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Phospholipase D: Its Role in Metabolic Processes and Development of Diseases

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Abstract—Phospholipase D (PLD; EC 3.1.4.4) is one of the key enzymes catalyzing hydrolysis of cell membrane phospholipids. This review considers and summaries current knowledge about six human PLD isoforms, their structure and a role in physiological and pathological processes. Comparative analysis of PLD isoforms structure is presented. The review considers the mechanism of hydrolysis and transphosphatidylation performed by PLD, the role of PLD1 and PLD2 in the pathogenesis of some types of cancer, infectious, thrombotic, and neurodegenerative diseases is analyzed. The prospects of development of PLD isoform-selective inhibitors are considered in the context of their clinical use and inclusion into various therapeutic schemes; the latter is especially important in the case of already developed PLD inhibitors. Phosphatidylethanol (PEth) formed in the human body during phospholipid transphosphatidylation catalyzed by PLD is considered as an alcohol abuse biomarker.

Keywords: phospholipase D (PLD), phospholipase D isoforms, phospholipase D inhibitors, phosphatidylethanol (PEth), alcohol abuse biomarkers

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1. PHOSPHOLIPASE D (PLD) AND ITS ENZYMATIC ACTIVITY

The current state of medicine is characterized by an increased interest in a detailed study of the role of various enzymes in physiological and pathological processes. One such enzyme is phospholipase D (PLD, EC 3.1.4.4), acting on cell membrane phospholipids. Hydrolysis of the phospholipid phosphodiester bond by PLD results in formation of phosphatidic acid (PA), a second messenger involved in various signaling pathways and a participant of many cellular processes [1].

PLD was originally discovered in carrots and cabbage leaves in the late 1940s [2, 3]. Subsequent studies revealed existence of various PLD isoforms belonging to the same superfamily of phospholipases D, which were found in viruses, prokaryotes and eukaryotes (yeast, sludge, plants, and mammals) [4]. PLD isoforms have characteristic highly conserved catalytic amino acid sequences HxKxxxxD(6x)GSxN (HKD, where H is histidine, K is lysine, D is aspartic acid, G is glycine, S is serine, N is asparagine, and x is any amino acid); they are located in II and IV motifs of the PLD active site and are necessary for realization of enzymatic activity (Fig. 1) [5, 6].

There are the enzymes that do not have the HKD sequence but they are capable of hydrolyzing phospholipids; although they do not belong to the PLD superfamily, nevertheless, historically some of them have been denominated as PLD. These include PLD produced by *Streptomyces chromofuscius*, *Corynebacterium*, *Arcanobacterium*, and even cytochrome P450 isoforms, CYP1A2 and CYP2E1, hydrolyzing phosphatidylcholine in hepatocytes [4, 7].

In addition to hydrolytic activity, PLD catalyzes transphosphatidylation reactions in the presence of such nucleophiles as various primary alcohols (ethanol, *n*-butanol), resulting in the formation of phosphatidylalcohols [4]. It is suggested that due to the similar structure of the active site, different PLD isoforms exhibit catalytic activity that follows the same two-step S_N2 ping-pong mechanism without the reversal of the substrate configuration. The PLD catalytic site is formed by two HKD sequences; imidazole group of the N-terminal HKD histidine residue nucleophilically attacks the phosphate atom of the substrate with formation of a covalently bound phosphatidyl-histidine intermediate (stage 1, Fig. 2). The C-terminal histidine residue serves as a proton donor for the leaving group (stage 2, Fig. 2). Since phosphatidylcholine is the most common phospholipid of cell membranes, choline is the most common leaving group. Subsequent nucleophilic attack with water or primary alcohol destroys the intermediate

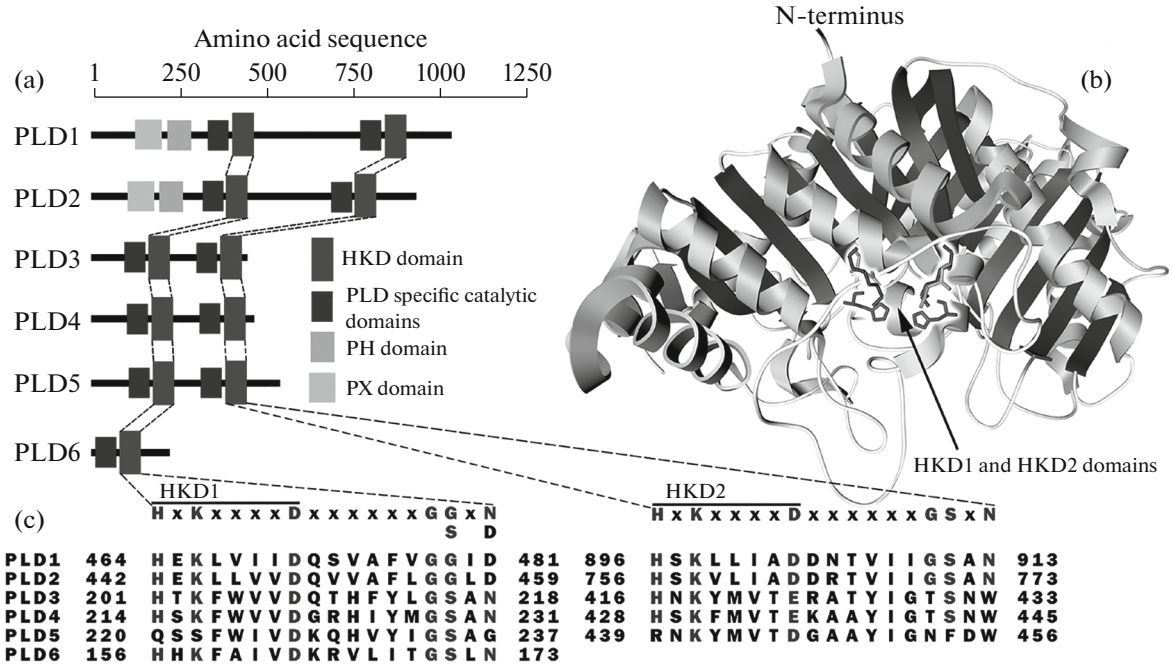

Fig. 1. The PLD structure. (a) Comparison of the domain structure of six PLD isoforms; (b) comparison of the conservative HKD domains of six PLD isoforms; (c) three-dimensional structure of PLD and its active center.

with release of phosphatidic acid or phosphatidyl alcohol (stages 3 and 4, Fig. 2) [4, 8–10].

2. PLD ISOFORMS

2.1. PLD1 and PLD2

Although six PLD isoforms have been recognized in mammals, PLD1 and PLD2 are the most studied enzymes [11]. The domain structure of PLD1 [12] and PLD2 [13] is basically the same: in addition to the HKD sequence, it includes highly conserved pleckstrin homology (PH) and phox homology (PX) domains, as well as a phosphatidylinositol-4,5-bisphosphate binding site (PtdIns(4,5)P_2) [6, 14]. The presence of the loop sequence in the PLD1 probably explains the lower basal activity of PLD1 as compared to PLD2 (this domain has an autoinhibitory effect) [15]. PLD1 and PLD2 are localized in virtually all mammalian tissues; PLD2 is located on the plasma membrane, while PLD1 is transported to plasma membrane from cell endomembranes in response to the effects of extracellular stimuli [6]. The mechanism of interaction of PLD1 and PLD2 with membranes is not fully understood; it is suggested that such interaction involves PX and PH domains, as well as the catalytic site sequence enriched with basic amino acid residues [16]. The PX domain contains a highly specific site for binding of membrane polyphosphoinositides, as well as a site for binding PA, phosphatidylserine and other negatively charged lipids [6]. Conservative cysteine residues (Cys240 and Cys241) present in the PH-domain can undergo palmitoylation, which also provides enzyme binding to the membrane [6]. PLD1 and PLD2 catalyze the reactions of phospholipid hydrolysis and transphosphatidylation. These isoforms play an important role in cytoskeletal rearrangement, exocytosis and endocytosis, cell proliferation and migration, autophagy and apoptosis, and intracellular transport between the Golgi apparatus and the endoplasmic reticulum [4, 11].

2.2. PLD3, PLD4 and PLD5

PLD3 [17] and PLD4 [18] are the transmembrane proteins located on the endoplasmic reticulum and their structure lacks the PH and PX domains. PLD3 is localized in many mammalian tissues, and the increase in the expression level of this isoform is observed in neurons and muscle cells during their differentiation [17]. PLD3 is widely expressed in brain cells. Rare genetic variants of PLD3 increase the risk of Alzheimer's disease [19]. At the same time, PLD3 plays an important role in the muscle tubule formation during myogenesis [17]. PLD4 is most widely expressed in spleen cells [18]. It has also been shown that PLD4 is involved in microglial phagocytosis [20]. Although PLD3 and PLD4 have the HKD sequences, no catalytic activity has been detected for these isoforms. PLD5 is the least studied isoform; it is suggested that PLD5 also lacks catalytic activity [11].

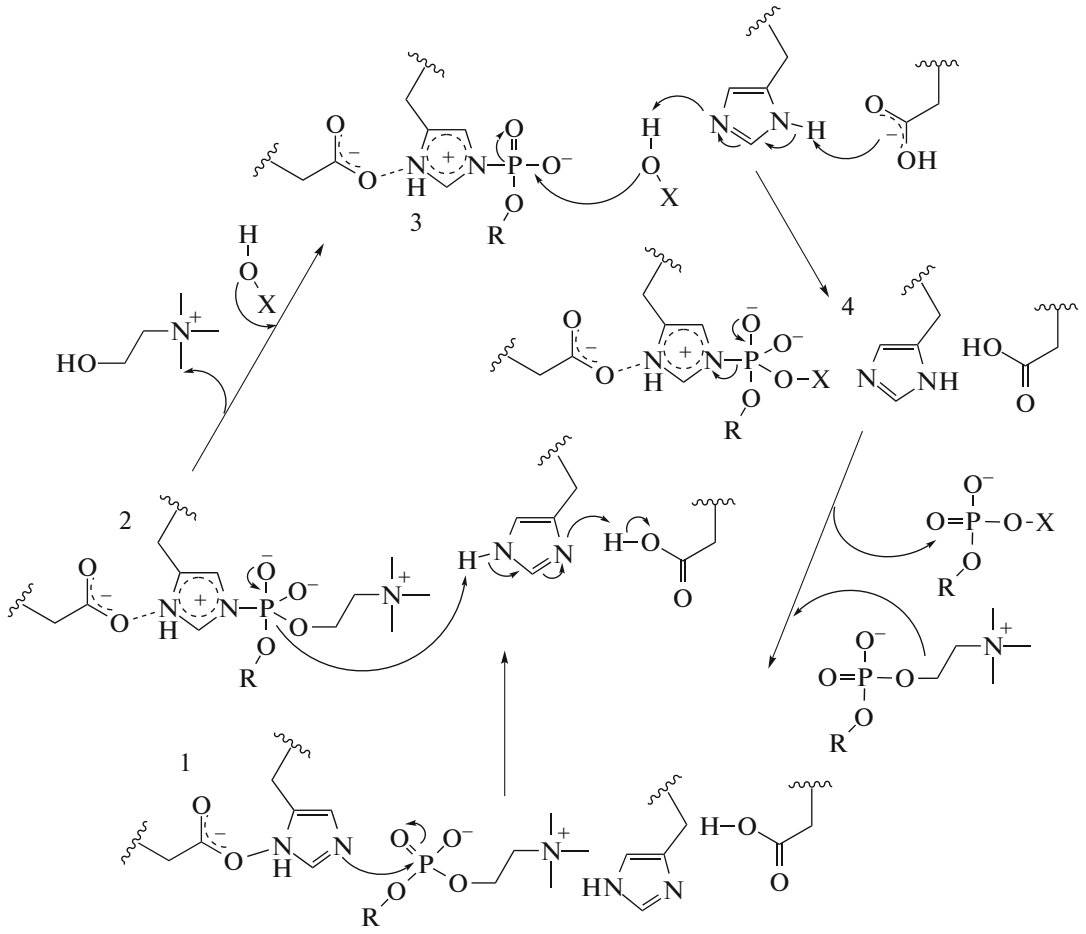

Fig. 2. Mechanism of S_N2-reaction catalyzed by PLD. R is a diacylglycerol residue, X is $(CH_2)_nCH_3$ or H. Depending on the nucleophile, the molecule of the primary alcohol or water attacking the phosphorus atom of the substrate in step 3, a transphosphatidylation or hydrolysis reaction occurs (modified from [4]).

2.3. PLD6

In contrast to the above-mentioned isoforms, PLD6 (MitoPLD) is located on the outer surface of the mitochondrial membrane and has only one HKD sequence in its structure [21]. PLD6 hydrolyses cardiolipin, the mitochondrial lipid, and also cleaves long-chain RNA with formation of piRNA (piwi-interacting RNA) during spermatogenesis, thus exhibiting endonuclease activity [22–26]. Phosphatidic acid formed during cardiolipin hydrolysis influences the processes of mitochondrial fusion and fission [25], while piRNAs are involved in transposon silencing for maintenance of the genome stability [27] (Table 1).

The activity of PLD1 and PLD2 is controlled by numerous intracellular and extracellular factors, because phosphatidic acid formed during phospholipid hydrolysis is a second messenger in various signaling pathways; in addition it can be further metabolized to diacylglycerol (DAG) and lysophosphatidic acid, which also act as signaling molecules [4, 28, 29]. It has been shown that polyphosphoinositides, particularly PtdIns(4,5)P_2, behave as a PLD cofactor increasing catalytic activity of this enzyme [6]. The direct allosteric activators of PLD are small monomeric G-proteins, GTPases, belonging to the Rho and ARF families of the Ras superfamily [4]. PLD is phosphorylated by kinases, mainly by protein kinase C, and also by serine-threonine, tyrosine protein kinases, calcium/calmodulin-dependent kinases [30]. PLD1 and PLD2 can also be activated indirectly via G-protein coupled receptors and receptors exhibiting tyrosine kinase activity stimulated by such receptor agonists such as hormones, chemotactic peptides, cytokines, growth factors, etc. [11, 31]. Mechanisms for regulation of PLD1 and PLD2 activity have been considered in more detail in a review by Kolesnikov et al. [6].

3. INHIBITORS OF PLD ACTIVITY

For more detailed studies of the physiological and possible pathological functions of PLD isoforms in animal organisms and for elucidation of the therapeutic efficacy of inhibitors of this enzyme, various

Table 1. Comparative analysis of PLD isoforms

Phospholipase D isoforms	Locus	Substrate specificity	Metabolites	Inhibitors	Intracellular localization
PLD1	NP_002653	Phosphatidylcholine, phosphatidylserine, other phospholipids	Phosphatidic acid, phosphatidyl alcohols	FIPI VU0359595 ML299	Cell endomembranes; transported to the plasma membrane in response to extracellular stimuli
PLD2	NP_002654	Phosphatidylcholine, phosphatidylserine, other phospholipids	Phosphatidic acid, phosphatidyl alcohols	FIPI VU0364739 ML298 ML395 ML299	Plasma membrane
PLD3	NP_001278240	Catalytic activity was not detected	–	–	Transmembrane protein located on the endoplasmic reticulum
PLD4	NP_620145	Catalytic activity was not detected	–	–	Transmembrane protein located on the endoplasmic reticulum
PLD5	NP_689879	Catalytic activity was not detected	–	–	No data
PLD 6	NP_849158	Cardiolipin; long-chain RNAs	Phosphatidic acid; piRNA	–	Outer surface of the outer mitochondrial membrane

approaches are used to suppress PLD activity. From the 1980s to the 2000s, primary alcohols, most often *n*-butanol, were used to block the cell signaling pathways involving PLD; then new techniques based on the mechanism of RNA interference, knockdown and/or knockout of genes, coding PLD were introduced [4, 11, 32].

Experiments performed on cell cultures and animal models, revealed that inhibition of PLD could be promising for the treatment of certain oncological, infectious, and autoimmune diseases, thrombotic conditions, neurodegenerative disorders; this stimulated development of PLD pharmacological inhibitors [11, 33, 34]. The development of direct inhibitors of PLD1 and PLD2 based on halopemide, a dopamine receptor antagonist, which was initially studied as an atypical antipsychotic agent, started in 2007 [35–37]. Currently, a halopemide derivative FIPI (5-Fluoro-2-indolyl des-chlorohalopemide) is the most widely used inhibitor in animal and cell culture studies [38, 39]. Although FIPI demonstrates more potent inhibition of PLD1 (IC_{50} = 1 nM) than PLD2 (IC_{50} = 44 nM), it is considered as a non-selective PLD inhibitor and is characterized by lack of selectivity for the pharmacological action (Fig. 3).

The need in selective inhibition of PLD1 and PLD2 resulted in the development of second generation of PLD inhibitors [33, 40, 41]. Introduction of a (S)-methyl group into the ethyl bridge of the halopemide molecule and also of the bromine atom and the phenylcyclopropane group significantly increased (by more than 1700 times) selectivity of the inhibitor VU0359595 to PLD1 (IC_{50} = 3.7 nM) as compared with PLD2 (IC_{50} = 6400 nM). A selective PLD2 inhibitor based on the triazaspirone skeleton, VU0364739 (JWJ), was also synthesized (PLD1 IC_{50} = 1500 nM, PLD2 IC_{50} = 20 nM). It has been shown that VU0359595 and VU0364739 are allosteric PLD inhibitors, and the pleckstrin homology domain plays the key role in binding of these molecules. The next stage in the development of PLD inhibitors, determined by the need in molecules that would act exclusively on PLD2, culminated in the development of the third generation of PLD inhibitors. This generation currently includes three molecules: ML298 (PLD1 IC_{50} = 20000 nM, PLD2 IC_{50} = 355 nM) and ML395 (PLD1 IC_{50} > 30000 nM, PLD2 IC_{50} = 380 nM), which are selective inhibitors of PLD2, and also ML 299, which inhibits both PLD1 (IC_{50} = 6 nM) and PLD2 (IC_{50} = 320 nM) (Fig. 4).

In addition, a new class of PLD inhibitors based on 1,3-disubstituted-4-amino-pyrazol-pyrimidine is currently developed [42]. The highest efficacy towards PLD1 was found in an inhibitor molecule containing 1-naphthyl group in position 3 and 4-methoxyphenyl group in position 1. An 4-amino-pyrazol-pyrimidine derivative containing phenyl radical in position 3 and 4-methoxyphenyl group in position 1 demonstrated the highest selectivity to PLD2.

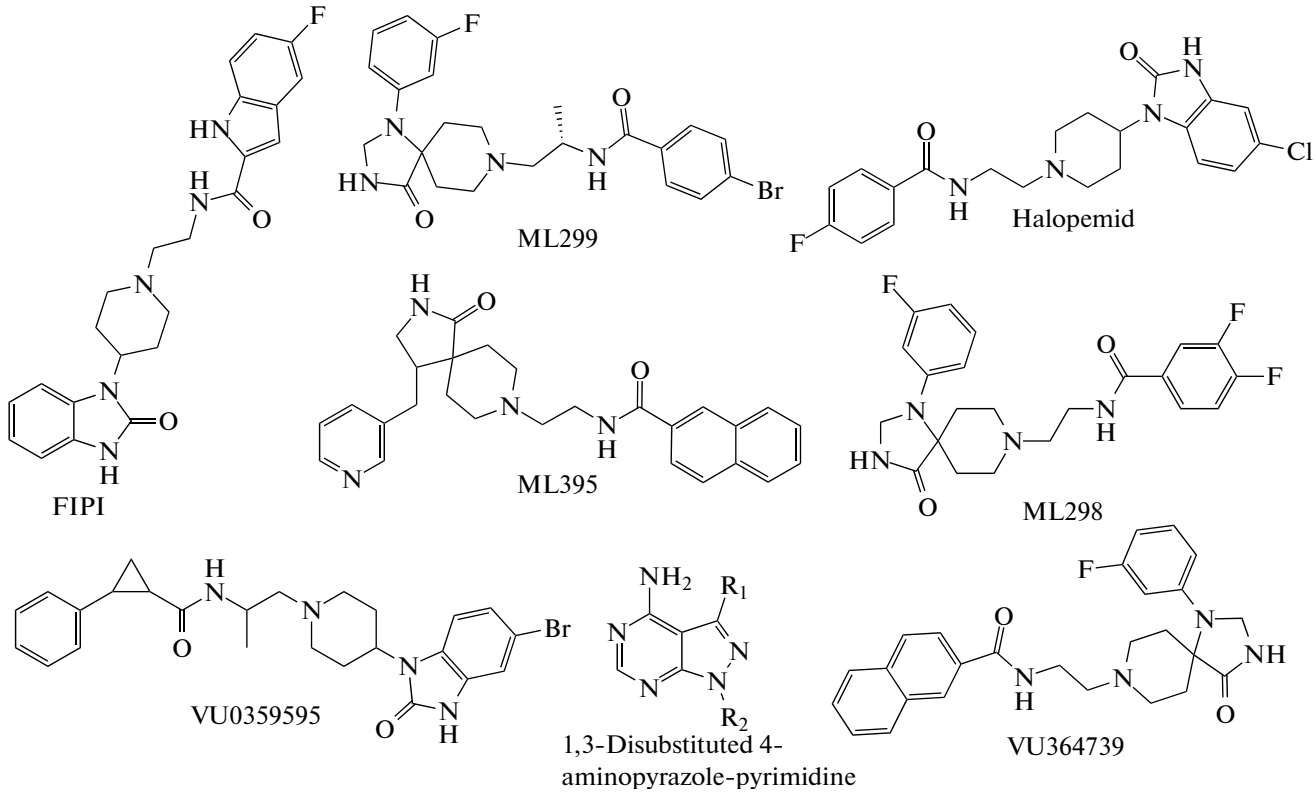

Fig. 3. Pharmacological inhibitors of PLD activity.

4. THE ROLE OF PLD IN THE DEVELOPMENT OF PATHOLOGICAL CONDITIONS AND THEIR TIMELY DIAGNOSTICS

Earlier we already mentioned that before the 2000s, primary alcohols were used in studies of the physiological role activity of PLD; they were able to affect cell processes nonspecifically and independently of PLD and therefore the results of many previous experiments on PLD inhibition could now be revised [11, 43, 44]. For example, in a number of studies it has been shown that PLD is necessary for superoxide anion radical formation by neutrophils stimulated with the bacterial tripeptide fMLP (*N*-formyl-methionyl-leucyl-phenylalanin) and for neutrophil degranulation, while PLD inhibition by ethanol or *n*-butanol suppressed these processes [45–47]. However, in 2013, several studies [48, 49] demonstrated that the pharmacological inhibition of PLD1 and PLD2 by FIPI and knockout of the PLD1 and PLD2 genes in $PLD^{-/-}$ mice did not affect superoxide formation and the neutrophil degranulation in the presence of fMLP. Consequently, the alcohols rather than PLD inhibition influenced neutrophil functioning.

To date, PLD1 and PLD2 are the most studied isoforms, so the possibilities of their pharmacological inhibition are actively studied to produce therapeutic effects in humans [11, 14].

4.1. Oncological Diseases

Many viewpoints exist on the role of PLD in the development of cancer and the potential for its pharmacological inhibition; however, they all need to be tested in animal models, since tumor development in vivo is a complex interrelated phenomenon [11]. Results of various studies confirm involvement of PLD1 and PLD2 in the pathogenesis of breast, ovarian, lung, colon, pancreas, kidney, prostate, and brain cancers [33]. It is known that the expression and activity of PLD1 are significantly increased in tumor cells [38, 50, 51]; PLD1 is involved in proliferation, invasiveness and viability of cancer cells [14]. PLD1 and PLD2 also participate in transduction of the vascular endothelial growth factor signal, which is released into the tumor by hypoxic stress to stimulate angiogenesis, which provides blood supply to tumor cells [52, 53]. It has been found that phosphatidic acid, which is mainly formed via PLD-mediated hydrolysis of phospholipids, has a mitogenic and anti-apoptotic effects, and under certain conditions this may become a factor in the development of neoplasms. Phosphatidic acid activates protein kinase mTOR (mammalian target of rapamycin), which regulates growth and proliferation of cells through the mTOR signaling pathway [54]. In addition, it has been shown that phosphatidic acid formed by PLD2 is necessary for the extracellular signal transduction via the Ras cascade [55]. Phos-

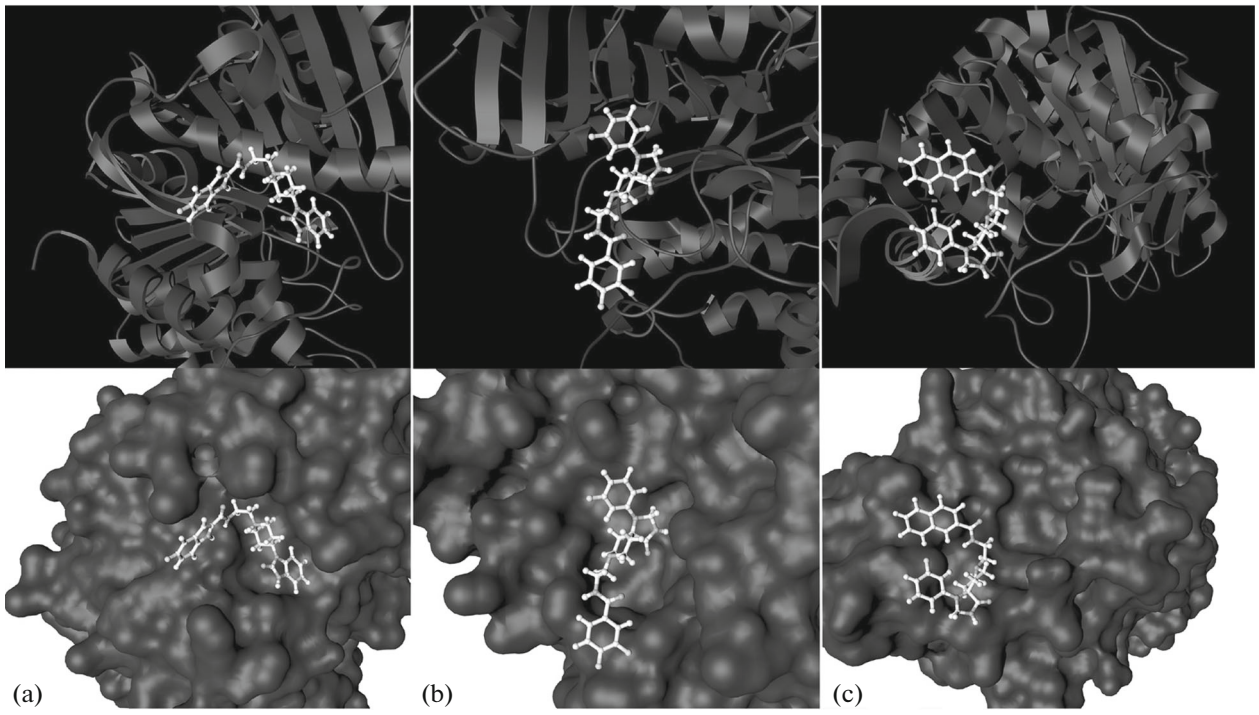

Fig. 4. Interaction of PLD2 with: (a) FIPI; (b) ML298; (c) VU0364739. ML298 and VU0364739 are allosteric inhibitors of PLD2, while FIPI interacts directly with the catalytic site.

phatidic acid interacts with the SOS factor (Son of Sevenless) and promotes its attachment to the plasma membrane followed by activation of the G protein Ras by exchanging GDP for GTP. Phosphatidic acid induces translocation of the serine-threonine kinase Raf-1 from the cytosol to the plasma membrane; it also activates the MAPK (mitogen-activated protein kinase) signaling cascade, which is responsible for the regulation of mitogenesis, cell proliferation and differentiation [56]. Lysophosphatidic acid formed from phosphatidic acid exhibits mitogenic effects, and it is also involved in cell proliferation and migration [57]. In addition to the mentioned above, PLD1 itself indirectly participates in tumor metastasis promoted by platelets [58]. However, the use of PLD inhibitors in oncology may be limited, since in some types of tumors, for which a decrease in cell mobility leads to a progression of tumor development, prescription of PLD inhibitors is contraindicated [11].

4.2. Influenza

In 2009, using RNA interference, the genes influencing replication of the influenza virus (and including PLD2 encoding genes) were identified in the human body [14, 59]. It has been shown [60] that PLD2 is involved in the process of endocytosis of the virus into the cell, thus promoting its escape from the effects of innate immunity factors. Based on these data, it is suggested that selective PLD2 inhibitors can exhibit antiviral activity by blocking endocytosis of the virus. Direct experiments have demonstrated [60] that inhibition of PLD2 by VU0364739 results in partial blockage of endocytosis of the influenza virus; on the one hand, this allows the immune system to fight effectively the infection and to stop replication of the virus, and on the other hand, does not completely blocks endocytosis in the cell. This determines low toxicity of VU0364739 and maintenance of normal cellular metabolism. The effect of PLD2 inhibitors can be explained by the presence of a large number of phosphatidic acid molecules in a certain region of the cell membrane; this causes formation of negative curvature, which is necessary for membrane fusion and fission during vesicular transport [61]. Thus, inhibition of PLD-mediated formation of phosphatidic acid promotes blockade of viral entry into the host cell. On the other hand, Ali et al. [62] demonstrated that inhibition of PLD2 had a negative impact on the functions of the immune system, suppression of neutrophil migration and phagocytosis. In this context, the question, whether PLD2 inhibitors should be used in the treatment of influenza still requires further studies.

4.3. Thrombotic Conditions

Another area of possible application of PLD1 and PLD2 inhibitors is the treatment of thrombotic conditions [11]. It is well known, that interaction of activated integrin $\alpha_{IIb}\beta_{III}$, located on the surface of plate-

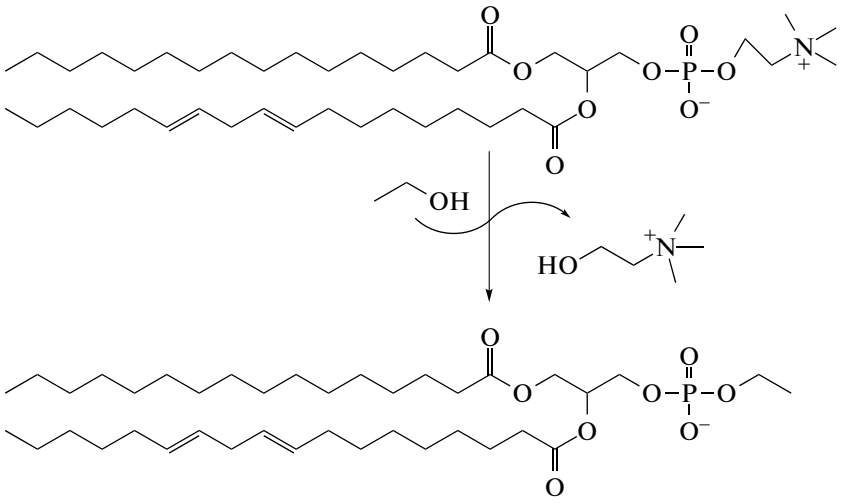

Fig. 5. PEth 16 : 0/18 : 2 formation from phosphatidylcholine catalyzed by PLD.

lets, with fibrinogen plays an important role in the process of thrombus formation. Since the activation of integrin $\alpha_{IIb}\beta_{III}$, is controlled by PLD, the therapeutic inhibition of PLD1 and PLD2 can be used for attenuation of thrombogenesis [63, 64]. It has been shown that FIPI can be successfully used in the prevention of arterial thrombosis and ischemic stroke [65]. In studies on mouse models, the use of PLD inhibitors did not increase the incidence of bleeding [65]; this favorably distinguishes them from various classes of antithrombotic agents used in clinical practice.

4.4. HIV

Taylor et al. [66] found that inhibition of PLD1 by VU0359595 reduced the ability of the HIV virus to replicate in $CD4^+$ T lymphocytes in vitro. This is due to the suppression of phosphorylation of extracellular signal-regulated kinases, which cause metabolic changes that occur when T cells are activated. Thus, inhibition of PLD1 does not allow the HIV virus to use metabolic resources of activated T lymphocytes and therefore PLD1 inhibitors can be considered as a new approach in antiretroviral therapy.

4.5. Alzheimer's Disease

As mentioned above, rare polymorphisms of PLD3 can lead to the development of late-onset Alzheimer's disease. It is suggested that the loss of PLD3 functioning increases the secretion of β-amyloid peptide. However, due to poorly understood role of PLD3, the pharmacological effect on this process is not possible at the moment. At the same time, experimental evidence exists that in the absence of PLD2, the β-amyloid peptide loses its ability to influence the electrophysiological activity of neurons, particularly, to suppress long-term potentiation. PLD2 inhibition prevents memory loss and promotes protection of synaptic endings in Alzheimer's disease [11, 33].

4.6. Alcoholism

Formation of phosphatidylalcohols catalyzed by PLD has practical application in the laboratory-diagnostics of alcohol abuse. It has been found that the transphosphatidylation reaction proceeds even with minimal amounts of alcohol, since the enzyme affinity to it is 1000 times higher than to water [67]. Thus, in the presence of ethanol PLD is involved in phosphatidylethanol (PEth) formation in the human body. PLD1 and PLD2 have been shown to catalyze transphosphatidylation in vitro [31], but the question on contribution of each of these isoforms to PEth formation in vivo still remains unanswered [68]. Numerous studies have been performed to develop methods of determination of blood PEth content to establish the fact of alcohol abuse [68, 69].

In contrast to such traditional indirect alcohol abuse biomarkers, as γ-glutamyltransferase (GGT), carbohydrate-deficient transferrin (CDT), mean corpuscular volume of erythrocytes (MCV), PEth formation is not affected by pathological processes occurring in the human body and unrelated to the use of alcohol [70]. Therefore, the specificity of PEth is 100% [71, 72], because its formation occurs during the conversion of phosphatidylcholine and other membrane phospholipids catalyzed by PLD only in the presence of ethanol (Fig. 5).

PEth includes 48 homologous glycerophospholipids possessing the same structure: a glycerol residue, a polar phosphoethanol head group and different fatty acid residues varied in lengths (from 14 to 22 carbon atoms) and an unsaturation degree (from 0 to 6 double bonds) [73]. The most common homologues are PEth 16 : 0/18 : 1 and PEth 16 : 0/18 : 2, which together

comprise about 60% of the total PEth amount; their sum correlates with the total PEth content in the human body better than the value of each of the two homologues separately [74].

Such diversity of homologues is explained by the fact that formation of phospholipids is greatly influenced by the nature of nutrition, as the food is the major source of fatty acids. Therefore, it is suggested that the individual profile of PEth homologues also depends on the composition of the diet [75]. It has been shown that the relationship between alcohol consumption and PEth in the human body is not affected by such factors as sex and age, as well as concomitant diseases such as hypertension and liver damage [76, 77]. Certain evidence also exists that PEth formation is not affected by hematologic indices: mean corpuscular volume, hematocrit, the number of erythrocytes [78]. It should be noted that in the laboratory diagnostics of PEth, the effect of the temperature storage regime of blood samples taken for analysis can influence the quantitative content of PEth. For example, under in vitro conditions at −20°C and at room temperature, the PEth level increases due to preserved PLD activity; this can lead to false positive results. However, at a temperature of 4°C (up to 72 h) and at a temperature of −80°C, an increase in the PEth level was not observed [79].

PEth formation occurs in various tissues, including the brain, liver, lymphocytes, platelets, and erythrocytes [80]. PEth synthesis is proportional to the ethanol concentration and can individually vary in dependence on PLD activity. The rate of PEth formation is influenced by the rate of ethanol absorption from the gastrointestinal tract, which depends on gender, the body fat percentage, the stomach contents, the genetically determined differences in alcohol dehydrogenase and acetaldehyde dehydrogenase [81].

PEth degradation involves phospholipase A_2, phospholipase C and phosphohydrolase [68]. However, in erythrocytes, the process of PEth degradation occurs slower probably due to the absence of phospholipase C [80]. This extends PEth half-life, which is about 4 days, and in the case of chronic abuse this biomarker accumulates in red blood cells; this allows PEth to be detected 28 days after the last intake of alcohol [76, 82, 83]. It is important to note that the elimination rate for PEth homologs differs during the withdrawal period. It was shown that PEth 16 : 0/18 : 2 was excreted faster from the body than PEth 16 : 0/18 : 1 [84].

Various studies provided convincing evidence for clear correlation between the amount of consumed alcohol and the blood PEth concentration, and the sensitivity of PEth as a biomarker of alcohol abuse ranged from 94% [71] to 100% [85]. The highest specificity and sensitivity parameters of PEth among all direct and indirect biomarkers of alcohol abuse make PEth the most accurate and objective biomarker. Most cases of the PEth analysis employ various modifications of liquid chromatography–tandem mass spectrometry [80]. However, taking into consideration the fact that most of the data on correlation between PEth concentrations and the amount of consumed alcohol have been obtained using relatively small population groups and using different methods of PEth determination, it is important to continue large-scale studies on representative population groups.

CONCLUSIONS

PLD isoforms are one of the key enzymes involved in the signal and metabolic pathways of the cell. PLD1 and PLD2 participate in cytoskeleton reorganization, exocytosis and endocytosis, cell proliferation and migration, autophagy, apoptosis and vesicular transport; they are also involved in pathogenesis of certain oncological and infectious diseases, thrombotic conditions and neurodegenerative disorders, and this opens new prospects in treating these diseases using PLD inhibitors. The product of the phospholipid transphosphatidylation reaction, PEth, is the most specific and sensitive biomarker of alcohol abuse. The latter expands the possibilities of laboratory diagnostics of alcoholism. Thus, the superfamily of phospholipases D can be regarded as an important object of biomedicine with a high prospect for further research.

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