

Update on Signal Transduction

The Role of Phospholipase D in Signaling Cascades¹

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Phospholipases hydrolyze phospholipids, which are the backbones of biological membranes. The activities of these enzymes not only have a profound impact on the structure and stability of cellular membranes but also play a pivotal role in regulating many critical cellular functions. The activation of phospholipases is involved in many cell-signaling cascades. These enzymes often execute their regulatory functions through the generation of second messengers that transduce biotic and abiotic cues into physiological responses.

Three classes of phospholipases, PLD, PLC, and PLA₂ (Fig. 1), have been studied extensively for their roles in generating lipid and lipid-derived messengers. PLC and PLA₂ are two well-documented signaling enzymes in animal systems. PLC produces the second messengers DAG and inositol phosphate, and PLA₂ catalyzes the rate-limiting step in eicosanoid synthesis and regulation. In the last several years, PLD has been identified as an important signaling enzyme that produces both PA and a free-head group such as choline (Fig. 1). This activity has been proposed to play a role in mediating a wide range of cellular processes, including hormone action, membrane trafficking, cell proliferation, cytoskeletal organization, defense responses, differentiation, and reproduction (Rose et al., 1995; Cockcroft, 1997; Colley et al., 1997; Exton, 1997; Fan et al., 1997; Ritchie and Gilroy, 1998).

Significant progress was made recently toward understanding the structure, regulation, and function of PLD. Plant PLD is an "old" enzyme receiving renewed attention, mainly because of its potential role in transmembrane signaling. It was discovered in plants about one-half century ago, and some distinct and perplexing properties of its activity were soon noted (for review, see Heller, 1978; Wang, 1997). This conventional PLD is widespread in plant tissues and has been purified from several species; however, its regulation and physiological function remained an enigma. The molecular cloning of the first eukaryotic PLD from plants helped to propel the investigation to the molecular realm (Wang et al., 1994; Hammond et al., 1995; Rose et al., 1995).

The identification, cloning, and expression of novel types of plant PLDs established that they are a family of heterogeneous enzymes that differ in catalytic and regulatory

properties (Pappan et al., 1997a, 1997b, 1998; Qin et al., 1997). In addition, the regulated activation of PLD was recently documented in several plant systems, including wounding, hormone action, and plant-pathogen interactions (Ryu and Wang, 1996; Fan et al., 1997; Lee et al., 1997; Ritchie and Gilroy, 1998). The genetic manipulation of PLD in the cell was achieved in plants, mammals, and yeast, and this has provided new insights into the involvement of PLD in cellular functions (Rose et al., 1995; Colley et al., 1997; Fan et al., 1997). With these developments, the role of PLD in signaling cascades has become a topic that attracts increasing attention in various systems (for review, see Wang, 1997; Chapman, 1998; Munnik et al., 1998).

THE PLD MULTIPLE GENE FAMILY

Eukaryotic intracellular PLD, which was first cloned from castor bean (Wang et al., 1994), is a highly conserved gene family. The conservation of several regions of the PLD amino acid sequences led to the identification and cloning of PLDs from yeast and animals (Hammond et al., 1995; Rose et al., 1995). All cloned PLDs contain two HxKxxxxD motifs, which are separated by approximately 320 amino acids in plant PLDs. The conserved His, Lys, and Asp residues form a catalytic triad responsible for catalysis. The HxKxxxxD motif was also observed in two phospholipid-synthesizing enzymes, bacterial PS synthase and cardiolipin synthase, in endonucleases, and in other proteins of unknown function in viruses and bacteria. The characteristics of the HxKxxxxD motif are used to define the PLD superfamily (Sung et al., 1997).

Plant PLD is encoded by a multiple heterologous gene family. Four PLD cDNAs, designated PLD α , PLD β , PLD γ , and PLD γ 2, were isolated from Arabidopsis (Qin et al., 1997). The Arabidopsis genome project also yielded two PLD genes, for which cDNAs are not yet isolated. Multiple PLDs were cloned in rice and cabbage (Morioka et al., 1997; Pannenberg et al., 1998). Database searches in October 1998 found 15 complete PLD cDNA/gene sequences isolated from eight plant species (castor bean, Arabidopsis, cowpea,

Abbreviations: DAG, diacylglycerol; NAPE, N-acyl PE; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidyl 4-phosphate; PIP₂, PI 4,5-bisphosphate; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; PPI, polyphosphoinositide; PS, phosphatidylserine.

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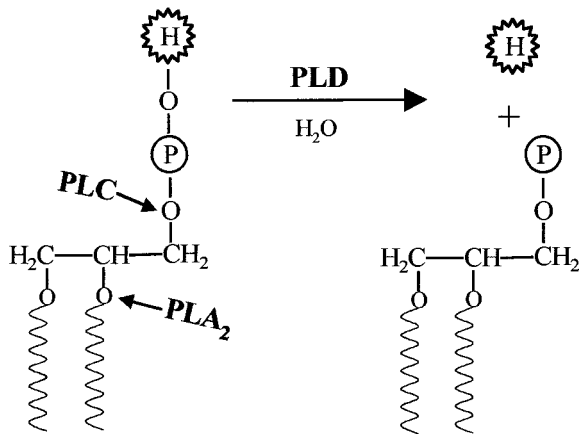


Figure 1. Sites of cleavage of phospholipids by PLD, PLC, and PLA₂, and the products of PLD, PA, and head group (H).

cabbage, tobacco, *Pimpinella brachycarpa*, rice, and maize). Alignments of these PLD sequences revealed several distinct clusters. Cluster I included Arabidopsis PLD α and all of the cDNA cloned to date from other plant species whose sequence identity was 75% to 90%. Therefore, PLDs of cluster I are grouped as PLD α , and this classification takes into account sequence similarity, catalytic properties (described in a later section), and gene structure.

Cluster II consists of Arabidopsis PLD β and the two PLD isolates on chromosomes II and IV (tentatively named PLD β 2 and PLD β 3), which share approximately 75% amino acid sequence identity. Cluster III has two members, Arabidopsis PLD γ 1 and PLD γ 2, which share more than 85% sequence identity. The overall sequence identity shows that PLD β and PLD γ are more similar to each other than either is to PLD α . Multiple PLD genes also occur in other systems. In mammalian cells, two distinct PLDs were cloned, PLD1 and PLD2. PLD1 has two alternative splicing variants, PLD1a and PLD1b (Hammond et al., 1995; Colley et al., 1997). Two PLDs were reported in yeast, but only the sequence of PLD1 was identified (Rose et al., 1995; Waksman et al., 1997).

The overall domain structures of plant PLDs are similar, but important differences occur in some of the motifs (Fig. 2). A C2 domain is present in all cloned plant PLDs, but not in animal or yeast PLDs. C2 is a Ca²⁺/phospholipid-binding fold, and Ca²⁺ binding is coordinated by four to five amino acid residues provided by bipartite loops (Ponting and Parker, 1996). PLD γ and PLD β conserve all of the Ca²⁺-coordinating acidic amino acids (Qin et al., 1997), whereas two of the acidic residues in the C2 domain of PLD α are substituted by either positively charged or neutral amino acid residues, indicating a possible change of affinity for Ca²⁺ in PLD α . A PPI-binding motif (RxxxxKxRR) and an inverted sequence (RKxRxxxxR) are present in PLD β near the catalytic domain of the C terminus (Qin et al., 1997). Three of these four basic consensus residues are conserved in PLD γ , whereas PLD α shows the least conservation of residues (some are replaced by acidic residues). PLD γ possesses a myristoylation consensus sequence that is not present in PLD α or PLD β (Qin et al., 1997).

DISTINCT CATALYTIC PROPERTIES OF DIFFERENT PLDS

Molecular analyses have documented not only the occurrence of multiple PLDs but also the structural variations that may underlie distinct biochemical properties. PLD activities from plants can be divided into three groups based on their differing requirements for Ca²⁺ in vitro. The first group is the conventional plant PLD that displays a striking Ca²⁺ requirement; it is most active at millimolar concentrations of Ca²⁺, with the optimal concentration ranging from 20 to 100 mM (Heller, 1978). PLD α expressed from the castor bean PLD α cDNA exhibits the characteristic activity of conventional PLD purified from plants (Dyer et al., 1994; Wang et al., 1994; Pappan et al., 1998). Antisense suppression of PLD α in Arabidopsis led to the loss of this conventional PLD activity (Pappan et al., 1997a), so the PLD α gene product must have been responsible for it. Additionally, three isoforms and two cDNAs of the conventional PLD were also identified in some plant species (Dyer et al., 1994; Young et al., 1996; Pannenberg et al., 1998).

The second group of PLDs includes those that are the most active at micromolar levels of Ca²⁺. The presence of such PLD activity was documented in transgenic Arabidopsis, in which the expression of PLD α was suppressed by an antisense gene (Pappan et al., 1997a). The cloning and analysis of PLD β from Arabidopsis provided unequivocal, molecular evidence for the new type of PLD (Pappan et al., 1997b). The PLD γ that was cloned later also exhibited a Ca²⁺ dependence similar to that of PLD β (Qin et al., 1997). These PLDs are PPI dependent and are stimulated by PIP₂ and to a lesser extent by PIP, but not by other acidic phospholipids such as PI, PS, PG, and PA.

Although the above PLDs require Ca²⁺ for activity, a third type that is independent of cations was reported in *Catharanthus roseus* suspension cells (Wissing et al., 1996). Another unique property of this PLD is its lack of transphosphatidylase activity: Two membrane-associated and two soluble variants of this activity have been noted. However, to our knowledge, it has not been purified to homogeneity, and no PLD cloned thus far exhibits such activity.

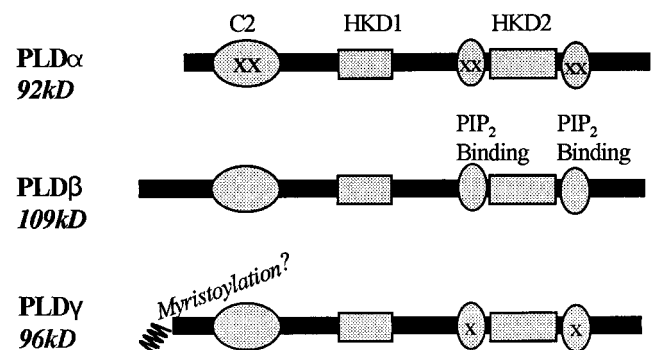


Figure 2. Domain structures of PLD α , PLD β , and PLD γ in Arabidopsis. XX in the PLD α C2 marks the loss of two acidic residues potentially involved in Ca²⁺ binding; XX in the PPI-binding motifs marks the loss of the number of basic residues potentially required for PPI binding.

This third type of PLD also differs in substrate specificity and preferences. Conventional PLD uses more than one phospholipid as a substrate. In general, PC, PE, and PG are good substrates, whereas PI, PS, cardiolipin, and plasmalogens are much less efficiently used, if at all (Heller, 1978; Dyer et al., 1994; Abousalham et al., 1997). PLD α , PLD β , and PLD γ all use PC, PE, and PG as substrates, but the reaction conditions required for PLD β and PLD γ are strikingly different from those for PLD α (Pappan et al., 1998). PLD β and PLD γ , but not PLD α , can use PS and NAPE as substrates. Although PLD β and PLD γ hydrolyze the same substrates, PLD γ prefers ethanolamine-containing PE and NAPE to other lipids, but PLD β does not. The Ca²⁺-independent PLD from *C. roseus* exhibits a unique substrate specificity (Wissing et al., 1996). It is PI specific, which is in contrast to cloned PLD α , PLD β , and PLD γ , which do not hydrolyze PI. These varied substrate specificities and preferences suggest that the activation of different PLDs may result in selective hydrolysis of membrane phospholipids.

REGULATION AND ACTIVATION OF PLD

Their distinct structural and biochemical properties suggest that PLD isoenzymes are subject to unique controls and activation mechanisms. The different Ca²⁺ requirements could mean that changes in the levels of cytoplasmic Ca²⁺ activate PLD isoenzymes differentially. However, the fact that the conventional PLD (PLD α) requires millimolar levels of Ca²⁺ in vitro casts doubt upon the significance of Ca²⁺ in controlling its activity in vivo. It is important to note that the optimal Ca²⁺ concentration was determined by using a single class of lipid substrate often in the presence of organic solvents or detergents such as SDS, which are artificial conditions. A recent study showed that PLD α was active at nearly physiological Ca²⁺ concentrations when it was assayed at an acidic pH (4.5–5.0) and in the presence of mixed lipid vesicles containing PIP or PIP₂ (K. Pappan and X. Wang, unpublished data). This suggests that even though the effect of Ca²⁺ on PLD α is complex, its activity can be increased by elevating cellular Ca²⁺ levels. On the other hand, PLD β and PLD γ were inactive at that pH and were most active at a neutral pH. These distinct pH optima may mean that changes in cellular pH have a different effect on PLD isoforms. At near-physiological concentrations of Ca²⁺, PLD β and PLD γ are neutral phospholipases, whereas PLD α is an acidic phospholipase that may be activated by cellular acidification.

The presence of a C2 domain in plant PLDs points to a specific mode of activation by Ca²⁺. C2 domains were identified in a number of signal transduction and membrane trafficking proteins, such as PKC, PLC, and PLA₂ (Ponting and Parker, 1996). This domain is important in the Ca²⁺-regulated translocation of proteins to membranes. Indeed, in the wound activation of PLD in castor bean, the Ca²⁺-mediated translocation of PLD from the cytosol to the membranes had already been proposed before the presence of a C2 domain on PLDs was recognized (Ryu and Wang, 1996). There is also data suggesting Ca²⁺-mediated activation of PLD in vivo (Munnik et al., 1998).

Another potential regulator of plant PLD is PPI. Not only do PLD β and PLD γ require PPIs for activity, PLD α activity is also stimulated by PPIs when low levels of Ca²⁺ are present (Qin et al., 1997). Binding assays have shown that PLD β , PLD γ , and PLD α are able to bind PIP₂. Two PLD regions may be involved in PIP₂ binding: one is the near N-terminal C2 domain and the other is the near C-terminal PPI-binding motifs that are missing in PLD α . PPIs are minor lipids and their levels are regulated dynamically. The activation of PLD is likely to be interconnected with the metabolism and signaling of PPIs.

It was also suggested that plant PLD is regulated by trimeric G-proteins based on its stimulation by mastoparan, cholera toxin, and alcohol (Munnik et al., 1995; Chapman, 1998). By comparison, the activation by small G-proteins such as ARF (ADP-Ribosylation Factor) and Rho is the best-characterized mechanism of regulation for mammalian PLD. ARF, Rho, and PKC synergistically activate PLD1 but not PLD2. These proteins may promote PLD1 activity via direct protein-to-protein interactions. Animal PLD is also stimulated by other factors, including Ca²⁺ flux, PKC, receptor-linked Tyr kinases, PIP₂, and gelsolin, and it is down-regulated by fodrin, clathrin assembly protein 3, synaptojanin, ceramide, and some lysophospholipids (Fig. 3; Cockcroft, 1997; Exton, 1997). LysoPE was also suggested to be a negative regulator of plant PLD (Ryu et al., 1997). The formation of DAG-PPI from PA is thought to attenuate plant PLD activation (Munnik et al., 1998). In addition, PLD gene expression and the differential appearance of PLD isoforms are involved in the long-term regulation of PLD (Dyer et al., 1994; Young et al., 1996; Fan et al., 1997).

The activation of PLD in animal systems was first identified just over 10 years ago, and it is now documented in more than 30 cell types stimulated by receptor-directed agonists and by other stimuli such as Ca²⁺ ionophores and phorbol esters (Cockcroft, 1997; Exton, 1997). Although, historically, the activation of PLD was observed first in plants, studies of PLD activation in plants now lag behind

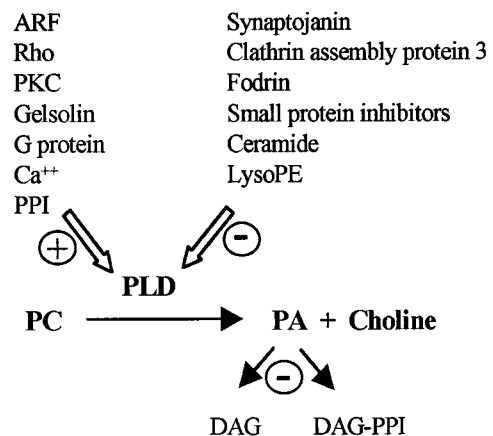


Figure 3. Schematic diagram of up- and down-regulation of PLD in plants and animals, showing that PLD signaling can be regulated by modulating PLD activity or by removing PA. The proteinaceous stimulators and inhibitors identified are mainly from animal systems.

those in animals. It has long been known that wounds and other stresses stimulate a rapid increase in PA and other lipid metabolites. These increases were regarded initially as autolysis resulting from the release of PLD and other lipolytic enzymes during cell damage. Recent studies have shown that wounding a tissue triggers a rapid activation of PLD-mediated phospholipid hydrolysis not only at the wound site but also at undamaged areas (Ryu and Wang, 1996). Stimulation of plant PLD has also been shown in response to treatments with ABA, light, fungal elicitors, and bacterial pathogens (Young et al., 1996; Fan et al., 1997; Chapman, 1998; Munnik et al., 1998; Ritchie and Gilroy, 1998).

DOWNSTREAM TARGETS OF PLD-DERIVED MESSENGERS

Identification of the downstream events of PLD activation is important to our understanding of PLD function. PA stimulation of signaling proteins is the most-studied mechanism of action in animals. One group of such proteins is protein kinases, including Ca^{2+} -dependent and independent kinases such as PKC, mitogen-activated protein kinases, and Raf-kinases (Cockcroft, 1997; Exton, 1997). PA can bind to Raf-kinase, but it is unclear how this binding may activate the enzyme. Recent reports indicate the presence of a PA-specific protein kinase that mediates the activation of NADPH oxidase (Waite et al., 1997). Other enzymes activated by PA are PIP-5 kinase, PLC, and PLA_2 , which are involved in lipid-signaling cascades. In addition to performing as a direct messenger, PA can be metabolized further to other lipid mediators (DAG, lysoPA, and free fatty acids; Fig. 4). The head group released by PLD can also have regulatory functions. The formation of

N-acylethanolamine by PLD was implicated in the responses of plants to fungal elicitation (Chapman, 1998).

Some of the cellular roles of PA may result from its effect on membrane properties and configuration rather than from its direct effect on proteins. PA is a nonbilayer lipid favoring hexagonal phase formation, particularly in the presence of Ca^{2+} (Cornell and Arnold, 1996). Thus, a rapid increase in the local concentration of PA may destabilize membranes. The activities of a number of signaling proteins, including G-proteins, PKC, PLC, PLA, PA phosphatase, DAG kinase, and PLDs, are sensitive to changes in membrane conformation (Cornell and Arnold, 1996; Pappan et al., 1998). An increase in PA also increases the net negative charge of membranes, which may alter protein-to-membrane interactions and the flux of ions such as Ca^{2+} . In addition, PA-mediated changes in membrane properties may be produced by altering membrane lipid composition, because PA is a central precursor in glycerolipid biosynthesis (Fig. 4).

INVOLVEMENT OF PLD IN SIGNALING PATHWAYS

It has been suggested that PLD plays a role in a broad range of cellular responses, but the requirement of PLD for a particular cellular function was not documented conclusively until recently. The molecular cloning of plant PLD helped to identify the first definitive requirement of PLD in a physiological process. It was noted that the sequence of the yeast sporulation-defective mutant SPO14 contains several regions of sequence similarity to the then newly cloned castor bean PLD, and this gene was later found to encode PLD1 (Rose et al., 1995). Both PLD1 activity and its presence in the nucleus are necessary for signaling the completion of meiosis (Sung et al., 1997). Whether plant PLDs are involved in a similar process is not known. Antisense suppression of plant PLD resulted in a loss of more than 90% of the $\text{PLD}\alpha$ in *Arabidopsis* flowers. But the fertility of $\text{PLD}\alpha$ -suppressed plants was not affected, indicating that a high level of $\text{PLD}\alpha$ is not essential for reproduction (Fan et al., 1997).

Recent studies provide strong evidence of a role for $\text{PLD}\alpha$ in ABA action. The expression of $\text{PLD}\alpha$ is up-regulated by ABA, as indicated by the increased levels of $\text{PLD}\alpha$ promoter activity, mRNA, protein, and membrane-associated activity in response to ABA treatments (Fan et al., 1997; Wang, 1997; Xu et al., 1997). Senescence of the leaves detached from the $\text{PLD}\alpha$ -deficient transgenic plants was retarded when they were incubated with ABA (Fan et al., 1997). These data indicate that $\text{PLD}\alpha$ is a mediator in ABA actions; the loss of $\text{PLD}\alpha$ activity in transgenic plants renders *Arabidopsis* less sensitive to ABA. A role for PLD/PA in ABA signaling was also indicated in an independent study that used a different system (Ritchie and Gilroy, 1998). ABA increased PLD activity after it was applied to barley aleurone protoplasts. Direct application of PA to aleurone protoplasts suppressed the production of α -amylase and increased the synthesis of an amylase inhibitor in a manner that mimicked the ABA antagonism of GA-induced events in barley aleurone. The fact that an ABA-mediated physiological process is changed by the

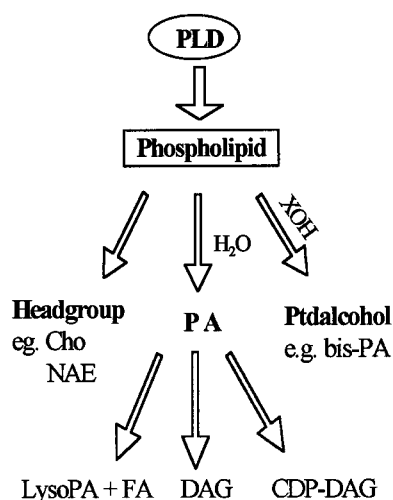


Figure 4. Direct and derived products of PLD activation. LysoPA and free fatty acid (FA) can be formed from PA by nonspecific acyl hydrolase or by PLA_2 . PA is dephosphorylated to DAG by PA phosphatase. CDP-DAG is the precursor for the synthesis of PS, PI, and PG. XOH, Primary alcohol used for transphosphatidylation; Ptd, phosphatidyl; NAE, *N*-acylethanolamine.

genetic and pharmacologic alteration of PLD activity suggests that PLD constitutes an early step in mediating ABA action.

PLD has also been implicated in the action and production of ethylene. Antisense suppression of PLD α decreases the rate of ethylene-promoted senescence in detached Arabidopsis leaves (Fan et al., 1997). In cultured carrot cells, PLD activation is thought to constitute a signaling step in the perception of an ethylene burst that occurs at the early stage of Glc starvation (Lee et al., 1998). LysoPE is proposed to retard senescence by blocking PLD α activity, which may be involved in promoting the burst of ethylene (Ryu et al., 1997).

The involvement of PLD in injury-induced lipid hydrolysis is perhaps the earliest result connecting PLD to a cellular process. PLD can be activated rapidly by stress injuries such as mechanical wounding, frost, and γ -irradiation (Voisine et al., 1993; Ryu and Wang, 1996). Apparently, wound activation of PLD results from its translocation to membranes, which is mediated by an increase in cytoplasmic Ca²⁺ upon wounding (Ryu and Wang, 1998). PLD activation is proposed to be an early event in the response of the plant to stress injuries, and the PLD-generated PA may serve as an effector or as a substrate for the production of other mediators such as DAG, polyunsaturated fatty acids, and oxylipins in defense signaling (Ryu and Wang, 1996, 1998).

The role of PLD in defense signaling extends to plant-pathogen interactions. In rice leaves challenged with the bacterial pathogen *Xanthomonas oryzae* pv *oryzae*, PLD α clustered at the region of the plasma membranes that came into contact with bacteria during hypersensitive interactions but not in the susceptible interactions (Young et al., 1996). In tobacco cells treated with the fungal elicitor xylanase, a rapid release of *N*-acyl ethanolamine was noted (Chapman, 1998), which probably resulted from hydrolysis by PLD γ or PLD β but not by PLD α , because the former two hydrolyze NAPE, and PLD γ prefers NAPE or PE over other phospholipids (Pappan et al., 1998).

One potential mechanism by which PLD participates in plant-defense responses is the regulation of NADPH oxidase, which is involved in reactive oxygen production. In neutrophils, the activation of PLD is known to mediate an oxidative burst, and PA is a potent activator of NADPH oxidase (Waite et al., 1997). NADPH oxidase is a complex composed of membrane-bound and cytosolic proteins. It becomes active when its cytosolic subunits translocate to the membrane, and the translocation of p47-*phox* is prompted by phosphorylation. Recent studies show that p47-*phox* is a substrate for the newly identified PA-activated protein kinase in animals (Waite et al., 1997). Plant NADPH oxidase and neutrophil NADPH oxidase seem to have the same subunit components. In addition, phosphorylation and translocation of plant p47-*phox* and p67-*phox* also occur in tomato cells treated with race-specific fungal elicitors (Xing et al., 1997). However, whether PLD and PA play a role in regulating plant NADPH oxidase activity is unclear. One study using soybean suspension-cultured cells failed to obtain evidence for

the involvement of PLD in the pathogen-elicited production of hydrogen peroxide (Taylor and Low, 1997).

FUNCTIONAL HETEROGENEITY AND CROSSTALK OF LIPID SIGNALING PATHWAYS

The occurrence of multiple PLDs with distinct regulatory and catalytic properties in the same organism suggests that each may have unique functions. Some evidence for distinct functions was obtained from the genetic manipulation of PLD in plant, animal, and yeast systems. The transfer of a PLD α antisense cDNA into Arabidopsis resulted in the loss of more than 95% of PLD α activity, but PLD β and PLD γ activities in the PLD α -deficient leaves were not reduced significantly (Pappan et al., 1997a). The PLD α antisense leaves displayed a marked retardation in ABA- or ethylene-promoted senescence, indicating that the loss of PLD α was not compensated for by PLD β and PLD γ (Fan et al., 1997). The yeast *SPO14* mutant was found to contain another PLD activity, designated PLD2, and thus disruption of the PLD1 function was not compensated for by the PLD2 gene (Waksman et al., 1997). Overexpression of mammalian PLD2 resulted in cytoskeletal reorganization, whereas an increase in PLD1 expression did not alter cell morphology (Colley et al., 1997).

It is now evident that more than one phospholipase is often involved in mediating a specific cellular response. PLD is thought to function as an integral part of a network involving other lipid-signaling enzymes such as PLA₂ and PLC (Fig. 5). Mitogenic signaling in animal cells, for example, involves both PIP₂-PLC and PC-PLD, and the activation of PLC results in the initial rise of DAG, whereas PLD coupled with PA phosphatase provides the sustained supply of DAG required for cell proliferation (Exton, 1997). On the other hand, PA is a stimulator of PLC, PLA₂, and PKC.

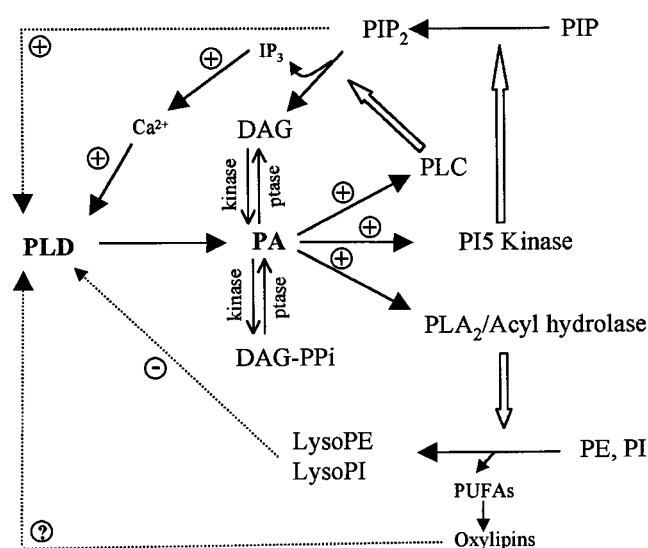


Figure 5. A working model depicting the networking of PLD activation with other lipid mediators and signaling enzymes. Plus signs indicate stimulation, and minus signs denote inhibition. IP₃, Inositol 1,4,5-trisphosphate; ptase, phosphatase; PUFAs, polyunsaturated fatty acids.

The network of PLD, PLC, and PLA₂ generates several potent lipid mediators, such as PA, lysophospholipids, DAG, and free polyunsaturated fatty acids. Stimulus-induced increases in these lipid metabolites have also been found in some plant systems. Moreover, the formation of PA was shown to precede that of DAG, lysophospholipids, and free fatty acids, suggesting a possible PLD-led activation of acyl hydrolases, PLC, and/or PA phosphatase (Voisine et al., 1993; Ryu and Wang, 1996, 1998; Lee et al., 1997). In addition, PA is a stimulator of PIP-5 kinase, which is responsible for the synthesis of PIP₂ (Fig. 5). On the other hand, PIP₂ is an activator of plant PLD β , PLD γ , and some PLDs from animals and yeast (Cockcroft, 1997; Qin et al., 1997). It has been proposed that activation of PLD and PIP-5 kinase in mammalian cells forms a positive feedback loop that leads to rapid generation of PA and PIP₂, which are involved in vesicular trafficking. In addition, crosstalk can occur within the PLD family, and the activation of one PLD may stimulate or attenuate the function of another.

OUTSTANDING QUESTIONS AND PROSPECTS

Recent advances in the investigation of PLDs in plants, animals, and yeast point to an important role for PLD in the mediation of cellular processes; however, many questions remain and a comprehensive understanding of PLD function is yet to be achieved. One major question addresses the molecular and cellular mechanisms by which PLD mediates the cellular functions. An answer requires identification of the cellular targets of PLD activation and the molecules that interact with PLD. Very little, if anything, is known about the downstream reactions or processes (e.g. kinases, phosphatases, ion channels, adapter proteins, and other targets) of PA, PA-derived mediators, and head groups in plant signaling. The paucity of information of the cellular effects of lipid messengers is the major impediment in lipid-signaling research in plants.

The finding of multiple PLD proteins indicates that the cellular regulation and the functioning of PLDs are complex. The limited biochemical and genetic data have suggested that the different PLDs may have unique functions. Defining the biochemical properties of each PLD is important to the understanding of its catalysis and regulation in the cell. *Arabidopsis* contains (potentially) six active PLDs; only three of them, PLD α , PLD β , and PLD γ , have been analyzed. Important insights into the cellular function of different PLDs can be obtained by determining the spatial and temporal expression and intracellular and cell/tissue localization. However, such information is presently available only for PLD α .

Although this article is concerned primarily with the role of PLD in signaling cascades, it is important to note that PLD can participate in other cell functions, such as membrane degradation and remodeling. The early studies of plant PLD functions dealt only with phospholipid breakdown during senescence, aging, and stress injuries; it was suggested that increases of PLD initiated a phospholipid degradation pathway (Voisine et al., 1993; Fan et al., 1997, and refs. therein). This catabolic role could be carried out by different PLD proteins, or the same PLD could exert

both degradation and signaling functions, depending on the severity of the stress.

With the availability of molecular information for the various PLDs, PLD isoenzyme-specific antibodies and DNA/RNA probes should be forthcoming and will be instrumental in addressing some of the above questions. In addition, the function of different PLDs can be studied effectively by generating and characterizing PLD antisense and knockout transgenic plants. Because of possible genetic redundancy, particularly for PLD β and PLD γ , producing double or triple mutants may be necessary for an unambiguous determination of the role of PLD in cellular metabolism. With our present knowledge of the molecular biology and biochemistry of this class of enzyme, we are poised for major advancements in the long-sought understanding of the physiological functions of PLDs and the membrane-lipid involvement in plant-signaling cascades.

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