

Aluminum fluoride inhibition of cabbage phospholipase D by a phosphate-mimicking mechanism

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Abstract Aluminum fluoride (AlF_4^-) inhibited phospholipase D (PLD) purified from cabbage in both PIP_2 -dependent and PIP_2 -independent assays, consistent with its previously observed effect on mammalian PLD. The possibility that AlF_4^- may exert this effect through its known phosphate-mimicking property was examined. Inorganic phosphate, as well as two phosphate analogs, beryllium fluoride and orthovanadate, also inhibited cabbage PLD. Enzyme kinetic studies confirmed that PLD followed Hill kinetics, characteristic for allosteric enzymes, with an apparent Hill coefficient (n_{app}) of 3.8, indicating positive cooperativity among multiple substrate-binding sites and suggesting possible functional oligomerization of the enzyme. AlF_4^- modification of PLD kinetics was consistent with a competitive mode of enzyme inhibition. It is therefore proposed that AlF_4^- , and other phosphate analogs, inhibits plant PLD by competing with a substrate phosphate group for a substrate-binding site, thereby preventing the formation of an enzyme-phosphatidyl intermediate. This may be a conserved feature of PLD superfamily enzymes.

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Key words: Phospholipase D; Aluminum fluoride; Cabbage

1. Introduction

Phospholipase D (PLD) hydrolyzes the terminal phosphodiester bond of membrane phospholipids, particularly phosphatidylcholine (PC), to release phosphatidic acid (PA) and a free polar head group. PA can give rise to additional regulatory molecules such as diacylglycerol and arachidonic acid. Mammalian PLDs thus play a central role in receptor-mediated signal transduction pathways. The enzyme also has important functions in controlling intracellular membrane/vesicle transport and cell proliferation [1,2]. Two major classes of mammalian PLDs have been identified, a PIP_2 -dependent class (with two members, PLD1 and PLD2) and a fatty acid (oleate)-sensitive form [3]. In a recent study on PLD in rat submandibular gland acinar cells, we found that both forms of the enzyme were potently inhibited by aluminum fluoride (AlF_4^-), by a GTP-binding protein-independent mechanism [4]. A direct inhibitory effect of AlF_4^- on the enzyme itself was suggested. This possibility was followed up in the present study, with the aims of determining whether AlF_4^- inhibition of PLD is universal in animal/plant forms of the enzyme and of generating new information on the nature of the inhibitory mechanism by using readily available purified plant (cabbage) enzyme in inhibition studies.

Plant PLD was first described in 1947 [5]. It is widespread in the plant kingdom and partially purified enzyme, particularly from cabbage and peanut [6–8], has been widely investigated. Unlike mammalian enzymes, plant PLDs are insensitive to GTP-binding proteins such as Arf and Rho and to GTP γ S stimulation [9]. Three plant PLD isoforms have recently been cloned from *Arabidopsis*. PLD α , the prevalent form of the enzyme, which has been extensively studied in plant species [10], is PIP_2 -independent and shows a high activity at mM Ca^{2+} concentrations [11]. Two additional forms, PLD β and PLD γ , require PIP_2 and are optimally active at micromolar Ca^{2+} levels [9,12].

PLD has recently been identified as one of a group of enzymes with diverse functions, termed the PLD superfamily [13]. Other members include cardiolipin synthases, phosphatidylserine synthases, pox viral envelope proteins, a bacterial endonuclease (Nuc) and the *Yersinia pestis* murine toxin (Ymt) [13–16]. The hallmark of the PLD superfamily is the presence of conserved HxKxxxxD motifs in the enzyme amino acid sequence [14,16]. It is believed that duplicated HKD motifs are key structural components of the active site (except in Nuc, which has a single HKD motif) and that members of the superfamily therefore share similar catalytic mechanisms [15,16]. Recent studies on two PLD-related enzymes, Nuc and Ymt, suggested a common catalytic action that proceeds via a phosphohistidine-linked phosphatidyl-enzyme intermediate [18,19]. Studies on the enzymology of plant PLD, and its inhibition by AlF_4^- and analogs, should therefore expand our understanding of the conserved mechanisms of action and of the regulation of superfamily members.

We propose that AlF_4^- is a competitive inhibitor of purified cabbage PLD, with a mechanism of action based on its phosphate-mimicking property.

2. Materials and methods

2.1. Materials

Dipalmitoyl-PC, dioleoyl-phosphatidylethanolamine (PE) and phosphatidylethanol (PEth) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dipalmitoyl-[2-palmitoyl-9,10- ^3H]choline and 1-palmitoyl-2-arachidonoyl-[arachidonoyl-1- ^{14}C]PE were from Dupont NEN, Mandel Scientific (Guelph, Ont., Canada). Phosphatidylinositol-4,5-bisphosphate (PIP_2) purified from bovine brain, PLD purified from cabbage or peanut, ultrapure sodium fluoride (NaF) and all other reagents were from Sigma-Aldrich (Oakville, Ont., Canada).

2.2. PIP_2 -dependent PLD assay

PIP_2 -dependent PLD activity was assayed in a cell-free system [4] containing phospholipid vesicles (PE/ PIP_2 /PC molar ratio 16:1.4:1) with a final concentration of 8.7 μM PC, prepared according to Brown et al. [20]. Ten μl lipid vesicles was added to the assay system (50 mM HEPES, 80 mM KCl, 3 mM MgCl_2 , 3 mM DTT, pH 7.0) which contained 2.5 μg cabbage PLD. The free calcium concentration was EGTA-buffered to 7.8 μM Ca^{2+} unless otherwise indicated. PLD

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activity was assayed either by the transphosphatidyl reaction in the presence of 1% ethanol, with dipalmitoyl-[2-palmitoyl-9,10- ^3H]PC as substrate as described previously [4], or by radiolabelled choline-release with dipalmitoylphosphatidyl-[methyl- ^3H]choline as substrate. The assays were conducted at 37°C for 20 min in a total assay volume of 60 μl . For the transphosphatidyl assay, ^3H -labelled PEth was separated by thin layer chromatography (TLC) and quantitated by scintillation counting as described previously [4]. For the choline-release assay, the reaction was stopped by adding 360 μl chloroform/methanol (2:1, v/v). After vigorous vortexing and centrifugation at 6000 $\times g$ for 5 min, radiolabelled choline was measured in 50 μl aliquots of the aqueous phase by scintillation counting.

2.3. PIP_2 -independent PLD assay

PIP_2 -independent PLD activity was assayed by a modified procedure of Wang et al. [10]. The reaction mixture contained 100 mM MES (pH 7.0), 1 mM SDS, 1% ethanol, 2.5 μg cabbage PLD and 0.2 mM dipalmitoyl-[2-palmitoyl-9,10- ^3H]PC with varying concentrations of CaCl_2 at the mM level. To assay PLD activity with PE as substrate, PC was replaced by unlabelled PE, plus 1-palmitoyl-2-arachidonyl,[arachidonyl-1- ^{14}C]PE in the assay system. The final concentration of PE was 0.4 mM. The assays were conducted at 37°C for 20 min in a total assay volume of 60 μl .

2.4. Kinetic study on cabbage PLD

The kinetic experiments were performed with the PIP_2 -independent PLD assay procedure. Two stocks of PC substrate were prepared by bath sonication. Stock one contained only [^3H]dipalmitoyl-PC (100 000 dpm/10 μl). Stock two contained the same concentration of [^3H]dipalmitoyl-PC and 1 mM cold dipalmitoyl-PC. Different concentrations of PC substrates were prepared by mixing these two stocks with brief sonication. Cabbage PLD (2.5 μg /assay) was pre-incubated with NaF plus AlCl_3 for 5 min before addition to the assay system. The reaction was carried out at 37°C for 5 min. The product, PA, was separated by TLC under the same developing solvents as those for PEth and quantitated by scintillation counting.

3. Results

3.1. Inhibition of plant PLD by AlF_4^- in the PIP_2 -dependent PLD assay

Under PIP_2 -dependent assay conditions, cabbage PLD exhibited a specific activity equal to 2.1 nmol PEth formation/min/mg. The inclusion of AlF_4^- (a combination of 3 mM NaF and 20 μM AlCl_3) potentially inhibited cabbage enzyme activity to 24% of the control value (Fig. 1A). Neither sodium fluoride nor aluminum chloride alone had this effect (Fig. 1A), indicating a requirement for the formation of AlF_4^- in the inhibitory response. Additional evidence for this requirement was the action of the aluminum ion chelator deferoxamine (DEF) in reversing the AlF_4^- inhibition of PLD (Fig. 1A). DEF itself had no effect on PLD (data not shown). The inhibitory effect of AlF_4^- (to 25% of control value) was further confirmed by a choline-release PLD assay on cabbage PLD (Fig. 1B). The effect was consistent with PLD purified from peanuts (inhibited to 16% control), indicating that inhibition by AlF_4^- may be universal for plant PLDs of a different origin (Fig. 1B).

Under the assay conditions used, cabbage PLD required μM levels of free calcium. Enzyme activity was abolished in the absence of added Ca^{2+} plus the presence of 1 mM EGTA. At a concentration of 300 nM free calcium in the assay buffer, PLD activity was only about 1/20 of that at 7.8 μM calcium (data not shown). Cabbage PLD activity was also concentration-dependent on PIP_2 , with a maximal response at about 12 μM phosphoinositide (Fig. 2A). The role of PIP_2 in PLD stimulation was examined by using neomycin, a high affinity ligand that selectively binds to polyphosphoinositides and blocks their binding to other targets [21]. The inclusion of

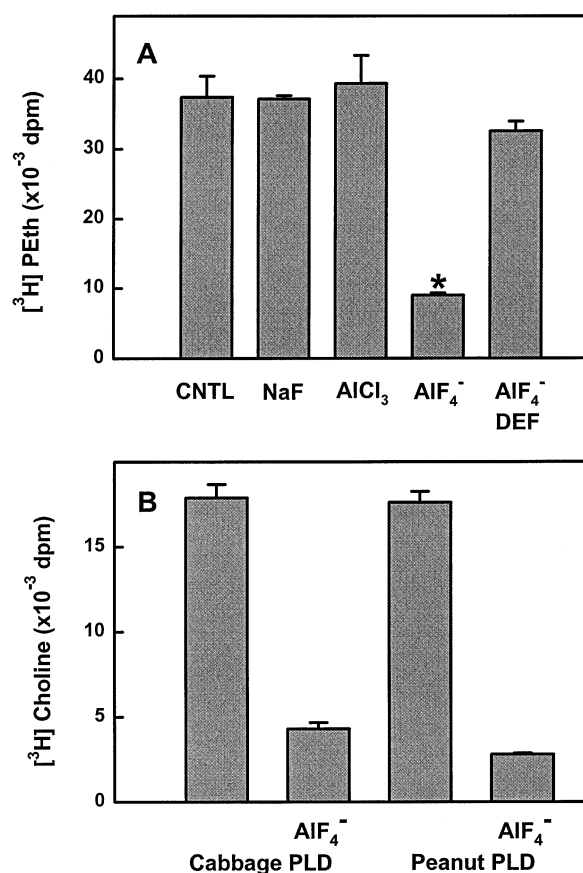


Fig. 1. AlF_4^- inhibition of cabbage PLD activity in a PIP_2 -dependent assay. A: Cabbage PLD (2.5 μg /sample) was assayed by the transphosphatidyl reaction in 1% ethanol over 20 min. The enzyme was pre-incubated with 3 mM sodium fluoride (NaF), 20 μM aluminum chloride (AlCl_3) or 3 mM NaF+20 μM AlCl_3 (AlF_4^-) in the presence or absence of 100 μM DEF for 5 min. B: Cabbage or peanut PLD (2.5 μg /sample) was assayed by the radiolabelled choline-release with or without AlF_4^- (3 mM NaF+20 μM AlCl_3) pre-incubation for 5 min. The assays were performed as described in Section 2. Values are means $\pm$ S.D., $n = 3$.

12 μM PIP_2 in mixed lipid vesicles enhanced PLD activity to approximately 7-fold the level found in samples lacking PIP_2 (Fig. 2B). One mM neomycin inhibited PIP_2 -activated PLD to 33% of the control value and AlF_4^- inhibited it to 9% (Fig. 2B). A combination of neomycin and AlF_4^- produced a maximal inhibitory effect (Fig. 2B). Similar results were again obtained by the choline-release assay (data not shown).

3.2. Effect of different phosphate analogs on PIP_2 -dependent cabbage PLD

AlF_4^- is a known phosphate analog. It stimulates heterotrimeric G proteins by mimicking the γ -phosphate of GTP and stabilizing the transitional state of the $\text{G}\alpha$ protein [22,23]. A phosphoenzyme intermediate has been proposed in the PLD substrate hydrolysis mechanism [17]. We therefore investigated the possibility that AlF_4^- inhibition of PLD may be caused by its phosphate-mimicking property. When the fluoride concentration was fixed at 3 mM, the inhibitory effect depended on the concentration of aluminum ions, with half maximal Al^{3+} about 15 μM (Fig. 3A). Similar dose-dependent curves were observed with two other known phosphate analogs, beryllium fluoride (BeF_3^-) and sodium orthovanadate

(Na_3VO_4), with half maximal inhibition doses of 3 mM NaF +50 μM BeCl_2 and 35 μM Na_3VO_4 , respectively (Fig. 3B,C). Furthermore, inorganic phosphate itself, in the millimolar concentration range, also inhibited cabbage PLD activity in a concentration-dependent way (IC_{50} 10 mM, Fig. 3D). These results suggest that AlF_4^- may inhibit PLD by a phosphate-mimicking effect and emphasize the central importance of a probable phosphate-related mechanism in the enzyme's action.

3.3. Effect of AlF_4^- and related phosphate analogs on cabbage PLD activity in a PIP_2 -independent PLD assay

Under the PIP_2 -independent assay conditions conventionally used for the plant enzyme, cabbage PLD was dose-dependently activated by Ca^{2+} over a 1–8 mM calcium concentration range (Fig. 4A). At 8 mM Ca^{2+} , the specific activity of PLD (dipalmitoyl-PC substrate) was 23 nmol PEth formation/min/mg protein. Enzyme activity was almost completely inhibited by AlF_4^- at all calcium concentrations (Fig. 4A). Consistent with the PIP_2 -dependent assay results (above), both BeF_3^- and Na_3VO_4 also inhibited PLD activity in the PIP_2 -independent assay (Fig. 4B). The effect of inorganic phos-

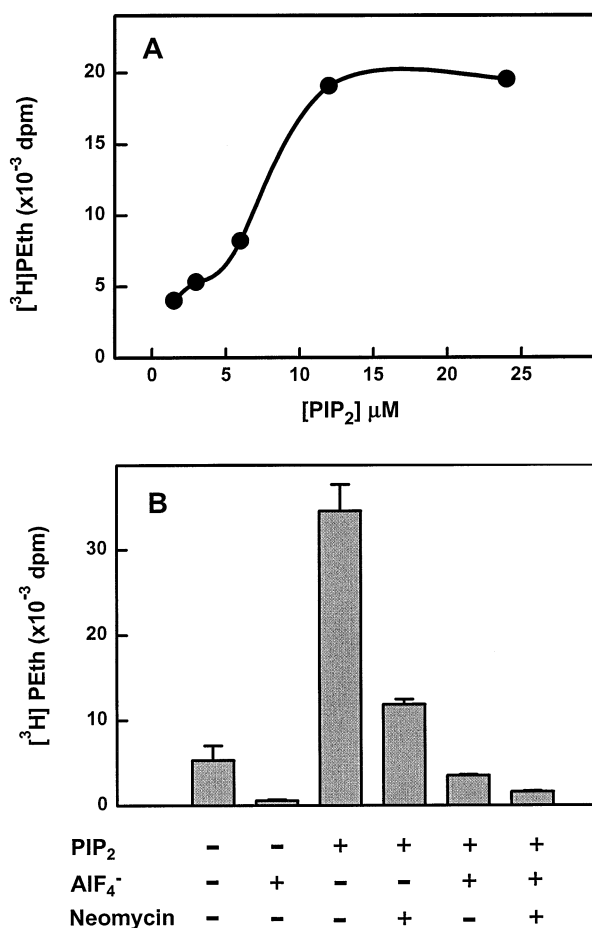


Fig. 2. PIP_2 -dependence of cabbage PLD. A: Cabbage PLD activity was assayed in mixed lipid vesicles (PE/ PIP_2 /PC) with PIP_2 concentrations from 1.5 to 24 μM . B: Cabbage PLD activity was assayed in the presence or absence of 12 μM PIP_2 . The enzyme was pretreated for 5 min with or without neomycin (1 mM) or AlF_4^- (3 mM NaF +20 μM AlCl_3) or neomycin+ AlF_4^- . Enzyme activity was measured by the transphosphatidyl reaction in 1% ethanol for 20 min. Values are means $\pm$ S.D., $n = 3$.

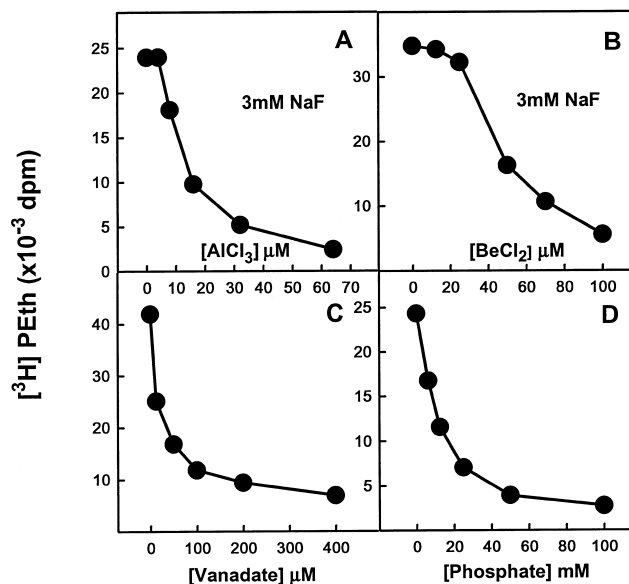


Fig. 3. Concentration-dependent inhibition of PIP_2 -dependent cabbage PLD activity by phosphate and phosphate analogs. Cabbage PLD (2.5 $\mu\text{g}/\text{assay}$) was pretreated for 5 min with (A) AlF_4^- (3 mM NaF +0–64 μM AlCl_3), (B) beryllium fluoride (3 mM NaF +0–100 μM BeCl_2), (C) sodium orthovanadate (0–400 μM), (D) inorganic phosphate (0–100 mM). Values are means from a representative experiment with variations of less than 12%, $n = 3$.

phate could not be tested under this assay condition, since phosphate precipitates mM levels of calcium [24]. In addition to its utilization of PC substrate, plant PLD is able to hydrolyze PE [25]. Cabbage PLD hydrolyzed PE at about 40% of the rate of PC cleavage (Fig. 4B). PE hydrolysis was also inhibited by three phosphate analogs with potencies comparable to those shown with PC substrate (Fig. 4B). This suggests a consistent mode of inhibitory action on the PLD hydrolysis of both phospholipid substrates.

3.4. Kinetics of cabbage PLD

The rate of PA formation from PC substrate by cabbage PLD was linear for approximately 20 min (data not shown). PA production was therefore measured over a reaction time of 5 min to represent the initial reaction rate. The velocity versus substrate concentration experiments generated data that did not fit conventional Michaelis-Menten kinetics, but, instead, produced a sigmoidal curve characteristic of Hill kinetics (Fig. 5). This is consistent with a recent study on cabbage PLD which first proposed Hill kinetic behavior for this enzyme [26]. The Hill model represents the kinetics of allosteric enzymes, in which the sequential binding of substrate molecules to multiple substrate-binding sites increases the binding affinities of the remaining vacant sites (cooperative binding) [27]. The Hill plot ($\log v/[V_{\max} - v]$ versus $\log [S]$) of cabbage PLD kinetic data showed an approximately straight line (Fig. 5, inset). The calculated dissociation constant, K' , was 0.65 mM and the apparent Hill coefficient (n_{app}) was 3.8. The effects of two different concentrations of AlF_4^- on the kinetics of PLD were examined. Both concentrations of AlF_4^- displaced the velocity/substrate concentration curves to the right in a dose-dependent way. The inhibitory potency of AlF_4^- decreased with an increasing PC substrate concentration, suggesting a competitive mode of inhibition under Hill kinetic behavior [25].

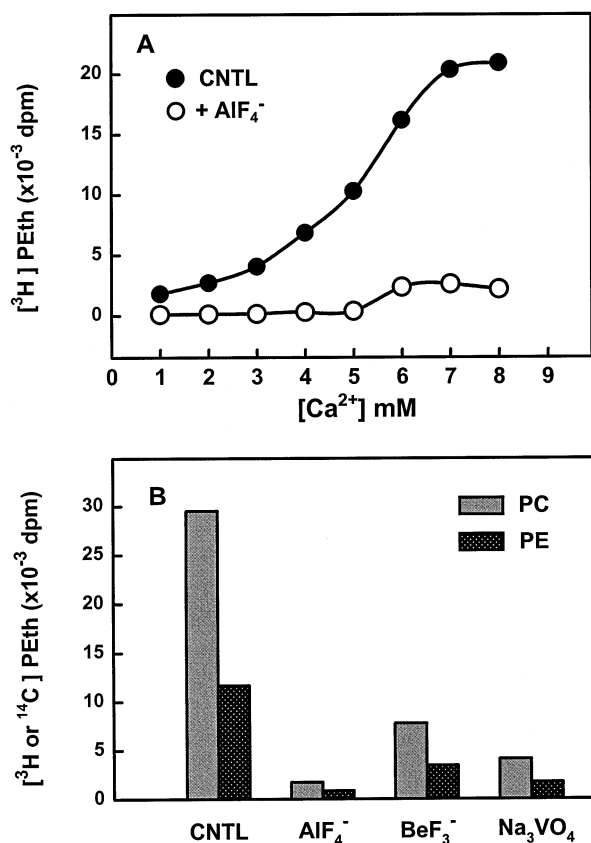


Fig. 4. Inhibition of PIP₂-independent cabbage PLD activity by AlF₄⁻ and other phosphate analogs. A: PLD activity (20 min) over a range of 1–8 mM Ca²⁺ concentrations. Cabbage PLD (2.5 µg/sample) was pre-incubated with or without AlF₄⁻ (3 mM NaF+20 µM AlCl₃) for 5 min. B: PLD activity was assayed with PC or PE substrates. The enzyme was pre-incubated for 5 min with or without AlF₄⁻ (3 mM NaF+20 µM AlCl₃), BeF₃⁻ (3 mM NaF+20 µM BeCl₂) or Na₃VO₄ (200 µM). Values are means from a representative experiment with variations less than 10%, *n* = 3.

4. Discussion

The present study sought to determine whether the inhibition of mammalian PLD by AlF₄⁻ [4] was consistent for plant PLD and, if so, to examine possible mechanisms of inhibition. The inhibitory effect of AlF₄⁻ was observed on both PIP₂-dependent and PIP₂-independent forms of cabbage PLD, acting on PC substrate, and on a PIP₂-independent form of the enzyme, acting on PE substrate. Therefore, disruption of polyphosphoinositide-binding to PLD, a potential regulatory mechanism [12], may be discounted as the common AlF₄⁻ mode of action.

The possibility that AlF₄⁻ may act through a calcium-related mechanism to inhibit PLD was considered. Plant PLDs contain a Ca²⁺/phospholipid-binding C₂ domain [28] and enzyme activation requires calcium. Cabbage PLD was sensitive to µM levels of Ca²⁺ in the PIP₂-dependent assay and to mM Ca²⁺ levels in the PIP₂-independent assay. AlF₄⁻ inhibited PLD in both assay systems. However, it also inhibits mammalian PLD, which lacks the calcium-binding C₂ domain, as well as mammalian oleate-sensitive PLD, assayed in the absence of free Ca²⁺ [4]. It is probable, therefore, that AlF₄⁻ inhibits PLD by a mechanism that does not affect Ca²⁺-binding to the enzyme. Also compatible with our pre-

vious observations on salivary gland PLD [4], a mechanism that involves the well-documented property of AlF₄⁻ to activate heterotrimeric GTP-binding proteins [29] is ruled out, since plant PLDs are insensitive to guanine nucleotide stimulation [9].

Many of the effects of AlF₄⁻, including the activation of G proteins, are exerted through its capacity to mimic a phosphate group [29] and AlF₄⁻ has been reported to affect enzymes with phosphotransfer activities (GTPase, ATPase, phosphatase) [22]. AlF₄⁻ may therefore inhibit PLD by acting as a phosphate analog. Supportive evidence for this is provided by our observation that two additional phosphate analogs, beryllium fluoride and orthovanadate, also inhibited both PIP₂-sensitive and -insensitive forms of PLD in concentration-dependent responses. Inorganic phosphate itself also inhibited PLD in the PIP₂-dependent assay (this could not be tested in the PIP₂-independent assay, where mM Ca²⁺ is precipitated by inorganic phosphate). The differences among the concentrations of phosphate (mM) and the other three analogs (µM) required to inhibit PLD (Fig. 3) may simply reflect the relative binding affinities of these compounds to the enzyme [27]. The three phosphate analogs also inhibited cabbage PLD conversion of PE substrate, as well as PLD in extracts of rat salivary glands [4], suggesting a universal, phosphate-mimicking mechanism of inhibition of different forms of PLD.

The PLD superfamily is characterized by a pair of conserved HKD domains in the active site and its members are thus expected to share a common catalytic mechanism [13,16]. A stereochemical study on cabbage PLD indicated the formation of a phosphatidyl-enzyme intermediate [17]. Recent studies on two family members, the bacterial endonuclease, Nuc, and a murine toxin, Ymt, demonstrated that catalysis proceeds via a two-step mechanism involving a phosphohistidine-linked phosphoenzyme intermediate [18,19]. A similar mechanism in cabbage PLD is suggested by the observation that the enzyme was inhibited by two histidine-modifying reagents, diethylpyrocarbonate and *p*-bromophenacyl bromide [30]. The only phosphate group in phospholipid PLD sub-

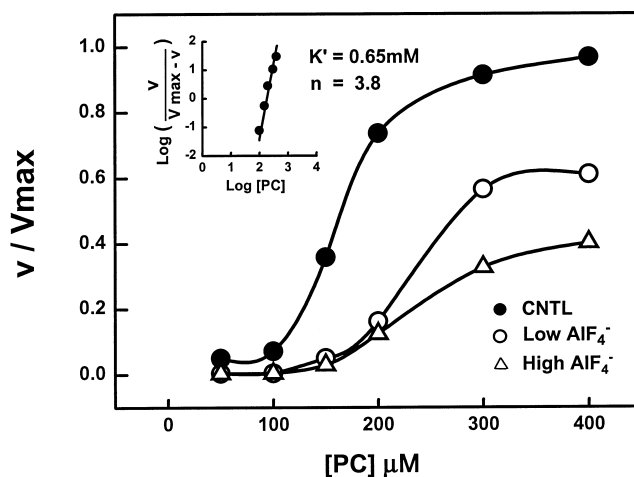


Fig. 5. Effect of AlF₄⁻ on the kinetics of cabbage PLD (PIP₂-independent assay). Two concentrations of AlF₄⁻ (1 mM NaF+5 µM AlCl₃ or 3 mM NaF+20 µM AlCl₃) were pre-incubated with the enzyme for 5 min. The assay was conducted for 5 min and PA was measured to reflect PLD activity. Inset: the Hill plot from the logarithmic form of the Hill equation.

strates is that linking the phosphatidyl group to the polar head group (e.g. choline, ethanolamine). It is therefore likely that enzyme inhibition by AlF_4^- or the other phosphate analogs examined is caused by their binding to the phosphate-binding site of the catalytic domain of PLD (possibly to a histidine residue) to prevent the formation of a phosphatidyl-enzyme intermediate. This model is supported by the competitive mode of inhibition of PLD by AlF_4^- observed in the enzyme kinetics experiments in the present study. It is also consistent with the recent observation that another phosphate analog, tungstate (WO_4^{2-}), which is a competitive inhibitor of the PLD superfamily member Nuc [18], binds Nuc at one histidine and one lysine residue on each of the two conserved HKD motifs on the catalytic site [31].

PLD contains two copies of the substrate-binding HKD motif, both of which are required for enzyme activity, which associate to form a single active site [33]. Functional dimerization of PLD has thus been suggested through a 'head to tail' interaction of two molecules to produce an enzyme with two active sites and four HKD motifs [32]. Supporting this idea, structural studies on the PLD superfamily members Nuc and Ymt found that both exist as dimers [19,31]. Dittrich et al. [26] demonstrated that cabbage PLD followed Hill kinetics, which are characteristic of cooperative allosteric enzymes with multiple substrate-binding sites [27]. The present study on cabbage PLD confirms the Hill model, which has been proposed to describe the kinetic behavior of lipases in general [33]. The calculated Hill coefficient ($n_{\text{app}} = 3.8$) indicates positive cooperativity among multiple binding sites, suggesting functional oligomerization of the plant enzyme. This would be consistent with the dimerization of the PLD superfamily members, Nuc and Ymt, discussed above. Oligomerization of cabbage PLD, and of PLD superfamily enzymes in general, may therefore be a requirement for activation.

In summary, we propose that AlF_4^- acts as a competitive inhibitor of cabbage PLD by occupying a phosphate-binding locus on the active site(s) to prevent the formation of a phosphatidyl-enzyme intermediate. Taken together with our previous observations on the inhibition of different forms of PLD by AlF_4^- in salivary glands [4], these findings suggest that this may be a universal mechanism of inhibition of PLDs of animal and plant origin. Aluminum- and beryllium fluoride, as well as metal oxoanion analogs of phosphate (vanadate, molybdate, tungstate), are known as potent inhibitors of phosphomonoesterases, e.g. PTPases [34,35]. The present study indicates for the first time that they also inhibit phosphodiesterases, such as PLD, and should thus prove to be useful probes in studying the enzymology of the PLD superfamily.

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