CMLS, Cell. Mol. Life Sci. 62 (2005) 2305–2316
1420-682X/05/202305-12
DOI 10.1007/s00018-005-5195-z

CMLS Cellular and Molecular Life Sciences

Review

Phospholipase D: a lipid centric review

G. M. Jenkins* **and M. A. Frohman**

Department of Pharmacology and the Center for Developmental Genetics, University Medical Center at Stony Brook, 495 CMM, Stony Brook, New York 11794-5140 (USA), Fax: +1 631 632 1692, e-mail: jenkins@pharm.sunysb.edu

Received 9 May 2005; received after revision 9 June 2005; accepted 30 June 2005
Online First 7 September 2005

Abstract. Phospholipase D (PLD) hydrolyzes the phosphodiester bond of the glycerolipid phosphatidylcholine, resulting in the production of phosphatidic acid and free choline. Phosphatidic acid is widely considered to be the intracellular lipid mediator of many of the biological functions attributed to PLD. However, phosphatidic acid is a tightly regulated lipid in cells and can be converted to other potentially bioactive lipids, including diacylglycerol and lysophosphatidic acid. PLD activities have been described in multiple organisms, including plants, mammals, bacteria and yeast. In mammalian systems, PLD activity regulates the actin cytoskeleton, vesicle trafficking for secretion and endocytosis, and receptor signaling. PLD is in turn regulated by phosphatidylinositol-4,5-bisphosphate, protein kinase C and ADP Ribosylation Factor and Rho family GTPases. This review focuses on the lipid precursors and products of mammalian PLD metabolism, especially phosphatidic acid and the roles this lipid performs in the mediation of the functions of PLD.

Key words. Phospholipase D; phosphatidic acid; diacylglercol; phosphatidylcholine; signal transduction.

Introduction

Classical phospholipase D (PLD) enzymes hydrolyze phosphatidylcholine (PC) to yield phosphatidic acid (PA) and free choline (see fig. 2). More broadly, PLD performs a transphosphatidylation reaction using water or primary alcohols such as ethanol or 1-butanol as the nucleophile to generate PA, phosphatidylethanol or phosphatidylbutanol (PBut), respectively. Ethanol and 1-butanol are preferentially used over water as the nucleophile by 1000-fold or more [1, 2]. PLD superfamily members are found in organisms ranging from viruses to bacteria, yeast, plants and animals. In mammalian cells, PLD activity is found in most cell types with the exception of leukocytes and a few lymphocyte lines [3, 4, see also 1999 BBA issue 1439]. The following review briefly covers the history of the PLD field and structure, localization, activation and regulation of mammalian PLD1 and PLD2 [reviewed in 2, 5-8], followed by an in-depth discussion of the lipid precursors and products of PLD enzymatic action and the functions of the lipid products, including their actions on downstream targets.

History

A PLD-type activity was first described by Hanahan and Chaikoff in carrot extracts in 1947 [9, 10]. PLD activity was not demonstrated in a mammalian system until 1975, when Saito and Kanfer described a release of choline and ethanolamine from rat brain preparations. Interestingly, the enzymatic activity was found to cleave both PC and phosphatidylethanolamine (PE) [11]. Classical biochemical preparation of the enzyme yielded several activities with varying responses to Ca^{2+} concentrations, detergents, pH, unsaturated fatty acids and phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) [reviewed in 7]. The first

* Corresponding author.

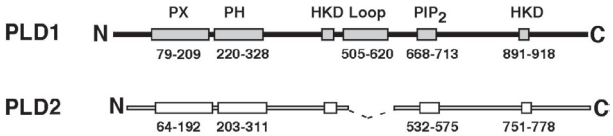

Figure 1. Basic structure of PLD1 and PLD2. Linearized cartoon of structure showing amino (N) and carboxy (C) terminals with conserved domains as boxes. Domains shown are the catalytic HKD motif (HKD), phox consensus sequence (PX), plekstrin homology (PH), phosphatidylinositol bisphosphate (PIP_2) and PLD1 loop region.

purified PLD enzymes were independently isolated from cabbage [12] and castor bean [13]. The purification of the two plant PLD enzymes led to the cloning of PLDα from the castor bean [14], followed by rice, maize, tomato and others [15–17].

SPO14, a yeast sporulation mutant identified through a screen of mutated strains that were defective in meiosis, was found to have sequence similarity to plant PLDα and to exhibit PLD activity [18 20]. This protein was activated by $PI4,5P_2$ [18] similar to a reported mammalian activity [21], but unlike the mammalian activity was unresponsive to ADP Ribosylation Factor (ARF) [22]. Cloning of *SPO14* facilitated the subsequent cloning of a mammalian 120-kDa protein designated as PLD1 [23] that was stimulated by $PI4,5P_2$ and GTPγS-loaded ARF [23]. A splice variant, still of unknown biological significance, was subsequently found, necessitating the designation of the original enzyme as PLD1a and the splice variant as PLD1b [24, 25]. Both enzymes were similarly activated by protein kinase C (PKC), Rho GTPases and ARF GTPases [24 26]. A second mammalian PLD enzyme was discovered and designated as PLD2 [26 28]. Initial characterization of PLD2 revealed that it exhibited a significantly higher basal activity and was not activated by ARF or Rho GTPases. However, PLD2 did require $PI4,5P_2$ [27]. PLD1 and PLD2 comprise the classical mammalian PLD enzyme family.

Mammalian PLD structure

The defining feature of PLD enzymes is the catalytic motif designated HKD, denoting the HxxxxKxD sequence, where the amino acids are histidine (H), any amino acid (x), lysine (K) and aspartic acid (D). Mammalian PLD1 and PLD2 both contain two HKD motifs, which are critical for enzymatic catalysis both in vitro and in vivo, as evidenced by the observation that point mutations in the motif disrupt PLD activity [29]. The mechanism of PC catalysis will be discussed later in this review.

Other highly conserved regions of the PLD genes are the phox consensus sequence (PX), the plekstrin homology (PH) domain and the $PI4,5P_2$ binding site (fig. 1). The PH domain is thought to function in the localization of the protein, as either careful deletion or point mutation causes mislocalization of the proteins [30, 31]. More recently,

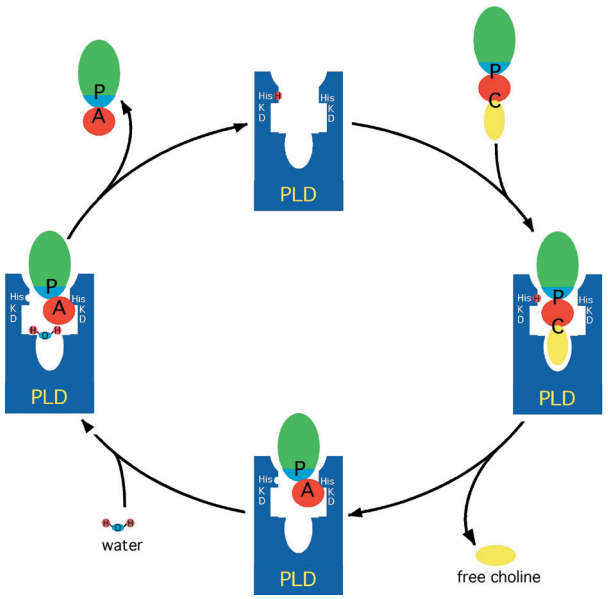

Figure 2. Cartoon of PLD hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. Shown in white are the essential amino acids of the two PLD catalytic domains: histidine (His), lysine (K) and aspartic acid (D). The steps of the process are summarized. Top center, open configuration with hydrogen (H) bound to amino-terminal histidine. Top right, PC enters PLD active pocket. Right center, PC in the active pocket. Bottom right, release of free choline. Bottom center, PA-PLD intermediate with PA bound to the carboxy-terminal histidine. Bottom left, water enters the active pocket. Left center, PA-PLD intermediate with water as the nucleophile in the active pocket. Left top, release of PA and regeneration of open state with bound hydrogen. Mechanism discussed in more detail in the text, and the lipids are color-coded to their structures in Figure 3.

the PH domain was demonstrated to mediate the PLD1 association with lipid rafts to facilitate an early step in the recovery of the enzyme to endosomes after stimulated translocation to the plasma membrane [32]. However, deletion of the PH domain demonstrated it is not required for enzymatic activity and furthermore did not alter the dependence on $PI4,5P_2$ for catalysis [33, 34]. This finding led to the discovery of the $PI4,5P_2$ binding motif [30, 32, 35]. The PX domain is thought to mediate protein-protein interactions or to bind phosphatidylinositol phosphates (PIP) [reviewed in 36]. Recently, the PLD PX domain was described to bind preferentially to phosphatidylinositol trisphosphate ($PI3,4,5P_3$), in comparison with other PIP species, including PI5P, as determined by a surface plasmon resonance assay [37]. In contrast, PX was determined to bind PI5P preferentially in a liposome binding assay, and the domain binding of PI5P, a lipid required for endocytosis, has been proposed to facilitate internalization of PLD1 from the plasma membrane [32]. Finally, PLD1 has a conserved loop region that is not found in PLD2. The loop region has been proposed to function as a possible negative regulatory element, as deletion of this region from PLD1 increased its basal activity threefold [33].

Overview of PLD localization

Study of the subcellular localization of PLD1 and PLD2 has yielded varying descriptions. Reports on PLD1 in numerous cell lines have described perinuclear localization suggestive of a Golgi, endoplasmic reticulum or late endosomal distribution [27, 33, 38, 39]. A contrasting report described PLD1 localizing to only the late endosomes and lysosomes [40]. Recently, PLD1 localized to the sorting/recycling endosomes and early endosomes with some PLD1 in the Golgi [32]. However, several groups have not found Golgi localization [40, 41]. PLD1 has also been localized to the plasma membrane in unstimulated cells [42]. Several papers now report that upon stimulation, PLD1 translocates to the plasma membrane [32, 43, 44]. With the emerging concept of dynamic cycling of PLD1 inside the cell, some of the varying reports of localization may be due to differential rates and number of vesicles cycling in the cell lines used and thus differential regulation of PLD1 localization.

PLD2 is most often reported to localize to the plasma membrane [27, 45, 46], but has also been localized to the cytosol [45], submembraneous vesicular compartments [47] and co-localized with β-actin [48]. PLD2 has been described as translocating to membrane ruffles in response to serum [27] and epidermal growth factor (EGF) [45].

Intracellular regulators of PLD

Mammalian PLD activity is regulated by many factors, including phosphoinositides, PKC, ARF and Rho GTPases and non-PKC protein phosphorylation. Each of these regulators will be briefly discussed [reviewed in 2, 7, 49, 50].

Phosphoinositides are lipids related by the base molecule of phosphatidylinositol (PI). PI can be phosphorylated to form PIPs, PIP_2s and PIP_3. As mentioned above, mammalian PLDs have a $PI4,5P_2$ binding domain. PLD activities from HL60 cellular extracts are stimulated by $PI4,5P_2$ [21, 51], and both PLD1 and PLD2 enzymatic activities are dependent upon $PI4,5P_2$ [23, 25, 27, 28, 52]. PIP_3 activates PLD with similar potency to $PI4,5P_2$, but with lessened efficacy [25]. Clearly, the phosphoinositides are important mediators of PLD activation and regulation.

PLD activation has long been observed in response to PKC activating factors such as calcium ionophores and phorbol esters such as phorbol 12-myristate-13-acetate (PMA), which is a stable analogue of diacylglycerol (DAG). The PKC regulation of PLD activation is very intertwined and complex due to the large variety of PKC enzymes that may regulate PLD [reviewed in 7]. A few basic points on the interactions between PKC and PLD are presented. Much of the regulation of PLD by the classical PKCs was thought to involve the direct phosphorylation of PLD [reviewed in 53]. In support of this model, studies employing downregulation of PKC by chronic PMA treatment rendered PLD unresponsive to activation [5, 54]. However, the classical PKCs also effect PLD activation independent of their kinase activity [25, 55, 56], and the activation may be mediated by the N-terminal portion of PLD [57]. PKC inhibitors, such as staurosporine and calphostin C, block PLD activation [58 61]. Care must be taken though, as more recent studies have shown that calphostin C has a direct inhibitory effect on PLD independent of its inhibition of PKC [62]. Some studies suggested the inhibition of PLD via PKCδ, thus antagonizing activation by PKCα [63]. Overall, the regulation of PLD by PKC appears most likely to involve direct interaction, phosphorylation and possibly other indirect mechanisms.

ARF was initially identified as an activator of a PLD activity extracted from HL60 cells [21, 51]. ARF directly activates PLD1 [23] and can activate PLD2 [28, 34]. However, in vitro assays have identified accessory factors, including guanine nucleotide exchange factors [reviewed in 64] and have indicated indirect regulatory pathways [65, 66]. Use of the ARF inhibitor Brefeldin A and expression of dominant negative ARF1 or ARF6 blocked stimulation of PLD by various means [67 69]. Evidence for the role of ARF in PLD activation is most strong in vesicular transport [reviewed in 70].

Rho GTPases such as RhoA, Cdc42Hs and Rac1 activate PLD in many cell types and tissues [71 74]. In the presence of GTPγS, these Rho proteins interact directly with purified PLD1 [25, 75, 76]. Pretreatment of *Clostridium botulinum* C3 toxin or *Clostridium difficile* toxins, all of which inactivate Rho proteins, blocks PLD activation [77 81]. Mutation of the Rho binding site on PLD1 [82, 83] demonstrated direct binding of RhoA and PLD1, and defined possible in vivo roles for the interaction [84]. However, the myriad possibilities of indirect Rho activation of PLD complicates the study of PLD as a downstream effector of Rho proteins.

PLD is likely to be regulated by protein phosphorylation beyond that mentioned for PKC kinase activity. Several studies using protein kinase inhibitors have implicated both receptor and non receptor tyrosine kinases in this regulation, along with serine/threonine kinases, calcium calmodulin dependent protein kinase and cyclic AMP (cAMP) kinases [85 88]. The role for phosphorylation of PLD by the above kinases remains poorly defined, but is likely to be involved either directly or indirectly in PLD function.

PLD as a lipid-modifying enzyme

Mammalian phospholipase D enzymatic action produces PA (fig. 2), which is a bioactive lipid. In contrast, free choline is not thought to fulfill any intracellular signal-

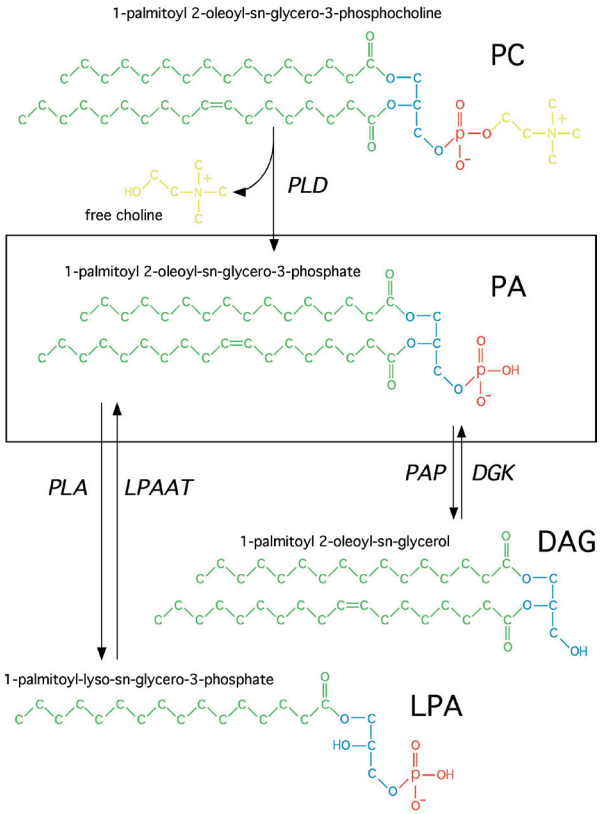

Figure 3. Generation of (16:0/18:1) phosphatidic acid (PA) from (16:0/18:1) phosphatidylcholine (PC) and interconversion of PA to (16:0/18:1) diacylglycerol (DAG) and (16:0) lyso-phosphatidic acid (LPA). Enzymes abbreviated are phospholipase D (PLD), phosphatidic acid phosphohydrolase (PAP), diacylglycerol kinase (DGK), phospholipase A (PLA) and lyso-phosphatidic acid acyltransferase (LPAAT). Fatty acid acyl chains are in green, glycerol backbone in blue, phosphate in red and choline in yellow.

ing roles [89]. The in vivo nucleophile is water, which attacks the diester phosphate group of PC. Both HKD motifs of PLD are needed for the enzymatic action of hydrolysis [29]. The current model of PC catalysis is a two-step mechanism based upon the crystallization of a bacterial endonuclease, which has a divergent HKD motif [90 92]. Initially, the amino-terminal HKD motif is proposed to be protonated on the histidine. Subsequently, PC enters the active pocket of PLD containing both HKD motifs. Next, free choline is released by liberation of the amino-terminal proton, and a PLD-PA intermediate is thought to be formed with the histidine of the carboxy-terminal HKD domain. Finally, the amino histidine re-acquires a proton from a water molecule, leaving the hydroxyl group to attack the PLD-PA intermediate and release PA [29, 90]. The use of ethanol or 1-butanol allows for a cumulative measurement of PLD activity, as the non-endogenous PBut or phosphatidylethanol thus formed are relatively stable lipids [93].

Structure of PA

PA is composed of three distinct parts (fig. 3). The basis of the molecule is the three-carbon glycerol backbone. The hydroxy groups of carbons 1 and 2 are ester-linked to fatty acids forming the non-polar acyl chains of the lipid. The fatty acids can vary in length (14–22 carbons long endogenously) [94] and the number of double bonds present in the carbon chain (saturation). The terminal carbon 3 is ester-linked to a phosphate group forming the small, negatively charged polar headgroup of PA. PC has the addition of an ester-linked choline to the phosphate headgroup of PA, thus forming the large polar headgroup of this lipid.

Further conversion of PA

The PLD-produced PA can be converted into DAG by the family of enzymes known as phosphatidic acid phosphohydrolases (PAPs) [95 97], which are highly active in vivo. As discussed in more detail below, activation of PLD results in the increase of both PA and DAG. The DAG produced can be reconverted to PA via phosphorylation by diacylglycerol kinases (DGKs). The regulation of PA and DAG levels appears to be tightly controlled via the activities of PAP and DGK. Furthermore, PA can be deacylated by a phospholipase A (PLA) activity to form lysoPA (LPA), a monoacylated form of PA [97], and subsequently reconverted to PA by lysophosphatidic acid acyltransferases (LPAAT). Figure 3 summarizes the above lipid conversion pathways and details the lipid structures of PC, PA, DAG and LPA.

PLD substrate specificity

The initial mammalian PLD activity described was reported to use both PC and PE as in vitro substrates [11]. In the first report on the cloned and purified recombinant isoforms, reactivity against PC but not PE was observed [23]. More recently, PLD-enriched lysates were reported to exhibit robust activity on PC, marginal activity on PE and no activity on phosphatidylinositol, although since the PLD was not purified, it is not clear whether the minor PE-hydrolyzing activity was directly mediated by the classical PLD isoforms [94]. However, several early papers, especially Pai et al., clearly demonstrated PLD enzymatic activity on PC in vivo, solidifying PC as the natural substrate [98 102], and subsequent work using the recombinant PLD1 and PLD2 proteins has confirmed this [94].

These early papers also partially defined the species of PC acted upon by PLD in vivo in terms of their fatty acid composition, mostly through the study of the PA and DAG produced. Cockcroft and Allan [103] described a rapid increase in PA, followed by an increase in DAG in response to cytochalasin and formylmethionyl-leucyl-

phenylalanine (now thought to activate PLD) treatment of neutrophils. Analysis of the fatty acid composition of the PA and DAG revealed a composition mainly of palmitate (16 carbons long with no double bonds, 16:0), stearate (18:0) and oleate (18:1). In contrast, the PI fatty acid composition of stearate (18:0) and arachidonate (20 carbons long with four double bonds 20:4) indicates that the PA and DAG produced were not likely to be from cleavage of PI [103]. In response to phorbol diesters, Besterman et al. [101] showed a rapid production of DAG and PA (15–80 s) that had fatty acid compositions resembling that of endogenous PC. Although not mentioned, the responsible enzyme activated by the phorbol diester, perhaps through PKC, is presumed to be PLD [101]. Subsequently, vasopressin was found to rapidly (2–5 min.) produce PA and DAG in a PLD-dependent manner. Analysis of the PA and DAG produced indicated that the fatty acid composition of these lipids was stearate, (18:0) and arachidonate (20:4) [100].

The most recent and comprehensive studies on PLD hydrolyzed and produced lipids, including PBut, which is exclusively formed by PLD, have clearly defined the fatty acid composition of these lipids [94, 104]. Stimulation of Swiss 3T3 cells by bombesin or LPA resulted in PA and PBut with acyl chains that are predominantly saturated or monounsaturated. In this case, the acyl chains that were derived from PLD activation were palmitate (16:0), stearate (18:0) and oleate (18:1) [104]. In addition, the DAG produced by PAP dephosphorylation of PA is different from polyunsaturated DAG produced via phospholipase C cleavage of $PI4,5P_2$ [104]. PLD was shown to hydrolyze all forms of PC in both in vitro and in vivo assays [94]. The in vitro specificity of PLD hydrolysis of egg PC was preferential to species of 16:0/18:2, 16:0/18:1 and 18:0/18:2 or 18:1/18:1. However, the in vivo specificity for PC was limited to mainly monounsaturated or saturated acyl chains (16:0/18:1, 18:0/18:1 and 18:1/18:1 or 18:0/18:2) and was determined by analysis of the PBut formed. The diacyl profile of PBut formed was comparable to that of endogenous PC [94]. Interestingly, endogenous PA levels were not significantly altered with incubation of 1-butanol, indicating that a majority of cellular PA is not derived from PLD action; however, the speciation of this PA was similar to that of PA produced via PLD [94]. Overall, PLD seems to hydrolyze PC with either monounsaturated and/or saturated acyl chains, forming PA of the same diacyl composition.

PA as mediator of PLD function

The PA generated via PLD activity can play multiple roles in cellular function. PA can function in signal transduction events, such as activation of a phosphoinositide kinase, or as a lipid-binding partner to several proteins, thus effecting protein recruitment. PA can also be an intermediate for the production of bioactive DAG or LPA via the mechanisms previously discussed. Finally, PA production has been shown to be important in vesicle trafficking, endocytosis and secretion. The role for PA in these processes is likely to be a combination of such activities as second messenger, lipid anchor and possibly fusogenic lipid. The following sections will focus upon these cellular functions.

PA in signal transduction

PA has been linked to many events of intracellular signal transduction through the wide use of 1-butanol as an inhibitor of PA accumulation via PLD. These pathways of activation include signaling through PI4P 5-kinase, activation of mammalian target of rapamycin (mTOR), and direct binding with Raf kinase, among other proteins.

The stimulation of type I PI4P 5-kinase, to produce $PI4,5P_2$ from PI4P, has emerged as a key downstream effect of PA generation via PLD. PA, but no other phospholipid tested including LPA, was initially found to activate PI4P 5-kinase in an in vitro assay of enzyme purified from bovine brain [105]. Further purification and in vitro assays revealed that PA stimulated only type I, but not type II PI4P 5-kinase [106]. Subsequent cloning of a third isoform of a type I PI4P 5-kinase confirmed the aforementioned activation of the enzyme by PA and linked the function to actin polymerization in vivo [107], in which PLD has been suggested to play a role [108]. The activation of PI4P 5-kinase in membrane ruffling mediated by EGF was described to proceed via activation and translocation of PLD2 to the ruffles resulting in PA production in the ruffles, where the PI4P 5-kinaseα is also localized [45]. LPA treatment of porcine aortic endothelial cells caused activation of type I PI4P 5-kinaseα that was determined to be dependent upon PLD production of PA [109]. In this paper, the authors showed that PA with mixed diacyl chain speciation from egg yolk extracts stimulated kinase activity. Furthermore, a dioctanoyl (8:0) PA had similar efficacy in stimulating PI4P 5-kinase [109]. The specificity of what type of PA can or cannot activate PI4P 5-kinase is still to be determined. The early findings probably used mixed acyl chain PA extracted from various sources. However, the comparable efficacy of the dioctanoyl PA does indicate that the length of the acyl chains need not be in the endogenous range of 14–22 carbons long [94].

PLD has been implicated in survival pathways that prevent apoptosis [110 112, 113 among others]. As an effector of cell cycle progression and cell proliferation, mTOR represents an attractive downstream effector of PLD's potential anti-apoptotic function. HEK293 cells transiently increased PA in response to serum stimulation, resulting in activation of mTOR and its downstream acti-

vation of S6 kinase1 (S6K1), whose blockage by 1-butanol implicates PLD involvement [114]. Furthermore, PA was found to directly interact with mTOR at the FK506 binding protein 12-rapamycin-binding domain of mTOR via in vitro binding assays [114, 115]. The binding was specific for PA among several phospholipids (PC, PS, PE, PI and PIPs) tested. Pretreatment with rapamycin, an inhibitor of mTOR, blocked PA interaction with mTOR, thus demonstrating competitive binding [114]. Overexpression of PLD2 was found to confer resistance to rapamycin blockage of cell proliferation, further involving PLD in mTOR regulation [116]. Lipopolysaccharide (LPS) stimulation caused an increase in PA, and this increase was determined to play a role in cytokine release through activation of mTOR. The cytokine release was potentiated by addition of propranolol (inhibitor of PAP) and blocked by 1-butanol, implicating PLD. The authors proposed PLD2 as the relevant enzyme, due to its high expression level as determined by Western blotting [117]. However, Fang et al. (2003) demonstrated in HEK293 cells that PLD1 was the enzyme responsible for mTOR signaling and suggested that Cdc42 is upstream of PLD1. Overexpression of the wild-type PLD1 increased S6K1 activity in response to serum stimulation, while overexpression of the catalytically inactive PLD1 decreased activity compared with control cells. In addition, the use of small interfering RNA (siRNA) against PLD1 in two different cell lines almost completely inhibited S6K1 activity in response to serum stimulation [118]. More recent work has agreed with PLD1 being the enzyme responsible for mTOR activation [119, 120]. Overall, mTOR is emerging as a well-defined target of PA produced by PLD1, and this effect has been determined to be parallel to the phosphoinositide 3-kinase survival pathway [114, 118 121].

In spite of the importance of PA in the mediation of various signaling events, the direct targets of PA remain elusive. A few proteins, including mTOR, which bind PA directly have been described. PA bound to agarose beads was found to pull down ARF, N-ethylmaleimide-sensitive factor and kinesin [122]. One of the first proteins to emerge as a well-defined PA binding protein was Raf-1 kinase [123, 124], and this protein was shown to translocate to PA-enriched membranes [125, 126]. Dioleoyl PA (18:1/18:1) was determined to interact directly with the protein via an in vitro assay using both phospholipids and sphingolipids such as PC, PS, PI, sphingosine and ceramide; however, cardiolipin also bound the protein. The carboxyl terminus of Raf-1 kinase was determined to contain a 35-amino acid segment that bound PA [125], and mutation of a conserved basic amino acid, arginine 398 to alanine, in this region reduced the PA binding. Also, fusion of the binding region to green fluorescent protein (GFP) was sufficient to translocate the recombinant protein to membranes [124]. Finally, a specific tetrapeptide motif was defined for the binding of PA to Raf-1 kinase [123]. Protein phosphatase-1γ (PP1γ) has emerged as a direct target for inhibition by PA [127]. Phosphatase activity of this protein was inhibited by PA, but no effect was found for DAG, PBut, LPA and phosphatidylethanol. Binding of the catalytic subunit of PP1γ was specific for PA since PS, PI, PC, PE and LPA, among other phospholipids, did not bind. The diacyl composition of PA needed for inhibition was not dependent upon the saturation of the fatty acyl chains [127]. A microdomain in the N-terminal region of PDE4A1 cAMP-specific phosphodiesterase bound PA, but not PC, PS, PIP or PIP_2 in an in vitro assay. TAPAS-1 (tryptophan anchoring phosphatidic acid selective binding domain 1) was defined in this amino-terminal microdomain and shown to provide the membrane association through PA for this phosphodiesterase [128]. Sphingosine kinase1 (SK1) also binds PA, and this direct interaction is needed for its translocation. SK1 is also proposed as an intracellular effector of PA [129].

PA as a lipid precursor

The conversion of PA by PAP produces DAG, which has long been studied as a lipid second messenger. PAP2b, a PAP isoform, co-localizes to PLD2-enriched membrane domains, thus possibly contributing to a rapid turnover of PLD-produced PA to DAG [95]. DAG has been repeatedly shown to activate the classical and most novel PKC families [reviewed in 130, 131]. The conversion of PA by PAP results in DAG species whose diacyl composition is primarily of the monounsaturated and/or saturated species as discussed in detail above [94, 104]. Significantly, the more saturated forms of DAG have been suggested to not activate PKC in vivo [104, 132]. Therefore, the possible role for these saturated DAGs produced from PA remains to be determined. Conversely, the production of DAG from PLD-derived PA may ultimately be a way to attenuate the PA signal.

PA can also be converted to mono-acylated LPA by phospholipase A activity [97]. LPA is a potent mitogen that performs key roles in cell proliferation, migration and survival [reviewed in 133]. The production of LPA from PLD-produced PA has not yet been fully demonstrated or intensively studied. The specific function of a monounsatured or saturated acyl species of LPA produced from PLD PA is potentially an interesting question.

PA in vesicle trafficking, secretion and endocytosis

PLD and its enzymatic product PA have been linked to vesicular traffic, secretion and endocytosis [reviewed in 6]. PLD is associated with Golgi vesicle formation through its activation by ARF. The use of primary alcohols including ethanol blocked protein transport from the

endoplasmic reticulum to Golgi, and the block was alleviated by exogenous liposomes containing PA [6, 134, 135]. PA recruitment of proteins has been suggested in the formation of secretory vesicles from the trans-Golgi network [136]. However, the role for PLD in vesicle formation is unclear to date, and the localization of PLD to the Golgi remains debated, as mentioned in the cellular localization section.

In contrast, the role of PLD in secretion and secretory vesicle formation is much more established. PLD plays a role in exocytosis [137 140] in an ARF-dependent manner [141, 142]. The role for PLD in secretion has been studied mainly in neuroendocrine cells [66, 136, 143, 144], but has also been studied in adipocytes [44, 145], pancreatic β cells [146] and mast cells [43, 147, 148]. In neuroendocrine cells ARF6 has been specifically demonstrated to act through PLD1 in a $PI4,5P_2$-dependent manner in the exocytic process [143, 149]. Recently, the PH domain of PLD1 was shown to be required for regulated exocytosis of neuroendocrine cells [46]. The inhibition of PLD has also been implicated in the sphingosine 1-phosphate-induced secretion of interleukin-8 from human bronchial epithelial cells [150].

PLD2 has recently emerged as an enzymatic mediator of receptor internalization via endocytosis. Initially, epidermal growth factor receptor (EGFR) internalization was found to be blocked by treatment with 1-butanol, and EGF binding to the receptor was determined to increase PA. These findings implicated PLD in this process. The internalization increased with overexpression of either PLD1 or PLD2 and decreased by overexpression of their catalytically inactive forms [151]. Examination of the μ-opioid receptor MOR1 uncovered a functional association between this receptor and PLD2. The authors showed that one receptor agonist that causes internalization activated PLD2, while one that does not cause internalization did not activate PLD2. Furthermore, the internalization was ARF-dependent [152]. Internalization of MOR1 was inhibited by either 1-butanol or overexpression of the catalytically inactive PLD2, thus defining the role of PLD2 in receptor internalization [152, 153]. In addition, both 1-butanol and siRNA against PLD2 blocked metabotropic glutatmate receptor endocytosis [154]. Overexpression of the catalytically inactive PLD2 and siRNA against PLD2 individually blocked angiotensin II receptor internalization [46]. Overall, receptor endocytosis appears to be a function for PLD2 in cells.

PA derived from PLD hydrolysis of PC has many functions which probably contribute to vesicle trafficking in cells. PA can act as a lipid anchor and recruiter for proteins, activate PI4P 5-kinase to form $PI4,5P_2$ and possibly undertake a biophysical role as a fusogenic lipid (fig. 4). $PI4,5P_2$, generated via PA activation of PI4P 5-kinase, recruits CAPS, thus enabling exocytosis of dense core vesicles [155, 156]. Furthermore, the role for PLD in fusion is compelling since it cleaves a non-fusogenic lipid, PC, to form a fusogenic lipid, PA. Due to its small headgroup and two fatty acyl chains, PA is thought to lower the activation energy required for negative curvature at the inward membrane curve [157]. PA can be further converted into two other fusogenic lipids, DAG [158] and LPA. LPA is thought to promote the opposite membrane curvature of PA [159]. The LPAAT enzyme, endophilin, has been reported to convert LPA to PA and is known to promote endocytosis [reviewed in 160]. In vitro reconstitution studies showed that the addition of PA to vesicles increases their rate of fusion [161]. Inhibition of PLD1 resulted in a lessening of the exposure of a glucose transporter on the external leaflet of the plasma membrane, but did not block the vesicle trafficking to the membrane. These findings indicate a possible block in fusion due to lack of PA generation [44]. Clearly, PLD-generated PA and its further metabolites such as LPA may function in both fusion with membranes and fission of vesicles from membranes.

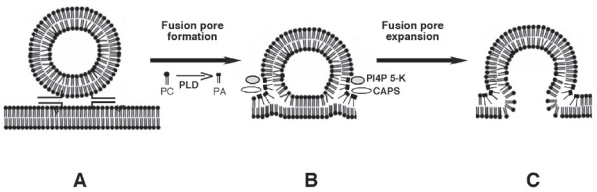

Figure 4. Vesicle fusion to a membrane. (A) Vesicle attached to the membrane. During fusion pore formation PLD is proposed to convert PC to PA. (B) Formation of a pore between the vesicle and membrane. At this point, PA is proposed to lower the energy of activation for the curvature of the inner membrane. In turn, PA may activate PI4P 5-Kinase (PI4P 5-K), increasing the membrane levels of $PI4,5P_2$ and thus recruiting CAPS. (C) Fully formed pore between vesicle and membrane.

Discussion

The study of mammalian PLD was significantly moved forward with the cloning of PLD1 and PLD2. This key event has led to the determination of many functional motifs and domains for PLD1 and PLD2. A great deal has been learned about the regulation of PLD enzymes from studies of these domains and has led to a further understanding of PLD enzymes and their possible in vivo roles. The ability of PLD to perform a transphosphatidylation reaction in vivo has caused the finding of a plethora of cellular stimuli which activate PLD. Unfortunately, this method does not determine which PLD isoform is activated, or whether the activation is sufficient or necessary for the given response to the various stimuli tested. However, the use of other experimental techniques has demonstrated a requirement for PLD activation in many cellular responses, such as signal transduction including

activation of PI4P 5-kinase, activation of mTOR for survival and a role in extracellular calcium entry into cells [162, 163]. PLD also has roles in vesicle formation and fusion in both endocytosis and secretion, cellular differentiation and proliferation. The study of PLD will be furthered with future development of specific inhibitors and a knockout mouse model. With several PLDs identified in mammalian cells and some unidentified PLD activities, discernment of the exact enzyme involved in a particular function has proven to be tricky. This problem is being overcome by the overexpression of catalytically active and inactive forms of the specific PLD enzymes in vivo. Furthermore, the rapid development of siRNA is leading to finer differentiation between the functions of PLD1 and PLD2, and may eventually lead to techniques to discern the relevance of PLD splice variants in a given cellular response. More careful study of PLD1 and PLD2 location and translocation is defining the subcellular compartments where these enzymes perform their functions and thus is contributing to a better understanding of their roles. Overall, the study of PLD is becoming much more focused on the specific PLD enzyme involved and subcellular location of action.

In contrast to the number of systems in which PLD is activated are the limited number of direct targets and direct functions for PA. Research is currently focusing on proteins that directly interact with PA, and this interaction can serve at least two functions: (i) regulation of enzymatic activities, and (ii) recruitment of proteins to specialized membranes. Also, theoretical models suggest that PA may play a biophysical role in mediating PLD functions such as fusion. The current and future development of PA sensors such as TAPAS1 and the yeast Opi1p [164] will help define where PA levels are changing in cells. These data will be compared to localization of PLD to better understand the coordination between enzyme, product and target. Additionally, the use of improved lipid measurements, such as tandem mass spectrometry, will allow for the discernment of increases in the molecular species of PA in a given situation, which will provide clues as to whether the PA is generated from diacylglycerol kinase activity on polyunsaturated PA or PLD cleavage of monounsaturated or saturated PC. Also, with the improved sensitivity of mass spectrometry analysis, one can potentially isolate defined subcellular compartments and measure PA levels and species therein. The further study of PA will lead to a more complete understanding of PLD.

Acknowledgements. This work was supported by grants from the American Diabetes Association and the National Institutes of Health (DK64166) to M.A.F. G.M.J received support from Institutional Postdoctoral National Research Service Award no. 2 -T32 DK07521 from the NIDDK, NIH. The authors would like to thank Dr. Guangwei Du, Dr. Ping Huang, Dr. Seok-Yong Choi and Dr. Margie LaLonde for input and proofreading of the manuscript.

1 Yang S. F., Freer S. and Benson A. A. (1967) Transphosphatidylation by phospholipase D. J. Biol. Chem. **242:** 477–484
2 Frohman M. A., Sung T. C. and Morris A. J. (1999) Mammalian phospholipase D structure and regulation. Biochim. Biophys. Acta **1439:** 175–186
3 Kinsky S. C., Loader J. E. and Benedict S. H. (1989) Phorbol ester activation of phospholipase D in human monocytes but not peripheral blood lymphocytes. Biochem. Biophys. Res. Commun. **162:** 788–793
4 Bradshaw C. D., Ella K. M., Qi C., Sansbury H. M., Wisehart-Johnson A. E. and Meier K. E. (1996) Effects of phorbol ester on phospholipase D and mitogen-activated protein kinase activities in T-lymphocyte cell lines. Immunol. Lett. **53:** 69–76
5 Exton J. H. (1999) Regulation of phospholipase D. Biochim. Biophys. Acta **1439:** 121–133
6 Jones D., Morgan C. and Cockcroft S. (1999) Phospholipase D and membrane traffic. Biochem. Biophys. Acta **1439:** 229–244
7 McDermott M., Wakelam M. J. and Morris A. J. (2004) Phospholipase D. Biochem. Cell Biol. **82:** 225–253
8 Liscovitch M., Czarny M., Fiucci G. and Tang X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. **345:** 401–415
9 Hanahan D. J. and Chaikoff I. L. (1947). The phosphorus-containing lipides of the carrot. J. Biol. Chem. **168:** 233–240
10 Hanahan D. J. and Chaikoff I. L. (1948). On the nature of the phosphorus-containing lipides of cabbage leaves and their relation to a phospholipide-splitting enzyme contained in these leaves. J. Biol. Chem. **172:** 191–198
11 Saito M. and Kanfer J. (1975) Phosphatidohydrolase activity in a solubilized preparation from rat brain particulate fraction. Arch. Biochem. Biophys. **169:** 318–323
12 Abousalham A., Riviere M., Teissere M. and Verger R. (1993) Improved purification and biochemical characterization of phospholipase D from cabbage. Biochim. Biophys. Acta **1158:** 1–7
13 Wang X., Dyer J. H. and Zheng L. (1993) Purification and immunological analysis of phospholipase D from castor bean endosperm. Arch. Biochem. Biophys. **306:** 486–494
14 Wang X., Xu L. and Zheng L. (1994) Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from *Ricinus communis*. J. Biol. Chem. **269:** 20312–20317
15 Ueki J., Morioka S., Komari T. and Kumashiro T. (1995) Purification and characterization of phospholipase D (PLD) from rice (*Oryza sativa* L) and cloning of cDNA for PLD from rice and maize (*Zea mays* L). Plant Cell Physiol. **36:** 903–914
16 Pappan K. and Wang X. (1999) Molecular and biochemical properties and physiological roles of plant phospholipase D. Biochim. Biophys. Acta **1439:** 151–166
17 Cha Y. R., Lee K. W., Moon Y. H., Kim J. C., Han T. J., Lee W. S. et al. (1998) Cloning of a cDNA encoding phospholipase D from Pimpinella brachycarpa. Mol. Cells **8:** 19–26
18 Rose K., Rudge S. A., Frohman M. A., Morris A. J. and Engebrecht J. (1995) Phospholipase D signaling is essential for meiosis. Proc. Natl. Acad. Sci. USA **92:** 12151–12155
19 Ella K. M., Dolan J. W., Qi C. and Meier K. E. (1996) Characterization of Saccharomyces cerevisiae deficient in expression of phospholipase D. Biochem. J. **314:** 15–19
20 Waksman M., Eli Y., Liscovitch M. and Gerst J. E. (1996) Identification and characterization of a gene encoding phospholipase D activity in yeast. J. Biol. Chem. **271:** 2361–2364
21 Brown H. A., Gutowski S., Kahn R. A. and Sternweis P. C. (1995) Partial purification and characterization of ARF-sensitive Phospholipase D from porcine brain. J. Biol. Chem. **270:** 14935–14943
22 Rudge S. A., Cavenaugh M. M., Kamath R., Sciorra V. A., Morris A., Kahn R. A. et al. (1998) ADP-Ribosylation factors do not activate yeast Phospholipase Ds but are required for sporulation. Mol. Biol. Cell **9:** 2025–2036
23 Hammond S. M., Altshuller Y. M., Sung T. C., Rudge S. A., Rose K., Engebrecht J. et al. (1995) Human ADP-ribosylation

factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. J. Biol. Chem. **270:** 29640–29643
24 Katayama K., Kodaki T., Nagamachi Y. and Yamashita S. (1998) Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver. Biochem. J. **329:** 647–652
25 Hammond S. M., Jenco J. M., Nakashima S., Cadwallader K., Gu Q., Cook S. et al. (1997) Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. J. Biol. Chem. **272:** 3860–3868
26 Kodaki T. and Yamashita S. (1997) Cloning, expression and characterization of a novel Phospholipase D cDNA from rat brain. J. Biol. Chem. **272:** 11408–11413
27 Colley W. C., Sung T. C., Roll R., Jenco J., Hammond S. M., Altshuller Y. et al. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr. Biol. **7:** 191–201
28 Lopez I., Arnold R. S. and Lambeth J. D. (1998) Cloning and initial characterization of a human phospholipase D2 (hPLD2). J. Biol. Chem. **273:** 12846–12852
29 Sung T. C., Roper R. L., Zhang Y., Rudge S. A., Temel R., Hammond S. M. et al. (1997) Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. EMBO J. **16:** 4519–4530
30 Sciorra V. A., Rudge S. A., Wang J., McLaughlin S., Engebrecht J. and Morris A. J. (2002) Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J. Cell Biol. **159:** 1039–1049
31 Sugars J. M., Cellek S., Manifava M., Coadwell J. and Ktistakis N. T. (2002) Hierarchy of membrane-targeting signals of phospholipase D1 involving lipid modification of a pleckstrin homology domain. J. Biol. Chem. **277:** 29152–29161
32 Du G., Altshuller Y. M., Vitale N., Huang P., Chasserot-Golaz S., Morris A. J. et al. (2003) Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. J. Cell. Biol. **162:** 305–315
33 Sung T. C., Zhang Y., Morris A. J. and Frohman M. A. (1999) Structural analysis of human phospholipase D1. J. Biol. Chem. **274:** 3659–3666
34 Sung T. C., Altshuller Y. M., Morris A. J. and Frohman M. A. (1999) Molecular analysis of mammalian phospholipase D2. J. Biol. Chem. **274:** 494–502
35 Sciorra V. A., Rudge S. A., Prestwich G. D., Frohman M. A., Engebrecht J. and Morris A. J. (1999) Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. EMBO J. **18:** 5911–5921
36 Xu Y., Seet L. F., Hanson B. and Hong W. (2001) The Phox homology (PX) domain, a new player in phosphoinositide signalling. Biochem. J. **360:** 513–530
37 Stahelin R. V., Ananthanarayanan B., Blatner N. R., Singh S., Bruzik K. S., Murray D. et al. (2004) Mechanism of membrane binding of the phospholipase D1 PX domain. J. Biol. Chem. **279:** 54918–54926
38 Freyberg Z., Sweeney D., Siddhanta A., Bourgoin S., Frohman M. and Shields D. (2001) Intracellular localization of phospholipase D1 in mammalian cells. Mol. Biol. Cell **12:** 943–955
39 Lucocq J., Manifava M., Bi K., Roth M. G. and Ktistakis N. T. (2001) Immunolocalisation of phospholipase D1 on tubular vesicular membranes of endocytic and secretory origin. Eur. J. Cell Biol. **80:** 508–520
40 Toda K., Nogami M., Murakami K., Kanaho Y. and Nakayama K. (1999) Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes. FEBS Lett. **442:** 221–225
41 Hughes W. E. and Parker P. J. (2001) Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity. Biochem. J. **356:** 727–736
42 Kim J. H., Kim Y., Lee S. D., Lopez I., Arnold R. S., Lambeth J. D. et al. (1999) Selective activation of phospholipase D-2 by unsaturated fatty acid. FEBS Lett. **454:** 42–46
43 Brown F. D., Thompson N., Saqib K. M., Clark J. M., Powner D., Thompson N. T. et al. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr. Biol. **8:** 835–838
44 Huang P., Altshuller Y. M., Chunqiu Hou J., Pessin J. E. and Frohman M. A. (2005) Insulin-stimulated plasma membrane fusion of Glut4 glucose transporter-containing vesicles is regulated by phospholipase D1. Mol. Biol. Cell **16:** 2614–2623
45 Honda A., Nogami M., Yokozeki T., Yamazaki M., Nakamura H., Watanabe H. et al. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell **99:** 521–532
46 Du G., Huang P., Liang B. T. and Frohman M. A. (2004) Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. Mol. Biol. Cell **15:** 1024–1030
47 Divecha N., Roefs M., Halstead J. R., D'Andrea S., Fernandez-Borga M., Oomen L. et al. (2000) Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. EMBO J. **19:** 5440–5449
48 Lee S., Park J. B., Kim J. H., Kim Y., Kim J. H., Shin K. J. et al. (2001) Actin directly interacts with phospholipase D, inhibiting its activity. J. Biol. Chem. **276:** 28252–28260
49 Exton J. H. (2002) Regulation of phospholipase D. FEBS Lett. **531:** 58–61
50 Liscovitch M., Czarny M., Fiucci G., Lavie Y. and Tang X. (1999) Localization and possible functions of phospholipase D isozymes. Biochim. Biophys. Acta **1439:** 245–263
51 Brown H. A., Gutowski S., Moomaw C. R., Slaughter C. and Sternweis P. C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell **75:** 1137–1144
52 Colley W. C., Altshuller Y. M., Sue-Ling C. K., Copeland N. G., Gilbert D. J., Jenkins N. A. et al. (1997) Cloning and expression analysis of murine phospholipase D1. Biochem. J. **326 (Pt 3):** 745–753
53 Exton J. H. (1998) Phospholipase D. Biochim. Biophys. Acta **1436:** 105–115
54 Exton J. H. (1997) New developments in phospholipase D. J. Biol. Chem. **272:** 15579–15582
55 Zhang Y., Altshuller Y. M., Hammond S. M., Hayes F., Morris A. J. and Frohman M. A. (1999) Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. EMBO J. **18:** 6339–6348
56 Chen J. S. and Exton J. H. (2004) Regulation of phospholipase D2 activity by protein kinase C alpha. J. Biol. Chem. **279:** 22076–22083
57 Singer W. D., Brown H. A., Jiang X. and Sternweis P. C. (1996) Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. J. Biol. Chem. **271:** 4504–4510
58 Pai J. K., Pachter J. A., Weinstein I. B. and Bishop W. R. (1991) Overexpression of protein kinase C beta 1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. Proc. Natl. Acad. Sci. USA **88:** 598–602
59 Lopez I., Burns D. J. and Lambeth J. D. (1995) Regulation of phospholipase D by protein kinase C in human neutrophils. Conventional isoforms of protein kinase C phosphorylate a phospholipase D-related component in the plasma membrane. J. Biol. Chem. **270:** 19465–19472
60 Briscoe C. P., Martin A., Cross M. and Wakelam M. J. (1995) The roles of multiple pathways in regulating bombesin-stimu-

lated phospholipase D activity in Swiss 3T3 fibroblasts. Biochem. J. **306:** 115–122
61 Balboa M. A., Firestein B. L., Godson C., Bell K. S. and Insel P. A. (1994) Protein kinase C-α mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. J. Biol. Chem. **269:** 10511–10516
62 Sciorra V. A., Hammond S. M. and Morris A. J. (2001) Potent direct inhibition of mammalian phospholipase D isoenzymes by calphostin-c. Biochemistry **40:** 2640–2646
63 Hornia A., Lu Z., Sukezane T., Zhong M., Joseph T., Frankel P. et al. (1999) Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. Mol. Cell. Biol. **19:** 7672–7680
64 Moss J. and Vaughan M. (1998) Molecules in the ARF orbit. J. Biol. Chem. **273:** 21431–21434
65 Lambeth J. D., Kwak J. Y., Bowman E. P., Perry D., Uhlinger D. J. and Lopez I. (1995) ADP-ribosylation factor functions synergistically with a 50-kDa cytosolic factor in cell-free activation of human neutrophil phospholipase D. J. Biol. Chem. **270:** 2431–2434
66 Caumont A. S., Galas M. C., Vitale N., Aunis D. and Bader M. F. (1998) Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. J. Biol. Chem. **273:** 1373–1379
67 Dascher C. and Balch W. (1994) Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatu. J. Biol. Chem. ***269*:** 1437–1448
68 Rumenapp U., Geiszt M., Wahn F., Schmidt M. and Jakobs K. H. (1995) Evidence for ADP-ribosylation-factor-mediated activation of phospholipase D by M3 muscarinic acetylcholine receptor. Eur. J. Biochem. **234:** 240–244
69 Shome K., Nie Y. and Romero G. (1998) ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. J. Biol. Chem. **273:** 30836–30841
70 Singer W. D., Brown H. A. and Sternweis P. C. (1997) Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Ann. Rev. Biochem. **66:** 475–509
71 Singer W. D., Brown H. A., Bokoch G. M. and Sternweis P. C. (1995) Resolved phospholipase D activity is modulated by cytosolic factors other than ARF. J. Biol. Chem. **270:** 14944–14950
72 Siddiqi A. R., Smith J. L., Ross A. H., Qiu R. G., Symons M. and Exton J. H. (1995) Regulation of phospholipase D in HL60 cells. Evidence for a cytosolic phospholipase D. J. Biol. Chem. **270:** 8466–8473
73 Malcolm K. C., Ross A. H., Qiu R. G., Symons M. and Exton J. H. (1994) Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. J. Biol. Chem. **269:** 25951–25954
74 Bowman E. P., Uhlinger D. J. and Lambeth J. D. (1993) Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. J. Biol. Chem. **268:** 21509–21512
75 Hodgkin M. N., Clark J. M., Rose S., Saqib K. and Wakelam M. J. (1999) Characterization of the regulation of phospholipase D activity in the detergent-insoluble fraction of HL60 cells by protein kinase C and small G-proteins. Biochem. J. **339 (Pt 1):** 87–93
76 Bae C. D., Min D. S., Fleming I. N. and Exton J. H. (1998) Determination of interaction sites on the small G protein RhoA for phospholipase D. J. Biol. Chem. **273:** 11596–11604
77 Malcolm K. C., Elliott C. M. and Exton J. H. (1996) Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of Clostridium botulinum C3 exoenzyme. J. Biol. Chem. **271:** 13135–13139
78 Schmidt M., Rumenapp U., Bienek C., Keller J. and von Eichel-Streiber C. (1996) Inhibition of receptor signaling to phospholipase D by Clostridium difficile toxin B. Role of Rho proteins. J. Biol. Chem. **271:** 2422–2426
79 Schmidt M., Rumenapp U., Nehls C., Ott S., Keller J., Von Eichel-Streiber C. et al. (1996) Restoration of Clostridium difficile toxin-B-inhibited phospholipase D by phosphatidylinositol 4,5-bisphosphate. Eur. J. Biochem. **240:** 707–712
80 Meacci E., Vasta V., Moorman J. P., Bobak D. A., Bruni P., Moss J. et al. (1999) Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A459 cells. J. Biol. Chem. **274:** 18605–18612
81 Ohguchi K., Nakashima S., Tan Z., Banno Y., Dohi S. and Nozawa Y. (1997) Increased activity of small GTP-binding protein-dependent phospholipase D during differentiation in human promyelocytic leukemic HL60 cells. J. Biol. Chem. **272:** 1990–1996
82 Yamazaki M., Zhang Y., Watanabe H., Yokozeki T., Ohno S., Kaibuchi K. et al. (1999) Interaction of the small G protein RhoA with the C terminus of human phospholipase D1. J. Biol. Chem. **274:** 6035–6038
83 Du G., Altshuller Y. M., Kim Y., Han J. M., Ryu S. H., Morris A. J. et al. (2000) Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. Mol. Biol. Cell **11:** 4359–4368
84 Xie Z., Ho W. T. and Exton J. H. (2002) Functional implications of post-translational modifications of phospholipases D1 and D2. Biochim. Biophys. Acta **1580:** 9–21
85 Gustavsson L., Moehren G., Torres-Marquez M. E., Benistant C., Rubin R. and Hoek J. B. (1994) The role of cytosolic Ca2+, protein kinase C and protein kinase A in hormonal stimulation of phospholipase D in rat hepatocytes. J. Biol. Chem. **269:** 849–859
86 Kanaho Y., Nishida A. and Nozawa Y. (1992) Calcium rather than protein kinase C is the major factor to activate phospholipase D in FMLP-stimulated rabbit peritoneal neutrophils. Possible involvement of calmodulin/myosin L chain kinase pathway. J. Immunol. **149:** 622–628
87 Kiss Z. and Deli E. (1992) Regulation of phospholipase D by sphingosine involves both protein kinase C-dependent and -independent mechanisms in NIH 3T3 fibroblasts. Biochem. J. **288:** 853–858
88 MacNulty E. E., McClue S. J., Carr I. C., Jess T., Wakelam M. J. and Milligan G. (1992) Alpha 2-C10 adrenergic receptors expressed in rat 1 fibroblasts can regulate both adenylylcyclase and phospholipase D-mediated hydrolysis of phosphatidylcholine by interacting with pertussis toxin-sensitive guanine nucleotide-binding proteins. J. Biol. Chem. **267:** 2149–2156
89 Pelech S. L. and Vance D. E. (1984) Regulation of phosphatidylcholine biosynthesis. Biochim. Biophys. Acta **779:** 217–251
90 Stuckey J. A. and Dixon J. E. (1999) Crystal structure of a phospholipase D family member. Nat. Struct. Biol. **6:** 278–284
91 Leiros I., Secundo F., Zambonelli C., Servi S. and Hough E. (2000) The first crystal structure of a phospholipase D. Struct. Fold Des. **8:** 655–667
92 Gottlin E. B., Rudolph A. E., Zhao Y., Matthews H. R. and Dixon J. E. (1998) Catalytic mechanism of the phospholipase D superfamily proceeds via a covalent phosphohistidine intermediate. Proc. Natl. Acad. Sci. USA **95:** 9202–9207
93 Wakelam M. J. O., Hodgkin M. and Martin A., (1995) The measurement of phospholipase D-linked signaling in cells. In: Signal Transduction Protocols, pp. 271–278, Kendall D. A. and Hill S. J. (eds), Humana Press, Totowa, NJ
94 Pettitt T. R., McDermott M., Saqib K. M., Shimwell N. and Wakelam M. J. (2001) Phospholipase D1b and D2a generate structurally identical phosphatidic acid species in mammalian cells. Biochem. J. **360:** 707–715
95 Sciorra V. A. and Morris A. J. (1999) Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. Mol. Biol. Cell **10:** 3863–3876
96 Cook S. J. and Wakelam M. J. (1992) Epidermal growth factor increases sn-1,2-diacylglycerol levels and activates phospholipase D-catalysed phosphatidylcholine breakdown in Swiss 3T3

cells in the absence of inositol-lipid hydrolysis. Biochem. J. **285 (Pt 1):** 247–253
97 Brindley D. N. and Waggoner D. W. (1996) Phosphatidate phosphohydrolase and signal transduction. Chem. Phys. Lipids **80:** 45–57
98 Daniel L. W., Huang C., Strum J. C., Smitherman P. K., Greene D. and Wykle R. L. (1993) Phospholipase D hydrolysis of choline phosphoglycerides is selective for the alkyl-linked subclass of Madin-Darby canine kidney cells. J. Biol. Chem. **268:** 21519–21526
99 Daniel L. W., Waite M. and Wykle R. L. (1986) A novel mechanism of diglyceride formation. 12-O-tetradecanoylphorbol-13-acetate stimulates the cyclic breakdown and resynthesis of phosphatidylcholine. J. Biol. Chem. **261:** 9128–9132
100 Bocckino S. B., Blackmore P. F., Wilson P. B. and Exton J. H. (1987) Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. J. Biol. Chem. **262:** 15309–15315
101 Besterman J. M., Duronio V. and Cuatrecasas P. (1986) Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for generation of a second messenger. Proc. Natl. Acad. Sci. USA **83:** 6785–6789
102 Pai J. K., Siegel M. I., Egan R. W. and Billah M. M. (1988) Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. J. Biol. Chem. **263:** 12472–12477
103 Cockcroft S. and Allan D. (1984) The fatty acid composition of phosphatidylinositol, phosphatidate and 1,2-diacylglycerol in stimulated human neutrophils. Biochem. J. **222:** 557–559
104 Pettitt T. R., Martin A., Horton T., Liossis C., Lord J. M. and Wakelam M. J. (1997) Diacylglycerol and phosphatidate generated by phospholipases C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. J. Biol. Chem. **272:** 17354–17359
105 Moritz A., De Graan P. N. E., Gispen W. H. and Wirtz K. W. A. (1992) Phosphatic acid is a specific activator of phosphatidylinositol 4 phosphate kinase. J. Biol. Chem. **267:** 7207–7210
106 Jenkins G. H., Fisette P. L. and Anderson R. A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. J. Biol. Chem. **269:** 11547–11554
107 Ishihara H., Shibasaki Y., Kizuki N., Wada T., Yazaki Y., Asano T. et al. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. J. Biol. Chem. **273:** 8741–8748
108 Kam Y. and Exton J. H. (2001) Phospholipase D activity is required for actin stress fiber formation in fibroblasts. Mol. Cell. Biol. **21:** 4055–4066
109 Jones D. H., Morris J. B., Morgan C. P., Kondo H., Irvine R. F. and Cockcroft S. (2000) Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. J. Biol. Chem. **275:** 13962–13966
110 Martelli A. M., Bortul R., Bareggi R., Tabellini G., Grill V., Baldini G. et al. (1999) The pro-apoptotic drug camptothecin stimulates phospholipase D activity and diacylglycerol production in the nucleus of HL-60 human promyelocytic leukemia cells. Cancer Res. **59:** 3961–3967
111 Zheng X. L., Gui Y., Du G., Frohman M. A. and Peng D. Q. (2004) Calphostin-C induction of vascular smooth muscle cell apoptosis proceeds through phospholipase D and microtubule inhibition. J. Biol. Chem. **279:** 7112–7118
112 Kasai T., Ohguchi K., Nakashima S., Ito Y., Naganawa T., Kondo N. et al. (1998) Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jurkat T cells. J. Immunol. **161:** 6469–6474
113 Lee B. D., Kim J. H., Lee S. D., Kim Y., Suh P. G. and Ryu S. H. (2000) Hydrogen peroxide-induced phospholipase D2 activation in lymphocytic leukemic L1210 cells. J. Leukoc. Biol. **67:** 630–636
114 Fang Y., Vilella-Bach M., Bachmann R., Flanigan A. and Chen J. (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science **294:** 1942–1945
115 Chen J. and Fang Y. (2002) A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. Biochem. Pharmacol. **64:** 1071–1077
116 Chen Y., Zheng Y. and Foster D. A. (2003) Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene **22:** 3937–3942
117 Lim H. K., Choi Y. A., Park W., Lee T., Ryu S. H., Kim S. Y. et al. (2003) Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. J. Biol. Chem. **278:** 45117–45127
118 Fang Y., Park I. H., Wu A. L., Du G., Huang P., Frohman M. A. et al. (2003) PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr. Biol. **13:** 2037–2044
119 Kam Y. and Exton J. H. (2004) Role of phospholipase D1 in the regulation of mTOR activity by lysophosphatidic acid. FASEB J. **18:** 311–319
120 Hui L., Abbas T., Pielak R. M., Joseph T., Bargonetti J. and Foster D. A. (2004) Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53. Mol. Cell. Biol. **24:** 5677–5686
121 Chen Y., Rodrik V. and Foster D. A. (2005) Alternative phospholipase D/mTOR survival signal in human breast cancer cells. Oncogene **24:** 672–679
122 Manifava M., Thuring J. W., Lim Z. Y., Packman L., Holmes A. B. and Ktistakis N. T. (2001) Differential binding of trafficrelated proteins to phosphatidic acid- or phosphatidylinositol (4,5)-bisphosphate-coupled affinity reagents. J. Biol. Chem. **276:** 8987–8994
123 Ghosh S., Moore S., Bell R. M. and Dush M. (2003) Functional analysis of a phosphatidic acid binding domain in human Raf-1 kinase: mutations in the phosphatidate binding domain lead to tail and trunk abnormalities in developing zebrafish embryos. J. Biol. Chem. **278:** 45690–45696
124 Rizzo M. A., Shome K., Watkins S. C. and Romero G. (2000) The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. J. Biol. Chem. **275:** 23911–23918
125 Ghosh S., Strum J. C., Sciorra V. A., Daniel L. and Bell R. M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-0-tetradecanoylphorbol-13-acetate-stimulated madin-darby canine kidney cells. J. Biol. Chem. **271:** 8472–8480
126 Rizzo M. A., Shome K., Vasudevan C., Stolz D. B., Sung T. C., Frohman M. A. et al. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. J. Biol. Chem. **274:** 1131–1139
127 Jones J. A. and Hannun Y. A. (2002) Tight binding inhibition of protein phosphatase-1 by phosphatidic acid. Specificity of inhibition by the phospholipid. J. Biol. Chem. **277:** 15530–15538
128 Baillie G. S., Huston E., Scotland G., Hodgkin M., Gall I., Peden A. H. et al. (2002) TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP-specific phosphodiesterase that allows rapid, Ca2+-triggered membrane association with selectivity for interaction with phosphatidic acid. J. Biol. Chem. **277:** 28298–28309
129 Delon C., Manifava M., Wood E., Thompson D., Krugmann S., Pyne S. et al. (2004) Sphingosine kinase 1 is an intracellular effector of phosphatidic Acid. J. Biol. Chem. **279:** 44763–44774

130 Newton A. C. (1993) Interaction of proteins with lipid headgroups: lessons from protein kinase C. Annu Rev Biophys. Biomol. Struct. **22:** 1–25
131 Newton A. C. (1997) Regulation of protein kinase C. Curr. Opin. Cell Biol. **9:** 161–167
132 Hodgkin M. N., Pettitt T. R., Martin A., Michell R. H., Pemberton A. J. and Wakelam M. J. (1998) Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? Trends Biochem. Sci. **23:** 200–204
133 Moolenaar W. H., van Meeteren L. A. and Giepmans B. N. (2004) The ins and outs of lysophosphatidic acid signaling. Bioessays **26:** 870–881
134 Ktistakis N. T., Brown H. A., Waters M. G., Sternweis P. C. and Roth M. G. (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J. Cell Biol. **134:** 295–306.
135 Bi K., Roth M. G. and Ktistakis N. T. (1997) Phosphatidic acid formation by Phospholipase D is required for transport from the endoplasmic reticulum to the Golgi complex. Curr. Biol. **7:** 301–307
136 Chen Y. G., Siddhanta A., Austin C. D., Hammond S. M., Sung T. C., Frohman M. A. et al. (1997) Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. J. Cell. Biol. **138:** 495–504
137 Haslam R. J. and Coorssen J. R. (1993) Evidence that activation of phospholipase D can mediate secretion from permeabilized platelets. Adv. Exp. Med. Biol. **344:** 149–164
138 Stutchfield J. and Cockcroft S. (1993) Correlation between secretion and phospholipase D activation in differentiated HL60 cells. Biochem. J. **293:** 649–655
139 Lin P. Y., Wiggan G. A. and Gilfillan A. M. (1991) Activation of phospholipase D in a rat mast (RBL 2H3) cell line. A possible unifying mechanism for IgE-dependent degranulation and arachidonic acid metabolite release. J. Immunol. **146:** 1609–1616
140 Xie M. S., Jacobs L. S. and Dubyak G. R. (1991) Regulation of phospholipase D and primary granule secretion by P2-purinergic- and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells. J. Clin. Invest. **88:** 45–54
141 Jones D., Bax B., Fensome A. and Cockcroft S. (1999) ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer I. Biochem. J. **341:** 185–192
142 Fensome A., Cunningham E., Prosser S., Tan S. K., Swigart P., Thomas G. et al. (1996) ARF and PITP restore GTPγS-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP_2 synthesis. Curr. Biol. **6:** 730–738
143 Vitale N., Caumont A. S., Chasserot-Golaz S., Du G., Wu S., Sciorra V. A. et al. (2001) Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. EMBO J. **20:** 2424–2434
144 Vitale N., Chasserot-Golaz S., Bailly Y., Morinaga N., Frohman M. A. and Bader M. F. (2002) Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. J. Cell. Biol. **159:** 79–89
145 Emoto M., Klarlund J., Waters S., Hu V., Buxton J., Chawla A. et al. (2000) A role for phospholipase D in GLUT4 glucose transporter translocation. J. Biol. Chem. **275:** 7144–7151
146 Hughes W. E., Elgundi Z., Huang P., Frohman M. A. and Biden T. J. (2004) Phospholipase D1 regulates secretagogue-stimulated insulin release in pancreatic beta-cells. J. Biol. Chem. **279:** 27534–27541
147 Choi W. S., Chahdi A., Kim Y. M., Fraundorfer P. F. and Beaven M. A. (2002) Regulation of phospholipase D and secretion in mast cells by protein kinase A and other protein kinases. Ann. N. Y. Acad. Sci. **968:** 198–212
148 Choi W. S., Kim Y. M., Combs C., Frohman M. A. and Beaven M. A. (2002) Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. J. Immunol. **168:** 5682–5689
149 Vitale N., Chasserot-Golaz S. and Bader M. F. (2002) Regulated secretion in chromaffin cells: an essential role for ARF6-regulated phospholipase D in the late stages of exocytosis. Ann. N. Y. Acad. Sci. **971:** 193–200
150 Cummings R. J., Parinandi N. L., Zaiman A., Wang L., Usatyuk P. V., Garcia J. G. et al. (2002) Phospholipase D activation by sphingosine 1-phosphate regulates interleukin-8 secretion in human bronchial epithelial cells. J. Biol. Chem. **277:** 30227–30235
151 Shen Y., Xu L. and Foster D. (2001) Role for phospholipase D in receptor-mediated endocytosis. Mol. Cell. Biol. **21:** 595–602
152 Koch T., Brandenburg L. O., Schulz S., Liang Y., Klein J. and Hollt V. (2003) ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. J. Biol. Chem. **278:** 9979–9985
153 Koch T., Brandenburg L. O., Liang Y., Schulz S., Beyer A., Schroder H. et al. (2004) Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. J. Neurochem. **88:** 680–688
154 Bhattacharya M., Babwah A. V., Godin C., Anborgh P. H., Dale L. B., Poulter M. O. et al. (2004) Ral and phospholipase D2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis. J. Neurosci. **24:** 8752–8761
155 Grishanin R. N., Kowalchyk J. A., Klenchin V. A., Ann K., Earles C. A., Chapman E. R. et al. (2004) CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2 binding protein. Neuron **43:** 551–562
156 Olsen H. L., Hoy M., Zhang W., Bertorello A. M., Bokvist K., Capito K. et al. (2003) Phosphatidylinositol 4-kinase serves as a metabolic sensor and regulates priming of secretory granules in pancreatic beta cells. Proc. Natl. Acad. Sci. USA **100:** 5187–5192
157 Kozlovsky Y., Chernomordik L. V. and Kozlov M. M. (2002) Lipid intermediates in membrane fusion: formation, structure and decay of hemifusion diaphragm. Biophys. J. **83:** 2634–2651
158 Jun Y., Fratti R. A. and Wickner W. (2004) Diacylglycerol and its formation by phospholipase C regulate Rab- and SNARE-dependent yeast vacuole fusion. J. Biol. Chem. **279:** 53186–53195
159 Kooijman E. E., Chupin V., de Kruijff B. and Burger K. N. (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. Traffic **4:** 162–174
160 Reutens A. T. and Begley C. G. (2002) Endophilin-1: a multifunctional protein. Int. J. Biochem. Cell Biol. **34:** 1173–1177
161 Harsh D. M. and Blackwood R. A. (2001) Phospholipase A(2)-mediated fusion of neutrophil-derived membranes is augmented by phosphatidic acid. Biochem. Biophys. Res. Commun. **282:** 480–486
162 Carpio L.C. and Dziak, R. (1998) Phosphatidic acid effects calcium and proliferation in osteoblastic cells. Prostglandins Leukot. Essent. Fatty Acids **59:** 101–109
163 Walter, M., Tepel, M., Nofer J.R., Neusser M., Assman G. and Zidek, W. (2000) Involvement of phospholipase D in store-operated calcium influx in vascular smooth muscle cells. FEBS Lett. **497:** 51–56
164 Loewen C. J., Gaspar M. L., Jesch S. A., Delon C., Ktistakis N. T., Henry S. A. et al. (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. Science **304:** 1644–1647