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Critical Review

Phospholipid Signalling Through Phospholipase D and Phosphatidic Acid

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Summary

Phospholipase D (PLD) hydrolyzes the phosphodiester bond of the predominant membrane phospholipid, phosphatidylcholine producing phosphatidic acid and free choline. This activity can participate in signal transduction pathways and impact on vesicle trafficking for secretion and endocytosis, as well as receptor signalling. Phospholipids can regulate PLD activity directly, through specific intermolecular interactions, or indirectly, through their effect on the localization or activity of PLD's protein effectors. This short review highlights these various phospholipid inputs into the regulation of PLD activity and also reviews potential roles for PLD-generated phosphatidic acid, particularly a mechanism by which the phospholipid may participate in the process of vesicular trafficking.

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INTRODUCTION

In the late 1970s, work by Yasutomi Nishizuka helped trigger a new understanding in cellular signal transduction which still holds a pivotal place in our understanding of lipid signalling (*1, 2*). This work identified the role of the membrane glycerophospholipid, phosphatidylinositol (PtdIns) and its hydrolytic product, diacylglycerol (DAG), in activating the serine-threonine kinase, protein kinase C (PKC). Nearly 30 years on, it is clear that this 'classical' PtdIns-DAG-PKC cascade is only one arm of numerous inter-related lipid signalling cascades within the cellular 'lipidome' (*3*).

The lipidome consists of more than 1000 identified cellular lipids, of which phosphatidic acid (PtdOH) is one member (*3*). PtdOH has long been studied because it is a source of DAG but, recently it has become clear that this glycerophospholipid has more to offer than a potential alternate PKC activation pathway. PtdOH can be formed by the addition of phosphate to DAG, through the action of DAG kinases or by the action of lysophosphatidic acid acyltransferases on lysophosphatidic acid (Fig. 1). However, recently much interest has focused on the enzyme phospholipase D (PLD) which hydrolyses the relatively abundant phospholipid phosphatidylcholine (PtdCho) to also produce PtdOH (*4–7*). Studies on PLD have established that the enzyme itself is regulated by a series of lipid signalling cascades and recent studies discussed below have come closer to revealing a mechanism for the function of PtdOH.

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PHOSPHOLIPASE D IS AT THE CENTRE OF A PHOSPHOLIPID SIGNALLING NETWORK

Cellular PLD activity is tightly regulated *via* a number of mechanisms. These include *direct* control by membrane phospholipids, and *direct* inputs from a number of protein effectors. Some Ras-like small GTPases and PKCs, for example, have been demonstrated regulate PLD's activity *via* specific intermolecular interactions. However, phospholipids can also *indirectly* regulate PLD, since PKC and GTPase regulation of PLD can be highly dependent on the phospholipid environment.

Phosphatidylinositol Polyphosphates

Phospholipids of the phosphatidylinositol polyphosphate (PtdIns) class are essential cofactors for PLD. Derivatives of this class of phospholipid have been demonstrated to interact *directly* with PLD. Phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2) recognizes sequences towards the middle of PLD1 (*8*) whereas phosphatidylinositol(3,4,5)triphosphate (PtdIns(3,4,5)P_3) interacts with the amino-terminal Phox homology (PX) domain (Fig. 2) (*9*). These phospholipid interactions are required for enzyme activity from PLDs identified in mammals, plants and protozoa. The PtdIns

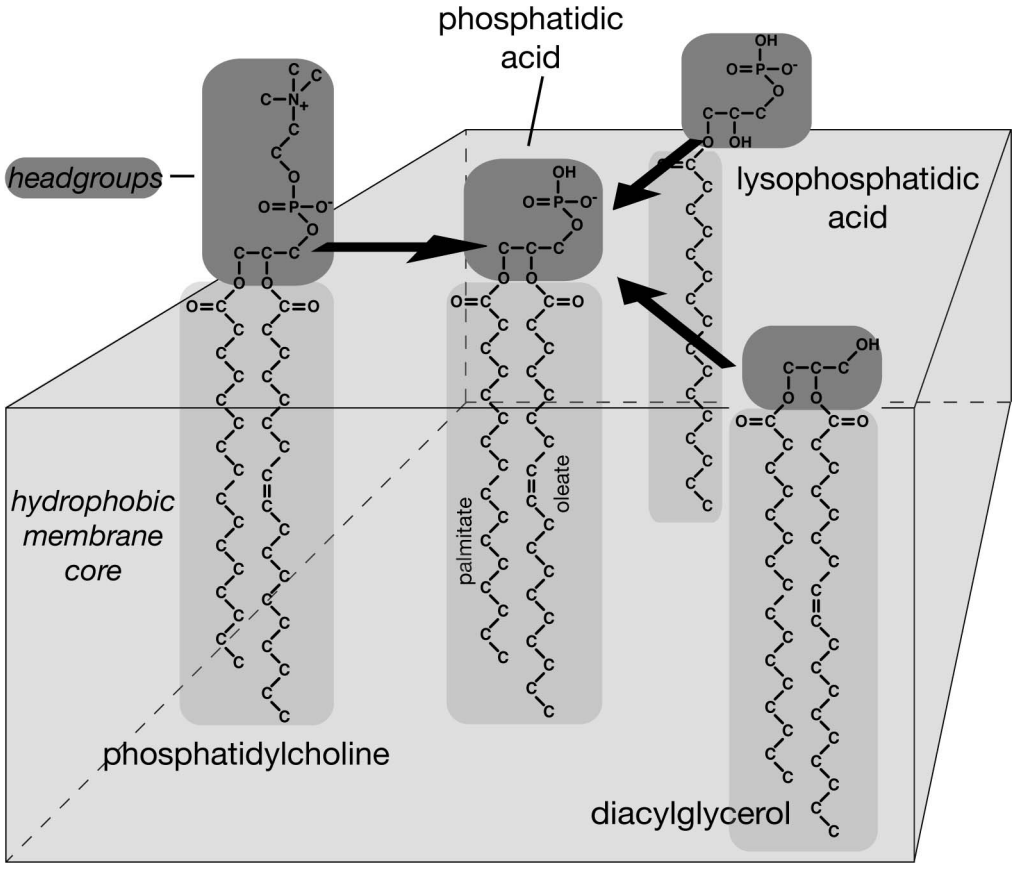

Figure 1. Phosphatidic acid species (1-palmitoyl 2-oleoly-*sn*-glycero-3-phosphate is shown) can be formed from the relatively rare phospholipids lysophosphatidic acid, by the action of lysophosphatidic acid acyltransferases, and diacylglycerol, by the action of diacylglycerol kinases. The action of phospholipase D (PLD) hydrolyses the abundant phospholipid phosphatidylcholine to produce PtdOH. Conversely, PtdOH can be converted to lysophosphatidic acid or DAG by the action of phospholipase A or PtdOH phosphohydrolases respectively.

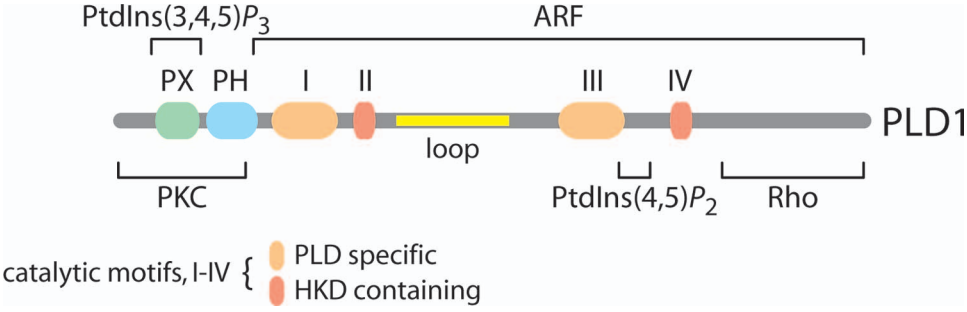

Figure 2. Schematic of motifs and domains within the mammalian (prototypical) PLD1. Phox homology (PX), plexstrin homology (PH) and motifs I-IV identified as essential for catalysis, including the HKD ($HxK(x)_4D(x)_6GSxN$) conserved motifs. The loop region is absent in PLD2, the only other mammalian PLD. Regions where phospholipid and protein effectors are known to directly interact are also highlighted.

derivative, phosphatidylinositol (3,4)bisphosphate (PtdIns(3,4)P_2) has no ability to activate any of these PLDs (*5*). Thus, production of very specific PtdIns polyphosphates, through the regulation of the activity of appropriate PtdIns-kinases and -phosphatases, is essential for PLD activity and subsequent PtdOH production.

Protein Kinase C

PKCs activate PLD independently of the catalytic activity of these kinases through *direct* interaction with regions in the extreme amino-terminus of PLD (Fig. 2) (*5*). This interaction between membrane bound PLD and usually cytosolic PKCs only occurs on recruitment of the PKC to the PLD containing membrane – a process dependent on appropriately localized production of DAG (*2*). Anionic phospholipids such as phosphatidylserine (and Ca^{2+} ions) further impact on the recruitment, membrane interaction and activity of PKCs *indirectly* affecting PLD activity. There is evidence that phosphorylation of PLD by PKC may inactivate the enzyme halting production of PtdOH (*10*). Thus, both production of DAG and the nature of the phospholipid environment contribute to activating and deactivating PLD and thus regulating PtdOH production.

ARF GTPases

ARF family small GTPases also *directly* activate PLD by interacting with regions in the middle and the amino-terminus of the phospholipase (Fig. 2) (*5*). Although ARF proteins are generally cytosolic, on binding GTP and becoming activated, they translocate to the membrane where they can recruit a number of proteins including phosphatidylinositol 4-phosphate 5-kinases, enzymes which are responsible for producing PtdIns(4,5)P_2 from phosphatidylinositol 4-phosphate (*11*). Thus, ARFs can be responsible for a *direct* and *indirect* phospholipid mediated activation of PtdOH production.

Rho GTPases

Rho family GTPases also *directly* activate PLD through interactions with multiple residues in the carboxyl-terminus of PLD (Fig. 2) (*5*). It has been proposed that phosphatidylinositol 4-phosphate 5-kinases can also be activated by Rho's regulation of Rho-kinase. Thus, Rho family GTPases can *indirectly* mediate PtdOH production by PLD activity *via* PtdIns(4,5)P_2 (*5*).

Other Lipid Mediators

Other lipids have been demonstrated to affect the ability of PLD to produce PtdOH. There is increasing evidence that sphingolipids may regulate this process. Ceramide appears to reduce cellular PLD1 activity *indirectly* but whether this is through disrupting PLD-effector interactions or by inhibiting membrane localization of PKCs, Rhos or ARFs is unclear (*12*). Sphingosine-1-phosphate has also been shown to activate PLD, although probably through a receptor-mediated signalling cascade rather than a *direct* interaction (*13*). Interestingly, an oleate stimulated PLD activity has also been described, however the protein responsible for this activity remains to be identified (*7*).

Thus, a network of lipid mediators including DAG, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, phosphatidylserine, sphingolipids and possibly oleate can contribute to the regulation of PtdOH production by PLD.

ROLE OF PHOSPHATIDIC ACID IN CELLULAR SIGNALLING

How do studies of PLD and its regulation improve our understanding of the role PtdOH? The first line of evidence comes from the cellular localization of PLD. Mammalian PLDs have been identified in a variety of locations, although the literature is complicated by data from different cell lines, antibodies (of variable quality) and by the use of over-expression constructs which sometimes mislocalize. In general, PLDs appear to be located on the plasma membrane and/or intracellular vesicles, suggesting a role for PtdOH in these locations. Likewise, a number of PLD activators, such as PKCs, ARFs and Rhos, are localized to these compartments, suggesting PtdOH production occurs at these sites.

Within the plasma or vesicle membrane, PtdOH produced by PLD has been associated with numerous cellular processes and although these processes undoubtedly overlap, they can perhaps be thought of as phospholipid metabolism, signal transduction, and vesicle trafficking (*6, 7, 14*).

Phosphatidic Acid in Phospholipid Metabolism

Cellular PtdOH is rapidly converted to DAG or lysophosphatidic acid by phosphohydrolases or phospholipases (Fig. 1). Phospholipase A isoforms convert PtdOH into lysophosphatidic acid (Fig. 1); however, whether PLD derived PtdOH can be so metabolised remains to be demonstrated *in vivo*. PtdOH phosphohydrolases can convert PtdOH into DAG (Fig. 1), a process known to activate PKC. Interestingly, a comprehensive analysis of the fatty acid composition of cellular DAGs and PtdOHs demonstrated that PLD-derived DAG was unlikely to activate PKC (*15*). Thus, while the enzymes exist to further metabolize PLD derived PtdOH, the biological relevance for these activities remain unresolved.

Phosphatidic Acid as a Signal Transducer

More definitive work has suggested that PtdOH is a signalling second messenger. PtdOH has been implicated as an activator of a number of key signalling kinases including the phosphatidylinositol 4-phosphate 5-kinases (type I), mTOR (mammalian target of rapamycin) kinase, sphingosine kinase 1 and Raf-1 protein kinase, where the latter two have also been demonstrated to translocate to regions rich in PLD derived PtdOH (*16, 17*). PtdOH has also been shown to bind to signalling enzymes including cAMP-specific phosphodiesterase (*18*) and protein phosphatase-1 (*19*). Many signalling studies have implicated PLD as a key component controlling biological processes such as cytoskeletal rearrangement, proliferation and cell survival. PtdOH's role in regulating phosphatidylinositol 4-phosphate 5-kinase is entirely consistent with these observations as PtdIns(4,5)P_2 has a well

defined role in regulating actin deposition (*20*). Similarly, the role of PtdOH in recruiting Raf-1 kinase to initiate proliferative MAP kinase signalling and mTOR, which phosphorylates S6 kinase 1 leading to a promotion of cell survival, are consistent with a mechanism by which PLD derived PtdOH directly interacts with signalling enzymes to transduce a cellular signal.

Phosphatidic Acid in Vesicular Trafficking

PLD derived PtdOH has been linked to vesicular trafficking processes including Golgi transport, endocytosis and exocytosis (*21*). The role of PLD derived PtdOH in exocytosis is particularly compelling as the enzyme has been shown to be required for key exocytotic processes in adipocytes (*22*) as well as neuroendocrine (*23*), mast (*24*) and pancreatic β-cells (*25*). Further significance is added to these observations as the multipotent phospholipid PtdIns(4,5)P_2, which in addition to activating PLD and thus producing more PtdOH, also regulates vesicle trafficking (*26*). Similarly, the ARF GTPases also have a clear role in regulating vesicle trafficking (*11*). In addition, both reside on the plasma membrane or intracellular vesicles, ideally located to promote vesicular trafficking events.

Recent work has provided some of the first real evidence of a mechanism by which PLD derived PtdOH regulates vesicle trafficking processes (*22*). These studies demonstrate that the insulin-stimulated translocation of GLUT4 glucose transporter containing vesicles to the plasma membrane is dependent on the activity of PLD located on the transport vesicles. As with many of the studies on PLD's role in exocytosis, this work demonstrated an increased consequence of exocytosis (in this case, glucose uptake through plasma membrane GLUT4) by increasing PLD activity and production of PtdOH. Conversely, decreasing PLD activity and PtdOH production resulted in reduced exocytosis (and glucose uptake). Importantly, the study went on to demonstrate that the block was not in intracellular trafficking of the GLUT4 vesicles or their recruitment to the plasma membrane but in the fusion process where the vesicle membrane becomes contiguous with the plasma membrane. PLD derived PtdOH appeared to be required for this process.

Membrane fusion is a remodeling process requiring energy to overcome the powerful hydrophobic effect of the core of the lipid bilayer and many proteins and lipids facilitating this fusion process have been described (*27–29*). PtdOH, with a very small negatively charged headgroup close to the acyl chains, generated in the *inner* cytoplasmic leaflet of fusing membranes is likely to induce negative membrane curvature and an environment favouring fusion (Fig. 3) (*30*). To test the

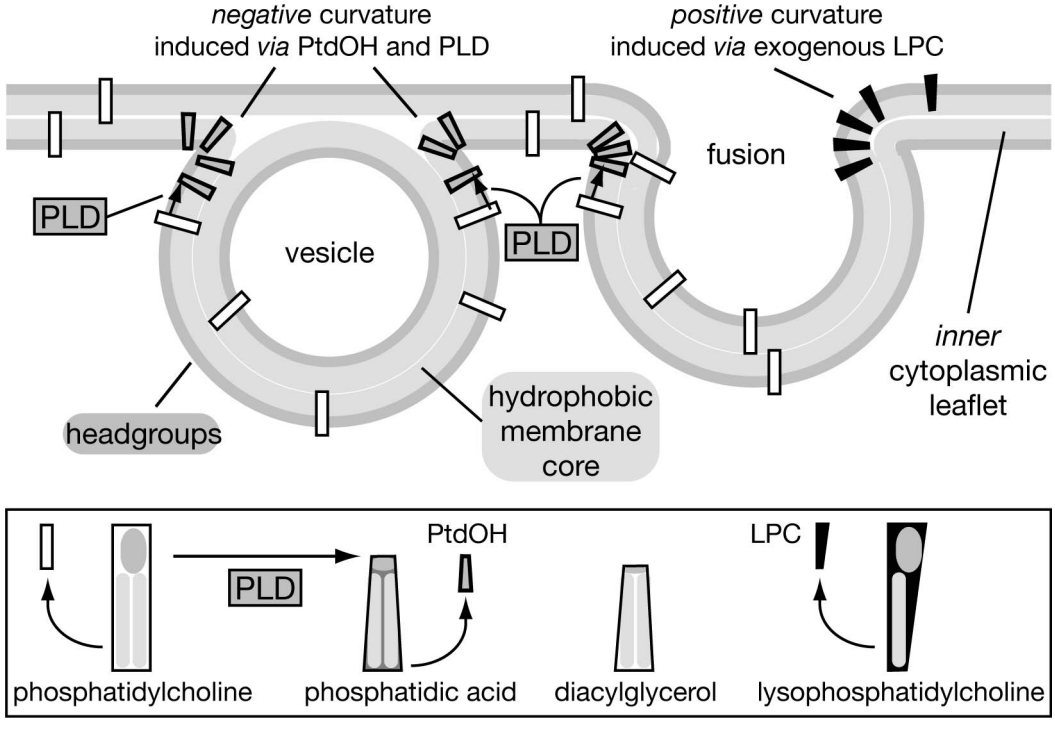

Figure 3. Cartoon showing membrane fusion proceeding through a hemifusion intermediate, detailing how the 'shape' of phospholipids (phosphatidylcholine, phosphatidic acid, diacylglycerol, lysophosphatidylcholine) may promote the curvature required to allow the complete fusion of two opposing lipid bilayers. PLD derived phosphatidic acid, PtdOH, could induce negative curvature on the inner, cytoplasmic, membrane leaflet. Exogenously added lysophosphatidylcholine, LPC, could induce positive curvature on the outer membrane leaflet.

hypothesis that PLD produced PtdOH physically promotes fusion, the phospholipid lysophosphatidylcholine, which induces positive membrane curvature, was added to the *outer* leaflet of the membrane (Fig. 3). Under these conditions lysophosphatidylcholine-induced outer leaflet positive curvature bypassed the block in exocytosis seen when PLD activity and PtdOH production were impeded (*22*). These data suggest a mechanism by which PLD derived PtdOH regulates fusion through promotion of the appropriate membrane curvature and fusagenic environment.

There are many parallels between the PLD-PtdOH dependent exocytotic processes described for GLUT4 vesicles in adipocytes and those seen in neuroendocrine cells, pancreatic β-cells and mast cells, and also in the other PLD-PtdOH dependent trafficking processes. Whilst it remains to be demonstrated that PtdOH is not 'transducing a signal' by recruiting effector molecules to promote fusion or being 'metabolized' to produce highly fusagenic DAG, it is attractive to propose that PLD derived PtdOH is itself produced to mediate the physical process of membrane fusion.

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