preliminary experiments with rat brain extracts and purified plant PLD, AlF_4^- also inhibited PLD activation (unpublished observation), so that this effect may represent a universal inhibitory mechanism on different forms of the enzyme. This is supported by our findings on GTPγS/Arf-sensitive and oleate-sensitive PLD, discussed above. The mode of action of aluminum fluoride in this effect is unknown. Since plant PLD requires only a combination of enzyme and simple PC substrate to cause activation [40], the enzyme itself may be the target for AlF_4^- action. Kinetic studies on purified PLD may clarify this.

Though it is well documented that AlF_4^- activates PLD in intact cells, we believe that this is the first report of its inhibition of the enzyme in cell extracts. This difference most likely reflects the fact that in extracts, aluminum fluoride acts on an inhibition-causing target which is shielded from it in intact cells, and which may be the enzyme itself. The use of AlF_4^- in PLD studies has potential value in expanding our knowledge of the enzyme's regulation and function.

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