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REVIEW

Molecular Structure of Phospholipase D and Regulatory Mechanisms of Its Activity in Plant and Animal Cells

{running title} PLD STRUCTURE AND ACTIVITY IN PLANT AND ANIMAL CELLS

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Abbreviations: Gα, α-subunit of heterotrimeric G-protein; PKC, protein kinase C; PLD, phospholipase D; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate.
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Abstract—Phospholipase D (PLD) catalyzes hydrolysis of phospholipids with production of phosphatidic acids, which often act as secondary messengers on transmission of intracellular signals. This review summarizes data of various leading laboratories on specific features of organization and regulation of PLD activity in plant and animal cells. The main structural domains of PLD (C2, PX, PH), the active site, and other functionally important parts of the enzyme are considered. Regulatory mechanisms of PLD activity are characterized in detail. Studies associated with molecular design, analysis, and synthesis of new nontoxic substances capable of inhibiting different PLD isoenzymes *in vivo* are shown to be promising for biotechnology and medicine.

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Key words: phospholipase D, domains, calcium, lipids, G-proteins, protein kinases, protein–protein interactions

Phospholipase D (EC 3.1.4.4) (PLD) is an enzyme widely distributed in plants [1, 2] and animals [3, 4]; it catalyzes hydrolysis of the phosphodiester bond in structural phospholipids (phosphatidylcholine, phosphatidyl ethanolamine, etc.) with production of phosphatidic acid and low molecular weight water-soluble compounds of choline or ethanolamine type [5]. Phosphatidic acid is considered to be not only a necessary structural element of membranes but also an important secondary messenger of hormone and stress signaling cascades [6, 7] and as a regulator of membrane transport [8]. Phosphatidic acid can also be generated by diacylglycerol kinases [9].

PLD was found and cloned in plants, and the model plant *Arabidopsis thaliana* has been shown to have 12 PLD genes [10, 11]. PLD is very sensitive to external and internal factors, and its multiformity is favorable for fine coordination of enzyme activity and features of cell metabolism. PLD is actively studied on different organizational levels in both plants and animals.

This review analyzes the data on PLD of plants and animals and also gives the authors' concept about the involvement of PLD in transmission of signals of such important phytohormones as cytokinins. In particular, known regulatory mechanisms of PLD activity are analyzed, and comparative characteristics of these enzymes in different cells are presented. A wide spectrum of factors known to play key roles in PLD regulation is considered, the organization of the enzyme is analyzed, its role in the regulation of cell metabolism is determined, and lines for studies on lipid signaling systems are described.

Studies in biochemistry and molecular genetics are favorable for determination of the role of PLD in plant and animal cells [12]. There are data on the involvement of PLD in malignancies [13], angiogenesis [14], leukocyte adhesion, and chemotaxis [15]; the functions of the enzyme are considered in platelets [16], nerve cells [17], myocardium [18], lungs [19], in transfer of intracellular membranes [20, 21], and signal transmission in animals [6, 22-25] and plants [2, 11]. Moreover, the therapeutic potential of PLD inhibitors has been considered [4]. However, the published data on the regulatory mechanisms of PLD activity in plants and animals and on the involvement of PLD in signal transmission have not been analyzed yet. The purpose of this review is to fill this gap and to inform readers about researches in this field.

PLD of animal cells is encoded by two genes and exists in two forms: PLD1 and PLD2 [26, 27]. PLD1 (120 kDa) is mainly present on endomembranes of the cell and is transported under the influence of extracellular stimuli. PLD2 (106 kDa) is located on the plasma membrane, has low *in vivo* activity, and is moderately stimulated by known activators of PLD1. Plants are found to have many genes of various PLD isoenzymes that encode high molecular weight multidomain proteins with molecular weights of 90-125 kDa. In *Arabidopsis* plants there are 12 genes of PLD: PLDα(3), β(2), γ(3), δ, ε, and ζ(2) [2]; in rice there are 17 genes of PLD: PLDα(8), β(2), δ(3), κ, ζ(2), and φ [28]. In *Arabidopsis* PLDα3 [29], PLDδ [11], PLDζ1 [2], and PLDε [30] are located mainly on the plasma membrane, PLDζ2 is connected with the tonoplast membrane [31], PLDα1

shuttles between the cytosol and membranes [2], whereas PLDγ1 is located on intracellular membranes [2]. The location of PLDβ is not strictly determined [2]. PLDα1 is the most active and PLζ1 and PLDε are the least active isoenzymes in plants. In the absence of activators and calcium in the incubation medium all PLD isoenzymes of plant cells are inactive *in vitro*.

SPECIFIC FEATURES OF PRIMARY STRUCTURE OF PLD

Active site of PLD. PLDs from animal and plant cells have a modular structure where sites responsible for catalysis are surrounded by regulatory sequences (figure). The active site of PLD consists of four conservative amino acid sequences (I-IV) among which II and IV are especially conservative in various organisms. Every motif of II and IV contains the sequence, HxKxxxxDxxxxxxGSxN (abbreviated HKD, where x are nonconservative amino acids), and due to this sequence PLD can catalyze hydrolysis of phospholipids [32]. Residues of histidine (H), lysine (K), and aspartic acid (D) are directly involved in hydrolysis of phosphodiester bonds of phospholipids. The histidine residue in this case acts as a nucleophile that attacks the phosphorus atom of the substrate [32-34]. The catalytic site of PLD is formed when the HKD sequences are in the correct proximity. The PLD-catalyzed hydrolysis of the phosphodiester bond is supposed to occur in two stages by the so-called ping-pong mechanism: successive attack of the substrate by imidazole residues of histidines, each of which is a component of the catalytic sequences of HKD. As a result, the phosphatidic acid residue of the cleaved phospholipid covalently binds transiently with the imidazole group of histidine of PLD producing a short-lived intermediate that can be easily cleaved on attack by a water molecule [35, 36].

{Figure} Features of organization of phospholipase D of animal and plant cells. DRY, binding site of α-subunit of the heterotrimeric G-protein; Gβγ, β- and γ-subunits of the heterotrimeric G-protein; I and III, conservative domains; PtdIns(5)P, phosphatidylinositol-5-phosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate

Key:
1) Plants
2) PLD
3) P
4) actin
5) Animals
6) acylation
7) PKCδ PKCα
8) tubulin
9) casein kinase
10) cyclin-dependent kinase (PLD2)
11) ribosomal S6 kinase 2
12) interaction with choline,
13) translocation to caveola
14) active site sequence

15) binding site of oleic acid
16) binding domain of lipids and calcium ions
17) conservative domain
18) binding site of polyphosphoinositides
19) plextrin-homologous domain
20) binding site of Gα
21) phox-homologous domain
22) phosphorylation site
23) negative regulation and caspase cut site

Lipid-binding PLD sequences. Lipids can modify PLD in plant and animal cells. In particular, some phospholipids usually act not as substrates but as cofactors of the enzyme. The PLD activity depends on binding of the cofactor phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). In animals this is explained by the presence within the active site limits (between motifs III and IV) of a conservative sequence that includes residues of basic and aromatic amino acids [37, 38]. This sequence ensures high levels of PLD1 and PLD2 activities and also promotes the translocation of PLD1 to membranes in response to extracellular signals [39, 40].

The amino acid sequence of PLD enriched by basic and hydrophobic residues is involved in binding polyphosphoinositides [37]. This structure and its inverted repeat (RxxxxKxRR and RKxRxxxxR) in PLDβ of *Arabidopsis* and tomatoes surround the *C*-terminal sequence HKD [2, 41]. PLDβ1 contains all four residues of basic amino acids (mainly Arg) binding polyphosphoinositides, whereas one of them in PLDγ and PLDζ is replaced by a nonpolar or acidic residue. This structure in PLDδ has two conservative residues of basic amino acids, whereas in PLDα and PLDε there is only one such residue and the other residues are replaced by nonpolar or acidic ones [2, 11]. In addition to inverted repeats that bind polyphosphoinositides (called PBR2), PLDβ also contains another site binding these lipids (PBR1) that is located near the *N*-terminal sequence of HKD [2]. PBR1 is believed to be an independent binding site of PtdIns(4,5)P_2 and of some other phosphoinositides; it consists of highly conservative amino acid residues and contains many side chains of lysine, arginine, and histidine residues. The specific binding of PtdIns(4,5)P_2 with them plays an extremely important role in the regulation of plant PLDβ by this phospholipid because it promotes conformational rearrangements strengthening the enzyme association with the substrate [2, 41, 42]. PLDα and PLDδ contain amino acid substitutions in the abovementioned binding site of PtdIns(4,5)P_2 [2]. The phosphate group in the 4′-OH position of the inositol ring of PtdIns(4,5)P_2 plays a key role in formation of ionic bonds with lysine residues K437 and K440 of the PLDβ molecule [2].

Domain C2 interacting with lipid membranes depending on the Ca^{2+} concentration is another type of phosphoinositide-binding sequences of plant PLDs. This domain is not strictly specific to PtdIns(4,5)P_2. At low level of Ca^{2+} negatively charged PtdInsP and PtdIns(4,5)P_2 molecules attract positively charged amino acid residues R and K in the C2 domain and thus favor the binding with substrates of PLDα of *Arabidopsis* and catalysis by PLDβ and PLDγ [2, 43]. Binding sites of PtdIns(4,5)P_2 in domain C2 are also found in PLDα of cabbage [44], poppy [45], and tomatoes [46]. The C2 domain in PLDα of tomatoes binds with high affinity phosphatidic acid but

not phosphatidylcholine, but association with phosphoinositides is markedly weakened on increase in the level of their phosphorylation [46].

Domain PX. PLD of animals and PLDζ of plants contain the *N*-terminal domain PX (named after the protein p47phox that is a subunit of NADPH oxidase of phagocytes), which includes a conservative sequence enriched in proline residues [2, 28]. The PX domain of PLD1 selectively binds PtdIns(5)P, thus promoting the translocation of the enzyme to membranes of endocytic vesicles [39], and also interacts with PtdIns(3)P and PtdIns(3,4)P_2 on the membranes [47]. This domain contains two binding sites of membrane phospholipids: one of them is highly specific to PtdIns(3,4,5)P_3, PtdIns(3)P, PtdIns(5)P, and other phosphoinositides, whereas the other also binds phosphatidic acid, phosphatidylserine, and other negatively charged lipids. The concurrent binding of phospholipids with the abovementioned sites synergistically increases the affinity of domain PX for the membranes [48, 49]. The PX domain of PLD1 plays an important role in regulation of the enzyme translocation into endocytosis sites on the membranes under the influence of extracellular signals [40, 49]. In PLD1 and PLD2 this domain can stimulate the GTPase activity of the protein dynamin [50] and ensure the binding with PLD2 and activation of PLD2 of phospholipase Cγ1 [51], tyrosine kinase Syk [52], and protein kinase Cζ [53].

Domain PH. PLD1 and PLD2 of animals [38] and also PLDζ of plants [2, 28] include the domain PH (plextrin-homologous domain) responsible for specificity of localization of these enzymes in cells. The PH domain is located near the *C*-terminus of the PX domain. Deletions or point mutations in the domain of PLD1 and PLD2 do not influence the activity *in vitro* [47, 54], but inactivate the enzymes *in vivo* due to disturbance of their localization in cells [38, 40]. The PH domain of PLD2 binds PtdIns(4,5)P_2 with moderate affinity but with high selectivity [38]. Deletion of this domain in PLD1 lowers the binding of this phosphoinositide [47], but did not influence the *in vitro* activation of PLD1 and PLD2 by phosphoinositides [37, 38]. This suggests the presence in the PLD structure of other sites of association with PtdIns(4,5)P_2. The PH domain of PLD1 and PLD2 contains conservative cysteine residues (Cys240 and Cys241) that can be palmitylated and thus ensure the binding with the membranes independently of phosphoinositides [55-57], which stabilizes the interaction of the enzyme with the lipid bilayer [56]. Palmitylation promotes the translocation of PLD1 into endocytosis sites on the membranes under the influence of an extracellular stimulus [40] and the translocation of PLD2 from early endosomes onto the plasma membrane [38].

Loop-shaped sequence. The primary structure of PLD1 of animal cells is uniquely characterized by the presence of a loop-shaped domain consisting of 116 amino acid residues (a.a.). Insertion of this sequence into PLD2 does not change the activity of this isoenzyme. The loop-shaped domain of PLD1 contains a site of negative regulation, which possibly is responsible for the low basal *in vitro* activity of PLD1 as compared to that of PLD2 [54]. This conservative sequence belonging to PLD1 also contains sites of cleavage by caspases. The *in vitro* action of caspases on this sequence weakens the sensitivity of PLD1 to protein kinase C but increases its sensitivity to small GTPases [58].

Sites of Ca^{2+} binding. Based on specific features of the primary structure of the *N*-terminal domains binding lipids, PLD are subdivided onto

C2-PLD (PLDα, PLDβ, PLDγ, PLDδ, and PLDε of plants) and PX/PH-PLD (PLDζ of plants and PLD1/2 of animals) (figure). The presence of *N*-terminal conservative domain C2 consisting of 130 a.a. and binding Ca^{2+} and phospholipids is a unique feature of the majority of plant PLDs [2]. The binding of Ca^{2+} with the domain C2 provides for the PLD translocation to substrate on the membrane and induces changes in the enzyme conformation [46]. A direct binding of Ca^{2+} in three-looped structures of the C2 domain [11] has been shown for PLDα and PLDβ of *Arabidopsis* [2]. Calcium forms a coordination bond with four or five acidic amino acid residues in the calcium-binding loops (CBL) of the PLD domain C2. The main role in coordination of Ca^{2+} in the C2 domain of PLDβ, γ, and δ is played by two aspartic acid residues in the Ca^{2+}-binding loop 3 (CBL3), the only Asn residue in the Ca^{2+}-binding loop 2 (CBL2), and Asp in the Ca^{2+}-binding loop 1 (CBL1) [2, 43]. In PLDα two of these amino acids are replaced in CBL1 and CBL2 by basic or neutral residues [11, 43], whereas the C2 domain of PLDε does not have any amino acid binding with calcium [30]. In PLDα of other plants coordination bonds with Ca^{2+} are created with f asparagine and aspartic acid [59], glutamine and glutamic acid [41], and also their combination [42]. The above-described amino acid substitutions are responsible for the low affinity of PLDα and PLDε for Ca^{2+}, and therefore these isoenzymes hydrolyze phospholipids only in the presence of high concentrations of Ca^{2+} [2] or at low pH values increasing the affinity for calcium. The Ca^{2+} binding has been also observed in the active site of PLDα [60] and PLDβ, where it is stimulated by phosphatidylserine [61].

Other functional regions of PLD. PLDs of animal cells also contain other functional regions on the *N*- and *C*-termini. In particular, the *N*-terminal amino acid sequence of human PLD1 (residues 1-49 and 216-318 including the PH domain) plays a key role in the stimulation of this enzyme by protein kinase Cα [62]. Moreover, the sequence consisting of 155 *N*-terminal amino acids of human PLD1, especially Ser2, is involved in the interaction with polymerized F-actin [63]. The actin-binding domain of plant PLD located between catalytic subdomains in the limits of the conservative domain III contains asparagines and threonine residues that are directly involved in the actin binding [64]. The *C*-terminus of PLD can regulate the enzyme localization in the cells, contribute to its interaction with small GTPases of the RhoA family, and also play an extremely important role in stabilizing the active conformation of the catalytic site of PLD [65, 66].

In plants an amino acid sequence DRY located on the *N*-terminal end of the HKD *C*-subdomain is responsible for the binding of PLDα1 with the heterotrimeric G-protein subunit Gα. Except for PLDζ and PLDγ2, this sequence is also present in other PLD isoenzymes of *Arabidopsis* [67]. Potential phosphorylation sites of serine, threonine, and tyrosine residues are found in the primary structure of PLDγ of *Brassica oleracea* and also in homologs of poppy PLDα [45, 68].

REGULATORY MECHANISMS OF PLD ACTIVITY

Activity of PLD is controlled by hormones, neurotransmitters, and other physiologically active molecules influencing the enzyme through specific regulatory mechanisms. And PLD is often contributes to signal

transmission through production of phosphatidic acid as a secondary messenger. In particular, PLD is involved in transmission of signals of classic plant hormones such as abscisic acid [11] and cytokinin [69].

Regulation of PLD activity by calcium ions. Calcium plays an important role as a modifier of PLD activity in plant cells, in particular under the influence of cytokinin [70] and also as a cofactor of PLD isoenzymes containing the C2 domain [2, 43, 61]. Calcium ions activate PLDα and PLDβ through binding with the C2 domain. This leads to conformational changes in the enzymes that result in strengthening the substrate binding and weakening the association with PtdIns(4,5)P_2 [2]. Calcium also binds with the active site of PLDβ, which increases its affinity for the activator (PtdIns(4,5)P_2) as discriminated for the affinity for the substrate (phosphatidylcholine) [61]. In turn, the binding of PtdIns(4,5)P_2 with the active site of the enzyme increases its affinity for the substrate [2]. In plants there are PLDs with activities depending on millimolar and micromolar concentration of Ca^{2+} *in vitro*.

Phospholipases D of plant cells are active *in vitro* in the presence of Ca^{2+} concentrations of 10-100 mM and hydrolyze phospholipids independently of the presence in the substrate mixture of lipid cofactors (PtdInsP or PtdIns(4,5)P_2). In particular, Ca^{2+} (1-100 mM) *in vitro* activates PLDα isolated from cabbage [60], strawberry [42], *Vigna sinensis* [71], sunflower [72], and poppy [45]. The maximal activity of PLDα1 in *Arabidopsis* is observed at the Ca^{2+} concentration of 25 mM. On using pure phosphatidylcholine as a substrate, the activity of this enzyme is stimulated at Ca^{2+} concentrations of 20-100 mM [2]. At the Ca^{2+} concentration of 50 mM, the other PLD isoenzyme, PLDε, is also active [30]. Such high concentrations of Ca^{2+} activate those plant PLD isoenzymes that have the C2 domain with low affinity for Ca^{2+} [2].

Plant PLDs are also stimulated my micromolar concentrations of Ca^{2+} [5]. In the case of PLDβ, this is due to a high affinity of the C2 domain for Ca^{2+} [2]. Activities of PLDβ and PLDγ of *Arabidopsis* are maximal at 50 μM Ca^{2+}. PLDγ is less stimulated by Ca^{2+} than PLDβ and PLDα. Increase in Ca^{2+} concentration (>50 μM) is associated with a decrease in the activities of PLDγ and PLDβ [2, 43]. Activities of the PLDγ subtypes PLDγ1 and PLDγ2 depend on the Ca^{2+} concentration similarly [73]. Moreover, PLDα of castor bean in acidic medium (pH 4.5-5.0) is also stimulated *in vitro* at low Ca^{2+} concentration (50 μM), but on increase in the pH value the active concentration of Ca^{2+} increases to millimolar values [2]. The activity of isoenzyme PLDδ of *Arabidopsis* is observed *in vitro* at both micro- and millimolar Ca^{2+} concentrations. The activity of PLDδ in the presence of an activator (oleic acid) is maximal at 100 μM Ca^{2+} [11]. PLDε is also active *in vitro* at micromolar Ca^{2+} concentrations, but only in the presence of oleic acid [30].

Plant cells also contain calcium-independent PLDs: PLDζ1 of *Arabidopsis* [2] and PLD of mustard seeds [74]. Moreover, Ca^{2+} does not markedly change the *in vitro* activity of purified PLD1 from animal cells [26].

Modification of PLD activity by lipids. PLDs are regulated by various lipids, and among them polyphosphoinositides are considered to be especially important modifiers of cytoskeleton dynamics, membrane transport, and enzyme activity [75]. PLDs sensitive to these phospholipids are involved in transmission of intracellular signals of hormones, in

particular of cytokinins in plants [76-78]. In animal cells the catalytic activities of PLD1 and PLD2 are sensitive to PtdIns(4,5)P_2, which acts as their cofactor [26]. The binding of PtdIns(4,5)P_2 with PLD1 and especially with PLD2 is important for the stimulation of these enzymes [37, 55, 79]. PLD2 is allosterically activated by phosphatidylinositol-4,5-bisphosphate *in vitro* [37]. Optimal concentrations of this lipid provide for a positive effect of activators (proteins Rho, ARF1, and PKCα) on PLD1 *in vitro* promoting the PLD binding with a lipid bilayer [80]. The enzyme of PtdIns(4,5)P_2 biosynthesis, phosphatidylinositol-4-phosphate-5-kinase Iα, is favorable for an increase in PLD activity in Cos7 cells. The PLD2 activity increases under conditions of a simultaneous expression of the genes encoding PLD and the abovementioned phosphoinositide kinase [79]. As discriminated from other phosphoinositides, PtdIns(3,4,5)P_3 binds with residue Arg179 in the PX domain of PLD1 [49], stimulates the enzyme activity [49, 81], and also promotes the translocation of PKCζ into the cell nucleus [82], which results in activation of the nuclear PLD.

In plants polyphosphoinositides are important cofactors of PLDβ, PLDγ, and PLDζ, and in their absence these isoenzymes are inactive *in vitro*. PLDβ of *Arabidopsis* displays the most pronounced specificity. This isoenzyme is stimulated *in vitro* over a wide range of PtdIns(4,5)P_2 concentrations [11]. PLDβ is less sensitive *in vitro* to other phosphatidylinositol phosphates [2]. PtdIns(4)P and PtdIns(4,5)P_2 are additive in increasing the activities of PLDβ and PLDγ [2, 43]. As discriminated from PLDγ2, PLDγ1 is the most sensitive to PtdIns(4,5)P_2 [73]. PLDα does not depend on polyphosphoinositides in the presence of optimal Ca^{2+}concentrations but is stimulated by these lipids *in vitro* in acidic medium in the presence of micromolar Ca^{2+} concentrations [2, 11]. The activity of PLDδ of *Arabidopsis* depends on PtdIns(4,5)P_2 because this lipid increases the affinity of the enzyme active site for the substrate [11, 83]. The PLDζ depends only on PtdIns(4,5)P_2 [2], whereas PtdIns(3,4)P_2 is a cofactor of PLDε at micromolar concentrations of Ca^{2+} [30]. Thus, PtdInsP_2 acts as a cofactor of PLDβ, PLDγ, PLDε, and PLDζ of *Arabidopsis*. Note that PLD activity independent of PtdInsP_2 has been detected in mustard seeds [74].

Other lipids also are negative modifiers of PLD. In particular, C_2-, C_6-, C_8- and double-chained ceramides inhibit the activity of PLD by affecting its interactions with protein activators [84], acting on the catalytic site of PLD or on its binding with PtdInsP_2 [85, 86]. Lysophospholipids also specifically inhibit PLD *in vitro* independently of the small GTPase Arf and PtdInsP_2. They directly bind with PLD and noncompetitively inhibit the enzyme in animals [87] and plants [88].

Unsaturated fatty acids, products of lipid cleavage, are believed to be activators of animal PLD2 *in vitro*. This enzyme can be stimulated synergistically by PtdIns(4,5)P_2 [89, 90]. PLDδ and PLDε of *Arabidopsis* under *in vitro* conditions depend on oleic acid [11, 30], but this may be associated with modification of properties of the lipid bilayer with which these isoenzymes are associated [83]. The mechanism of the inhibitory effect *in vitro* of N-acetylethanolamines (N-laurylethanolamine, N-myristoylethanolamine) on the activity of PLDα from *Arabidopsis*, castor bean, and cabbage [91] is unknown.

Regulation of PLD activity by G-proteins. The cells of animals contain specific protein factors that are important for activation of PLD.

These factors are small monomeric GTPases of the Rho and ARF families of the Ras superfamily. Rho GTPases play an important role in the regulation of various processes in animal cells, including transcription, cell cycle, secretion, cell transformation, and cytoskeleton rearrangements [92]. Small GTPases of the Rho family are also considered as modifiers of PLD activity. Some of these proteins — Rac1, Cdc42, and especially RhoA — allosterically activate PLD1 increasing its catalytic activity by lowering the Michaelis constant [80]. In the presence of nonhydrolyzable analogs of GTP, small GTPases Rac1, Cdc42, and RhoA activate PLD1 [26, 93, 94] and PLD2 [95] *in vitro*. RhoA [96, 97], Rac1 [98], and Cdc42 [99] are PLD1 stimulators *in vivo*. The C3-exoenzyme from *Clostridium botulinum*, which inhibits the functioning of Rho proteins via their ADP-ribosylation, decreases the activity of PLD1 [82].

The binding of RhoA with the *C*-terminus of PLD1 [33, 80, 100] depends on residues of basic amino acids of the sequence 946-962 of the enzyme and on activation site I of the RhoA protein. A similar site of Cdc42 also plays an important role in the mechanism of binding and activation of PLD1 [101]. Other important binding sites of RhoA on the *C*-terminus of human PLD1 are residues Ile870, Gln975, and Asp999 [102]. The Rho proteins interact with PLD1 only in an active GTP-bound state [103]. Some Rho GTPases (RhoA, Rac1, and Cdc42) stimulate PLD1 synergistically with ARF1, but not with PKCα [80]. Although RalA GTPases cannot directly stimulate PLD *in vitro* and *in vivo*, they play an important role in the activation of PLD1 and PLD2 [104, 105] by forming a complex with Rho and ARF proteins [106, 107]. This confirms the synergic influence of two monomeric GTPases on PLD activity.

PLD activity also depends on some cytosolic GTPases representing ADP-ribosylation factors (ARF). The PLD1 activity *in vitro* increases 10-15-fold on incubation with ARF proteins (ARF1, ARF3, ARF4, ARF5, and ARF6) [26, 47, 108], whereas the activity of PLD2 increases 1.5-2-fold (but independently of GTP) [54]. ARF1 and ARF6 activate PLD1 *in vivo* [109, 110], whereas ARF6 activates PLD2 [111]. Comparatively to ARF6, ARF1 preferentially activates PLD1 *in vitro* [112], whereas ARF6, as differentiated from ARF1, markedly stimulates PLD2 *in vivo* [108]. ARF1 stimulates PLD1 *in vitro* synergistically with Rho proteins (RhoA, Rac1, Cdc42) [80, 112] and PKCα [80]. Synergistic effect of ARF1 and Rho is observed only at optimal concentrations of $PtdInsP_2$ [80].

The translocation of ARF to membranes plays an important role in the mechanism of activation of PLD. The translocation of ARF6 stimulates PLD1 [113] and PLD2 [108] *in vivo*. The importance of ARF protein exemplified by ARF1 is proved by its necessity for the activation of PLDs. ARF1 activates PLD1 *in vitro* by increasing the catalytic constant without changes in the K_m value [80]. The replacement of GDP by GTP in ARF6 is important for activation of PLD [114], and this explains the stimulatory effect of guanine nucleotide exchange factors on the activity of PLD1 *in vivo* [115, 116]. The *N*-terminus of PLD2 (amino acids 1-308) is essential for modification of this enzyme activity through ARF. The first 73 *N*-terminal amino acids of ARF are involved in the activation of PLD1 [117], whereas the sequence of amino acids 35-94 plays an important role in the interaction of these proteins [118]. Thus, small GTPases of the Rho and ARF families are key activators of PLD in animals.

Both α- and β/γ-subunits of heterotrimeric G-proteins (G_q, G_i, G_{12}) contribute to the PLD activation by various hormones in animal cells, although this process occurs *in vivo* with direct involvement of proteins Rho, protein kinase C [119, 120], ARF6, or tyrosine kinase Src [121, 122]. The direct influence of heterotrimeric G-proteins results in inactivation of PLD. Combination of Gβ1γ1 subunits suppresses PLD1 and PLD2, whereas Gβ1γ1 and Gβ2γ2 inhibit PLD1 stimulated *in vitro* and *in vivo*. These protein complexes interact with the *N*-terminal region of PLD within limits of the PX and PH domains (amino acid residues 3-311) [123]. In plant cells the binding of PLDα with the G-protein α-subunit (Gα) in the GDP-form suppresses PLD activity *in vitro* [67, 124]. Addition of GTP into the system restores the PLD activity. And the interaction of PLDα1 with Gα stimulates the GTPase activity of Gα that leads to disintegration of the PLDα–Gα complex [67]. Therefore, the inactive heterotrimeric G-protein is thought to suppress the membrane-associated PLDα, whereas stimulation of the G-protein by an extracellular signal promotes PLDα activation. Hence heterotrimeric G-proteins are thought to be important regulators of PLD in plant and animal cells.

Phosphorylation as a mechanism of PLD regulation. Phosphorylation/dephosphorylation of proteins at specific amino acid residues plays an important role in mechanisms of signal transmission, regulation of enzyme activities, and formation of protein complexes in both plant and animal cells [125-127]. Mainly Ser, Thr, His, Asp, and Tyr residues are phosphorylated. The Tyr residue is most frequently studied in the works in this field [127].

The stimulation of PLD in the cells by phorbol esters (in particular, by PMA, which is an analog of diacylglycerol activating protein kinase C) and also its suppression by specific inhibitors confirm the role of PKC as a key factor of PLD regulation *in vivo* [128]. PKCα and PKCβ1 stimulate PLD [129, 130], but PKCδ and PKCε can act as both positive and negative regulators of PLD1 and PLD2 *in vivo*. PKCδ can displace PLD1 from the complex with PKCα [131, 132]. Results of *in vitro* studies have shown that human and rat PLD1 are activated under the influence of PKCα, PKCβ1, and PKCβ2 independently of their kinase activity [26, 47, 130]. The activation of PLD through protein–protein interactions can also be realized by atypical PKCs: PKCι (PLD2) [133] and PKCζ (PLD1, PLD2) [82, 134].

Phorbol esters as PKC activators induce the PLD1 binding with PKCα that leads to activation of PLD1 [130, 135]. PLD2 can also bind with PKCα [136] and PKCδ [137]. The *N*- and *C*-terminal sequences of PLD are involved in the activation of PLD1 on binding protein kinase Cα [80]. PKCα decreases the Michaelis constant and increases the catalytic constant of the PLD1 reaction. PKCα activates PLD1 *in vitro* synergistically with Arf1, as discriminated from Rho. This occurs only at optimal concentrations of $PtdInsP_2$ [80]. The binding of active PKCα with PLD2 also stimulates PLD2 *in vivo*, whereas the phosphorylation of PLD2 *in vitro* in many Ser and Thr sites by PKCα inactivates it [136, 138]. However, the phosphorylation of Thr566 under the influence of PKCδ is accompanied by activation of PLD2 [131, 137]. Phosphorylation by PKCα and PKCβ of PLD2 at Ser and Thr leads to translocation of PLD2 to membranes and its activation *in vivo* [139]. However, PKCα and PKCβ phosphorylate and as a result inactivate PLD1 *in vitro* [140]. The phosphorylation of PLD1 at Thr under the influence of PKCα suppresses PLD1 *in vivo* [141], and the

translocation of PLD1 to plasma membranes and its resulting activation are due to phosphorylation of the enzyme by protein kinase Cα at Ser2, Thr147, and Ser561 [142].

Activation of growth factor receptors stimulates soluble tyrosine protein kinases of the Src family. Inhibitors of Src are known to suppress the PLD2 activation in PC12 cells caused by depolarization [143]. Members of the Src family p60Src and p56Lyn mediate PLD activation in platelets [144]. As discriminated from PLD1, PLD2 is phosphorylated on Tyr and is activated by other tyrosine kinases of the Src family – Fyn and Fgr [145]. However, tyrosine kinase v-Src phosphorylates PLD1 and PLD2 on Tyr in COS-7 cells without influencing their activity [146]. Thyroid gland carcinoma protein kinase RET/PTC also directly phosphorylates PLD2 on Tyr without its activation [147].

The PLD2 isoform expressed as a constitutively active enzyme in various cells [148] is a phosphotyrosine protein *in vivo* [149] and *in vitro* [150]. Its phospholipase activity is regulated dually by phosphorylation/dephosphorylation and also can induce cell proliferation by the abovementioned human PLD isoenzyme with involvement of Tyr179 and Tyr511 [150]. Mutations of this and other tyrosine residues – Tyr296 and Tyr415 – result in a decrease in the PLD2 activity [151], whereas the phosphorylation degree of each catalyzed by different enzymes can influence the functioning of PLD2. The phosphorylation of PLD2 at Y296 in the PH domain by the epidermal growth factor receptor provides for the low activity of the enzyme *in vitro* only in tumor cells. However, tyrosinases JAK3 and Src phosphorylating PLD2, respectively, at Tyr415 and Tyr511 are responsible for the high activity of this enzyme *in vitro* in less invasive cells and thus prevent the negative effect of the Tyr296 phosphorylation. The Tyr511 residue is a dual site relative to regulation of the PLD2 activity. Dephosphorylation at Tyr511 and Tyr296 by tyrosine phosphatases CD45 and PTP1B stimulates PLD, whereas dephosphorylation at Tyr415 decreases the activity of this enzyme *in vitro* [150]. Phosphorylated Tyr179 and Tyr511 in PLD2 promote its binding with the SH2 domain of the adapter protein Grb2, which ensures a high lipase activity of the enzyme in COS-7 cells [151]. Thus, it is concluded that protein kinases/phosphatases modify PLD through direct phosphorylation/dephosphorylation of this enzyme on Tyr residues.

PLD activity sharply increases in tumor cell lines in response to stress caused by removal of serum. Dephosphorylation of Tyr179 and Tyr511 in PLD2 can act as a signal directed to decrease the activity of PLD and also of the accompanying proliferative potential capable of preventing cell malignization [151, 152]. The increased expression and activity of PLD have been found in cells of many human tumors, which suggests a functional association of the PLD activity with oncological signals and tumorigenesis [153]. Results of these studies have allowed new nontoxic PLD inhibitors to be created as promising antitumor preparations [153, 154].

Ser/Thr-protein kinases also play an important role in the regulation of PLD in animal cells. The mechanism of activation of PLD by casein kinase II (PLD2) [155], cyclin-dependent protein kinase 5 (PLD2) [156], ribosomal S6-kinase 2 (PLD1) [157], and also by AMP-activated protein kinase (PLD1) [158] is largely due to phosphorylation of the specific residues Ser134 [156], Thr147 (in the PX domain) [157], and Ser505 [158].

However, it is still unclear whether the activation of PLD is caused by protein kinase Rho depending on the Rho protein [159] or by calcium/calmodulin-derived protein kinase II [144] phosphorylation of the enzyme and whether the PLD2 phosphorylation on the active Ser/Thr protein kinase AKT (Thr175 in the PX domain) [160] or by protein kinase A in the case of PLD1 [161] changes the enzyme activity. It is also unclear whether the activation of PLD [162] is associated with the enzyme phosphorylation on Ser and Thr by MAP kinases p38, ERK1, and ERK2 [163-165] or is caused only by protein–protein interactions.

Incubation of plasma membrane from cells of the plant *B. oleracea* with acidic phosphatase *in vitro* decreases the activity of $PtdInsP_2$-dependent PLD by 80%, whereas inactivation of the phosphatase partially restores the activity of the PLD. Moreover, PLDγ of this plant is bound by antibodies to phosphoserine/threonine/tyrosine [68]. The presence of phosphorylation sites in the structure of PLDγ of *B. oleracea* [68] determines the role of this posttranslational modification in the regulation of PLD activity in plants.

Influence of cytoskeleton and light on PLD activity. The cytoskeleton proteins are PLD modifiers. PLD activity also depends on the degree of actin polymerization. In the presence of active monomeric GTPases, monomeric G-actin inactivates PLD1 in the membrane fractions or negatively influences this enzyme resulting by immunoprecipitation [166, 167]. However, G-actin stimulates a purified recombinant enzyme PLD1 expressed in cells of insects [63]. A highly purified polymerized F-actin stimulates PLD1 *in vitro* but only in the presence of activators – G-proteins ARF and Rac1 [2, 63]. The translocation of PLD1 to actin filaments is caused by phosphorylation of the Ser2 residue on the PLD *N*-terminus by protein kinase C [63].

The polymerized F-actin stimulates plant PLD activity, whereas monomeric G-actin suppresses it [2]. In tobacco cells PLDβ1 occurs to be a partner of actin, and the interaction with actin is provided for by Asn323 and Thr382 of the phospholipase [64]. Thus, the interaction of actin and PLDβ1 creates a system of positive feedback in which F-actin activates phospholipase D and lipase-produced phosphatidic acid, in turn, activates F-actin in places of its interaction with cell membranes [2, 64].

The activity of animal PLD2 is suppressed *in vitro* under the influence of β-actin, which prevents the access of PLD2 to the substrate [166]. The protein α-actinin that links actin filaments suppresses PLD2 *in vitro* by affecting its binding with ARF1 [167]. The negative effect of β-actin is inhibited in the presence of ARF1, whereas the binding of β-actin with PLD2 is prevented by association of α-actinin with this enzyme [166]. The phosphorylated form of cofilin (a protein promoting actin polymerization and depolymerization) is specifically bound and stimulates the basal activity of PLD1 *in vitro* by promoting its translocation to the plasma membrane [168]. Gelsolin (a protein capping actin) suppresses PLD1 and PLD2 *in vitro* [169], but in the presence of nucleotide trisphosphate it binds with PLD and activates it [170]. The actin-binding protein fodrin [171] and also dimer and trimer of spectrin suppress PLD activity and are involved in local regulation of PLD by actin [171]. However, the effect of two latter proteins can be associated with disorders in the interaction of PLD1 with $PtdInsP_2$ [172].

Tubulin monomer inhibits PLD2 of animal cells due to direct and reversible binding with the enzyme [173]. In plants stabilizers and destabilizers of microtubules stimulate the *in vivo* accumulation of phosphatidic acids produced by PLD [174]. Therefore, it is concluded that cytoskeleton proteins can act as regulators of PLD activity in plant and animal cells and provide the necessary level and specific location in cells of phosphatidic acid production.

Plant PLDs are known to be so-called dark-induced proteins (din-proteins). The role of a photoregulatory receptor is played by phytochrome: both white and red lights suppress PLD activity in etiolated plantlets, whereas far red light restores it (a reversible photo-effect) [175, 176]. This indicates a possible involvement of the phytochrome receptor in the regulation of PLD activity. Moreover, the photo-dependence of PLD activity is associated with photosynthesis, because diuron stimulates and glucose suppresses, respectively, the enzyme activity in green and etiolated oat plantlets.

Involvement of PLD in hormone signaling. PLDs are involved in reception and intracellular transmission of hormonal signals in both animals and plants. In animals PLDs are activated under the influence of hormones (growth hormone, vasopressin, gonadotropin-releasing hormone, etc.) and of various neurotransmitters (histamine, bradykinin, noradrenaline, etc.) [177]. Interesting data were recently obtained in studies on transmembrane receptors of animal hormones. Phospholipase D was shown to be a key enzyme for many of them, being involved in their secretion, endocytosis, and signaling. Thus, the parathyroid hormone 1 type receptor (PTH1R) increased PLD activity on interacting with the ligand, PLD1 was involved in the internalization and intracellular traffic, and PLD2 was involved in receptor endocytosis [178]. The subclass of formyl peptide receptors (FPRL1) induced by an agonist also can activate PLD, and the phospholipase plays an important role in endocytosis, membrane recycling, and receptor signaling [179, 180]. PLD2 functioning in plasma membrane similarly simplifies endocytosis of angiotensin II receptor type 1 [40].

The involvement of PLD in the transmission of abscisic acid signal has been shown in plants [28, 181, 182]. Moreover, there are data on the involvement of PLD in the action of gibberellins [28] and in auxin transport [184, 185] (table). PLD also plays a role in the activation by cytokinins of the primary response genes [77, 186, 187], and a rapid increase in PLD activity is observed under the influence of cytokinins [69, 76]. The PLDs involved in transmission of signals of different phytohormones are also different. The abscisic acid signal is transmitted with involvement of phospholipase D isoforms α and partially β (table) [28, 181], whereas the cytokinin signal is transmitted not with involvement of the PLD α-isoform, but more likely with the involvement of PLDγ [76]. Thus, PLD is an important and specific factor in the signal transmission of various hormones in both animals and plants.

{Table} Features and role of different PLD isoenzymes in regulation of plant cell metabolism

Key:
1) PLD isoenzyme

2) Note: ABA, abscisic acid; Bs, biotical stress; G, gibberellin; Fz, freezing; Dr, drought; IAA, indolylacetic acid; Oxs, oxidative stress; Os, osmotic stress; W, wound; Ss, saline stress; P, mineral phosphate; Et, ethylene; N, mineral nitrogen; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.
3) Molecular weight, kDa
4) Substrate specificity
5) PC
5') PE
5") PG
6) Cofactors
7) calcium
8) mM
8') μM
9) $PtdInsP_2$
10) present
10') absent
11) oleic acid
12) Regulation mechanisms
13) phosphorylation
14) G-proteins
15) actin
16) Activity pH-optimum
17) Location in chromosomes
18) Sensitivity to hormones and stress of mutants by PLD
19) increase
20) decrease
21) knockout/knockdown
22) superexpression
23) ABA Et Glc Ss Os Dr Fz W G Bs Oxs IAA
24) shortage of N
25) transport of IAA
26) shortage of P

PLD is a widely distributed enzyme involved in the fine regulation of metabolism of cells with different organization. This enzyme is necessary for growth and development of animals and plants, especially under conditions of stress and illumination. The primary structure of PLD is known in detail, and regulatory mechanisms of activities of these enzymes and their interaction with other signaling systems of the cell are intensively studied. It has been shown that the activity of known forms of animal PLDs is modified by serine/threonine and tyrosine protein kinases, GTP-binding proteins (ARF and Rho), the cytoskeleton, polyphosphoinositides, protein–protein interactions, and hormones. In plants PLDs are directly regulated by Ca^{2+}, polyphosphoinositides, heterotrimeric G-proteins, cytoskeleton, some phytohormones, and possibly by protein kinases. However, mechanisms of *in vivo* regulation of individual PLD enzymes are studied insufficiently, especially in plants, despite the established biological role of many molecular PLD forms (table). In the majority of biological processes PLD activity is concurrently modified by a number of regulators, and their action can be additive, synergic, or opposing. New data on the role of PLD in the

regulation of proliferation and in carcinogenesis has stimulated intensive searches for new inhibitors of PLDs as promising antitumor preparations. To better understand the functional role of PLD isoforms in cells, *in vivo* studies are important, and these can be useful when associated with molecular design, analysis, and synthesis of new nontoxic substances inhibiting different PLD isoforms *in vivo*.

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