The Role of Phospholipase D in Regulated Exocytosis*

Received for publication, August 9, 2015, and in revised form, September 30, 2015 Published, JBC Papers in Press, October 2, 2015, DOI 10.1074/jbc.M115.681429

Tatiana P. Rogasevskaia1,2 and Jens R. Coorssen1,2
From the 1Department of Biology, Mount Royal University, Calgary T3E 6K6, Canada and 2Department of Molecular Physiology, School of Medicine and the Molecular Medicine Research Group, Western Sydney University, Penrith NSW 2751, Australia

Background: Phospholipase D (PLD)-derived phosphatidic acid (PA) is suggested to function in exocytosis.

Results: PLD activity on the plasma membrane was higher than on vesicles. PLD inhibitors suppressed some fusion parameters, but blockade of PA did not affect fusion.

Conclusion: PLD-derived PA has modulatory rather than direct effects on fusion.

Significance: Identifying critical mechanistic components may enable interventions to target diseases affecting exocytosis.

There are a diversity of interpretations concerning the possible roles of phospholipase D and its biologically active product phosphatidic acid in the late, Ca2+-triggered steps of regulated exocytosis. To quantitatively address functional and molecular aspects of the involvement of phospholipase D-derived phosphatidic acid in regulated exocytosis, we used an array of phospholipase D inhibitors for ex vivo and in vitro treatments of sea urchin eggs and isolated cortices and cortical vesicles, respectively, to study late steps of exocytosis, including docking/priming and fusion. The experiments with fluorescent phosphatidylcholine reveal a low level of phospholipase D activity associated with cortical vesicles but a significantly higher activity on the plasma membrane. The effects of phospholipase D activity and its product phosphatidic acid on the Ca2+ sensitivity and rate of fusion correlate with modulatory upstream roles in docking and priming rather than to direct effects on fusion per se.

Phosphatidic acid (PA) is an anionic phospholipid that has been implicated in a wide range of cellular processes. Although

* This work was supported by the National Health and Medical Research Council (NHMRC) of Australia (APP1065328), the Molecular Medicine Research Group and the School of Medicine (Western Sydney University), and the CIHR (Canada) (to J. R. C.). This work was also supported by the Department of Biology (Mount Royal University) and the Department of Physiology and Pharmacology (University of Calgary) (to T. P. R.). The authors declare that they have no conflict of interest with the content of this article.

1 To whom correspondence may be addressed. Tel.: 403-440-8860; E-mail: trogasevskaia@mrroyal.ca.
2 To whom correspondence may be addressed. Tel.: 61-2-4620-3802; E-mail: j.coorssen@uws.edu.au.

3 The abbreviations used are: PA, phosphatidic acid; PE, phosphatidylethanolamine; PLD, phospholipase D; CV, cortical vesicle; HLP, halopemide (N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-ethyl]-4-fluoro-benzamide); VU01, VU0155056 (N-[2-[4-(2-oxo-2,3-diydro-1H-benzol][1]-1-piperidinyl]-1-ethyl]-4-fluoro-benzamide); product number 857370P); VU02, VU028565-5 (N-[2-[4-oxo-1-phenyl-1,3,8-triazaspiro(4.5)decan-8-yl]decan-8-yl]-4-fluoro-benzamide); VU03, VU0359595 (1-[1-(8R)-N-[5-(2,3-dihydro-2-oxo-2,3-diydro-1H-benzol][1]-1-piperidinyl]-yl)propan-2-yl]-2-phenylcyclopropanecarboxamide); Fip1L, N-[2-[4-(2,3-diydro-2-oxo-2,3-diydro-1H-benzol][1]-1-piperidinyl]-ethy]-5-fluoro-1H-indole-2-carboxamide; PC, phosphatidylcholine; NBD-PC (–PA), 1-oleoyl-2-[11-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phosphocholine (–PA); QRC, quercetin-3-O-rhamnoside, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one; 3,3′,4′,5,6-pentahydroxyflavone; SRM, siramesine; CSC, cell surface complex; PM, plasma membrane; HPTLC, high performance thin layer chromatography; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; zmol, zeptomol; ymol, yoctomol; a minor component of the membrane, PA is a biologically active lipid involved in regulated exocytosis, trafficking, membrane dynamics, and responses to environmental stimuli (1–6). PA is also involved in the activation of sphingosine kinase, which is known to modulate intracellular Ca2+-release in neutrophils (7–9) and to activate proteins responsible for cell survival, including mTOR (10), Hsp22 (11), and Ras (12). Numerous studies suggest that the biological role for PA relates to its anionic properties (13, 14) and correlates with the amount of Ca2+ bound to the lipid (15). These anionic properties depend not only on the local pH and pKa but also on the presence of divalent metals that contribute to the deprotonation of PA (16). The presence of phosphatidylethanolamine (PE) within the lipid bilayer can also promote deprotonation of the PA headgroup (17). As a negatively charged lipid, PA has been suggested to be involved in the membrane fusion pathway, which raises a question concerning the optimal local composition of PA and other lipids at the fusion site (3, 18, 19). Anionic properties of PA also suggest the possibility of specific pH-dependent interactions with peripheral proteins and positively charged domains of critical integral proteins (20–22). These PA-protein interactions are of particular interest as PA may define binding sites for fusion-assisting proteins and modulate their local activity.

PA is synthesized de novo by the phospholipase D (PLD)-driven hydrolysis of phosphatidylcholine (PC), ensuring optimal local levels of PA. In addition to its anionic properties, PA is also known to have intrinsic negative curvature; this potential to form concave, inverted structures could change membrane topology, promoting generation of curved membranes and potentially contributing to the Ca2+-triggered membrane fusion pathway (3, 6, 13, 14, 17, 23–25). Thus, if this simple lipid functions in a variety of biological roles by virtue of its anionic properties and/or intrinsic negative curvature, what is the specific role of PA and local PA-generating enzymes in the fusion process? As members of the PLD family, PLD1 and PLD2 generate biologically active PA that is suggested to locally alter membrane surface charge and curvature, resulting in membrane instability due to differences in the headgroup size of adjacent lipids (26). Thus, PLD could potentially be critical for regulated exocytosis, and its role(s) in the membrane fusion pathway needs to be clarified.

Although a body of work has implicated both members of the PLD family in the regulation of different phases of exocytosis...
The Role of PLD-derived Phosphatidic Acid in Exocytosis

(27–29), there are conflicting interpretations as to the role of PA in late steps of regulated release. Considering the spatial and temporal resolution of most of the assays used in a variety of secretory cell types, roles in vesicle recruitment, docking, and perhaps a late priming step would seem to be the most consistent and directly interpretable functions. Nonetheless, despite a lack of direct curvature or lipid assessment or any analyses of fusion pore kinetics, interpretations tend to also invoke a local curvature role for PA in promoting or initiating membrane fusion. In contrast, in another well established model system (release-ready cortical vesicles (CV) isolated from sea urchin eggs, which enables direct coupling of functional and molecular analyses) fusion was inhibited by either exogenously added PA or endogenous PA generated by an exogenous PLD (30). Although these data do not support a direct role for PA in membrane merger, they point to an upstream regulatory role in the docking and/or priming steps of the regulated fusion pathway (30). However, we have also detailed caveats that must be considered in such experiments using exogenous lipids (or proteins), in particular with reference to anionic lipids (19). Thus, although an essential role for PLD in the fusion step of regulated exocytosis was excluded some time ago using human platelets (31), in terms of the more recent studies noted it was deemed important to further evaluate the function(s) of this critical enzyme in the late steps of the exocytotic pathway.

Selective small molecules provide an effective tool to study the biological effects of critical enzymes and lipids on the fusion process, particularly using the CV model system (19, 32, 33). This is also noteworthy considering the range of different inhibitors available to target PLD activity and thus inhibit PA generation (e.g. halopemide (HLP); N-[2-[(5,3-dihydro-2-oxo-2H-benzo(d)imidazol-1-yl)-1-piperidinyl]-ethyl]-4-fluoro-benzamide; product number 13205), FIPI (N-[2-(2,3-dihydro-2-oxo-1H-benzo(d)imidazol-1-yl)-1-piperidinyl]ethyl)-5-fluoro-1H-indole-2-carboxamide; product number 13563, VU0155056 (VU01; N-[2-[2-oxo-2,3-dihydro-1H-benzo(d)imidazol-1-yl]-piperidin-1-yl]ethy]-2-naphthamide; product number 857370P), and QRC (quercetin-3-O-rhamnoside, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one,3β,4,5,6-pentahydroxyflavone; product number Q4951) were from Sigma. The Amplex Red PLD activity assay was from Invitrogen. All other chemicals were of the highest grade available to target PLD activity and thus inhibit PA generation (30, 32, 33, 39, 40) with a variety of selectiv PLD1 and PLD2 inhibitors to quantitatively address functional and molecular aspects of the involvement of PLD-derived PA in regulated exocytosis. Fluorescently labeled PC was derived as previously described (33), with continuous slow and gentle mixing for either 20 min or 20 h at 7 °C. Butanol was delivered as 1–4% aqueous baseline intracellular media (BIM) solutions; SRM, FIPI, HLP, QRC, and VU compounds were delivered from dimethylformamide stock solutions to a final dimethylformamide concentration of ≤1% for QRC and ≤0.05% for other inhibitors. Selective inhibitor concentrations were identified from the literature (31, 34–38) and adjusted here after initial fusion trials and lipid assessments. Treated eggs were subsequently processed to isolate CSCs (sheets of plasma membrane (PM) with associated, fusion-ready CV) or CV for standard fusion experiments (42, 43). BIM (210 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 10 mM PIPES, 1 mM MgCl2, 0.05 mM CaCl2, 1 mM EGTA; pH 6.7) with added protease inhibitors and 2.5 mM ATP (44) was used for all CSC and CV incubations and fusion experiments. SRM was used for 30-min CV and CSC treatments at 25 °C (A405 0.4 and 0.3, respectively) and was delivered from 10 mM stock solutions to final concentrations of 100 nm–5 μM. Standard CV and CSC fusion assays were performed as previously detailed (32, 39, 42, 43, 45). Briefly, centrifugation of CV at 700 × g for 5 min was used to remove PM fragments and 1800–2500 g for 20 min to pellet CV. Suspended CVs (A405 0.4) were then centrifuged in microplates (700 × g) to induce CV-CV contact and docking. CSC fusion assays were carried out on free floating cortices (A405 0.3). CV “settle” fusion assays to assess the capacity of endogenous CV membrane components to independently establish intermembrane attachment (i.e. docking) were also carried out as described (30, 32, 33, 39, 42, 45); in short, suspended CV (A405 0.4) were pipetted into microplates and left to settle into contact for 1 h followed by standard challenge with Ca2+ to trigger fusion responses. The Wallac Victor2 V Microplate Reader (PerkinElmer Life Sciences) was used for all light scattering measurements to assess fusion responses.

The sigmoidal cumulative log-normal model was applied to fit Ca2+ activity curves (i.e. the extent and Ca2+ sensitivity of fusion) (46); initial kinetics were fit with the linear equation for Experimental Procedures

Materials—Sea urchins (Strongylocentrotus purpuratus) were purchased from WestWind SeaLab Supplies (Victoria, BC). Neutral and phospholipid standards, 18:1–12:0 NBD-phosphatidylcholine (NBD-PC; 1-oleoyl-2-[12-[7-nitro-2–1,3-benzoxadiazol-4-yl]amino[dodecanoyl]-sn-glycero-3-phosphocholine; product number 810133P, fatty acid-labeled fluorophore: excitation/emission 460 nm/534 nm), VU01, and VU02, and VU03 were from Avanti Polar Lipids (Alabaster, AL). HLP and FIPI were purchased from Cayman Chemical (Ann Arbor, MI). Dimethylformamide, n-buty alcohol (butanol; product number B7906), and quercetin (QC; quercetin-3-O-rhamnoside, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one,3β,4,5,6-pentahydroxyflavone; product number Q4951) were from Sigma. The Amplex Red PLD activity assay was from Invitrogen. All other chemicals were of at least analytical grade. Siramesine (SRM) was provided by H. Lundbeck A/S (Copenhagen, Denmark). 0.2-μm MCE filters were from Fisher.

Eggs, Cell Surface Complex (CSC), and CV Treatments, Preparations, and Fusion Assays—Sea urchin eggs collected in artificial sea water (41) were filtered through nylon mesh (100-μm pore size) to remove the jelly coat, centrifuged at 700 × g, and suspended in 20 ml (~1.6 × 10⁶ egg/ml) of filtered Ca2+-free artificial sea water (i.e. 0.2-μm MCE filter) in 50 ml Falcon tubes. Incubation of eggs with inhibitors was as previously described (33), with continuous slow and gentle mixing for either 20 min or 20 h at 7 °C. Butanol was delivered as 1–4% aqueous baseline intracellular media (BIM) solutions; SRM, FIPI, HLP, QRC, and VU compounds were delivered from dimethylformamide stock solutions to a final dimethylformamide concentration of ≤1% for QRC and ≤0.05% for other inhibitors. Selective inhibitor concentrations were identified from the literature (31, 34–38) and adjusted here after initial fusion trials and lipid assessments. Treated eggs were subsequently processed to isolate CSCs (sheets of plasma membrane (PM) with associated, fusion-ready CV) or CV for standard fusion experiments (42, 43). BIM (210 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 10 mM PIPES, 1 mM MgCl₂, 0.05 mM CaCl₂, 1 mM EGTA; pH 6.7) with added protease inhibitors and 2.5 mM ATP (44) was used for all CSC and CV incubations and fusion experiments. SRM was used for 30-min CV and CSC treatments at 25 °C (A₄₀₅ 0.4 and 0.3, respectively) and was delivered from 10 mM stock solutions to final concentrations of 100 nm–5 μM. Standard CV and CSC fusion assays were performed as previously detailed (32, 39, 42, 43, 45). Briefly, centrifugation of CV at 700 × g for 5 min was used to remove PM fragments and 1800–2500 g for 20 min to pellet CV. Suspended CVs (A₄₀₅ 0.4) were then centrifuged in microplates (700 × g) to induce CV-CV contact and docking. CSC fusion assays were carried out on free floating cortices (A₄₀₅ 0.3). CV “settle” fusion assays to assess the capacity of endogenous CV membrane components to independently establish intermembrane attachment (i.e. docking) were also carried out as described (30, 32, 33, 39, 42, 45); in short, suspended CV (A₄₀₅ 0.4) were pipetted into microplates and left to settle into contact for 1 h followed by standard challenge with Ca²⁺ to trigger fusion responses. The Wallac Victor2 V Microplate Reader (PerkinElmer Life Sciences) was used for all light scattering measurements to assess fusion responses.

The sigmoidal cumulative log-normal model was applied to fit Ca²⁺ activity curves (i.e. the extent and Ca²⁺ sensitivity of fusion) (46); initial kinetics were fit with the linear equation for
the first 1.5 s after rapid Ca\(^{2+}\) injection, with an intercept of 0.5 s. The final free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{\text{free}}\)] in baseline intracellular media buffer were measured using a Ca\(^{2+}\)-sensitive electrode (Nicossensors, Huntington Valley, PA) (32, 33, 42). NBD-PC was delivered to CV suspensions from 25 mM ethanol stock solutions to a final ethanol concentration of \(\approx 0.02\%\) and left to incorporate into CV membrane for 30 min at 25 °C. Excess NBD-PC was subsequently removed by washing in baseline intracellular media and incorporation into CV assessed by fluorescence measurements (Wallac Victor2 V Microplate Reader); parallel dilution series were used for quantitative assessment. Overall, each individual condition was assessed in replicates of four for end-point analyses and in sets of eight replicates for kinetic assays; total experimental replicates (n) are indicated in “Results.” CV in suspensions were counted using a hemocytometer (32, 47).

**Lipid Extraction and High Performance Thin Layer Chromatography (HPTLC)—** CV membrane lipids were extracted with chloroform/methanol (48) using established modifications (33, 40), dried under a vacuum, and stored under nitrogen at −30 °C before analysis. Dried lipid films were dissolved in 150 μl of chloroform/methanol (2:1, v/v) and quantitatively loaded onto silica gel 60 HPTLC plates (EMD Chemicals, Darmstadt, Germany) using the CAMAG LINOMAT IV (CAMAG, Wilmington, NC). Dilution series of lipid standards were applied and resolved in parallel. All lipid standards were the oleic acid (18:1Δ9) esters. Pre-washed HPTLC plates (methanol/ethyl acetate (6:4)) were activated at 110 °C for 30 min (32, 33, 39, 40). A two-step separation was used for phospholipids: to 90 mm with dichloromethane/ethyl acetate/acetone (80:16:4 v/v) and then to 90 mm with chloroform/ethyl acetate/acetone/isopropyl alcohol/ethanol/methanol/water/acetic acid (30:6:6:6:16:28:62 v/v) using the CAMAG AMD 2 multidetection HPTLC unit (32, 33, 40). Neutral lipids were resolved in 5 steps: to 40 mm, then 55 mm with dichloromethane/ethyl acetate/acetone (80:16:4 v/v), and then to 68, 80, and 90 mm with hexane/ethyl acetate (90:10, 95:5, and 100:0 v/v, respectively) (32, 33, 39, 40).

**Staining and Visualizing Chromatograms—** To visualize resolved neutral lipids and phospholipids, developed plates were stained with the fluorogenic reagent, copper sulfate (CuSO\(_4\)). Plates were dipped into 10% CuSO\(_4\) in 8% H\(_3\)PO\(_4\) aqueous solution and then charred for 10 min as described (32, 33, 40). The CuSO\(_4\)-based signal was optimally detected by the PROXPRESS multiwavelength fluorescent imager (PerkinElmer Life Sciences) with the 540/30 (excitation) and 590/20 (emission) filter sets. To quantify the amounts of lipid, the integrated fluorescence intensities of samples were compared with a parallel dilution series of lipid standards. All images were analyzed using ImageQuant 5.2 (GE Healthcare).

**PLD Activity Assay—** CV and CSC membranes were isolated by ultracentrifugation (200,000 × g, 90 min) after lysis (19, 49). PLD enzymatic activity was then assessed using the Amplex Red PLD assay kit (Invitrogen) according to the manufacturer’s instructions.

**Statistical Analysis—** All data are reported as the mean ± S.E. Two-sample two-tailed t tests were used to assess differences between multiple groups of experimental conditions (p < 0.05, unless stated otherwise).

**Results**

To initially assess the role of PA in triggered fusion we tested the effect of SRM, which is known to selectively block and sequester PA on the membrane (50). Treatments of CSC with 0.1–20 nM SRM did not inhibit fusion parameters (Fig. 1a). 100 nM–5 μM SRM suppressed the extent of fusion by 25.1 ± 2.3% and significantly right-shifted Ca\(^{2+}\) sensitivity from 45.4 ± 1.6 μM to 58.0 ± 1.9 μM [Ca\(^{2+}\)\(_{\text{free}}\)] with 100 nM SRM and to 80.0 ± 2.7 μM [Ca\(^{2+}\)\(_{\text{free}}\)] with 1–5 μM SRM (p < 0.05); although 1 nM–1 μM SRM did not have any significant effect, 5 μM SRM inhibited the initial fusion rate by 19.0 ± 1.4% s\(^{-1}\) (p < 0.05; Fig. 1a). To enable more direct access of the SRM to docking/fusion sites and to enable settle (i.e. “docking”) assays, we assessed CV fusion (30, 32, 33, 39). Treatments of CV with 0.1–100 nM SRM had no significant effects on any fusion parameters, although there appeared to be a slight inhibitory trend at the higher concentration (Fig. 1b): 1 and 5 μM SRM caused 15.5 ± 2.6% and 21.5 ± 2.7% inhibition of fusion extent, respectively, with no significant changes in Ca\(^{2+}\)-sensitivity. Initial fusion kinetics were only affected by 5 μM SRM, which caused 23.2 ± 3.0% inhibition of the rate of fusion. Settle assays showed
that treatments of CV with 0.1 nM–5 μM SRM resulted in a concentration-dependent inhibition of fusion extent (to a maximal inhibition of ~35% with 5 μM SRM) and a rightward shift in Ca2⁺ sensitivity from 25.4 ± 1.6 to 48.6 ± 2.2 μM [Ca2⁺]free (p < 0.05; Fig. 1c). Initial fusion kinetics in the settle assay were also inhibited in a concentration-dependent manner by SRM, from 84.4 ± 3.8%/s for controls to 60.6 ± 4.8%/s, 46.9 ± 1.4%/s, 42.4 ± 3.1%/s, and 37.8 ± 2.8%/s (p < 0.05) for 1 nM, 10 nM, 1 μM, and 5 μM SRM, respectively. There were no significant changes in the lipid profiles after any SRM treatments confirming that SRM did not extract lipids from the membrane. Thus, blocking PA had a minimal direct effect on fusion per se except at quite high concentrations of SRM but pronounced concentration-dependent effects in the settle assay; an upstream effect on docking and/or priming was indicated.

To define the native functional roles of PLD and the PA produced, a group of well characterized, structurally diverse enzyme inhibitors was tested. In addition, we used both our well established approach of acute in vitro CSC and CV treatments and ex vivo pharmacological manipulations of intact eggs with the select reagents to suppress PLD activities both locally and at the whole cell level. Treatments included the following inhibitors: HLP, FIP1, VU01, VU02, VU03, QRC, and butanol. Control CSC fusion yielded a characteristic sigmoidal Ca2⁺-activity curve with an EC₅₀ of 58.6 ± 4.2 μM [Ca2⁺]free (Fig. 2, a and b); despite 30-min incubations, none of the reagents effected any fusion parameter. To ensure full access of the reagents to the fusion site, we again used isolated CV, which also yielded well characterized sigmoidal Ca2⁺-activity curves (30, 32, 33, 39). Considering the breadth of reagents tested, the effects on fusion parameters were quite selective. Neither butanol, FIP1, HLP, nor VU compounds had a significant effect on fusion extent and Ca2⁺ sensitivity after 30 min treatments of CV (Fig. 2, c and d), yet all selectively inhibited the initial rate of CV fusion from 70.9 ± 3.6%/s for untreated controls to 50.4 ± 5.1 for butanol, 46.5 ± 2.4 for FIP1, 47.2 ± 4.0 for HLP, and 41.8 ± 3.0%/s, 34.3 ± 2.2%/s, and 42.0 ± 2.9%/s after treatments with VU01, VU02, and VU03, respectively (Fig. 2, c and d). QRC (100 μM) treatment resulted in a similar inhibition of initial kinetics (to 34.1 ± 1.8%/s) but also inhibited the extent of fusion by 20.4 ± 1.3% without affecting Ca2⁺ sensitivity (Fig. 2c). Relative to control CV, no changes were found in the lipid
profiles of membranes extracted from CV treated with FPII, HLP, or butanol (Table 1). In contrast, after treatments with 100 μM QRC or 2 μM VU compounds, inhibition of fusion parameters correlated with significant reductions in phosphatidylethanolamine (PE) and increases in the levels of lyso-PE (LPE). The amount of CV membrane PE decreased from 25.0 ± 1.1 amol/CV for untreated controls to ~22 amol/CV for QRC- and VU-treated CVs, and LPE levels increased from 7.8 ± 0.5 amol/CV to ~11.5 amol/CV (Table 1; p < 0.05, n = 9). The amount of PA was below detectable levels; a minimal on-plate detection limit of 25 ng; this indicated membrane PA levels of <0.78 amol/CV.

To further test the correlations between inhibition of PLD, specific lipid changes, and alterations to fusion parameters, ex vivo treatments of intact eggs were used. In all cases the fusion parameters of CV isolated from the treated eggs were substantially inhibited. Regardless of whether the eggs had been incubated with PLD inhibitors for 20 min or 20 h, there was a similar trend and level of inhibition of CV fusion (data not shown). Maximal effects on the extent, Ca²⁺ sensitivity, and initial fusion kinetics are summarized in Fig. 1. Treatment of eggs with 200 nM, 500 nM, and 2 μM FPII for 20 min caused a concentration-dependent inhibition of all fusion parameters. As an example, a 20-min treatment with 2 μM FPII resulted in a 36.3 ± 5.2% inhibition of the extent of fusion, an ~16 μM rightward shift in Ca²⁺ sensitivity (from EC₅₀ 30.2 ± 2.4 to 46.5 ± 2.6 μM [Ca²⁺]free), and ~31% inhibition of the initial fusion rate (from 76.3 ± 2.9%/s with control CV to 44.9 ± 2.0%/s; Fig. 3a). Egg incubations for 20 h with 500 nM–2 μM FPII resulted in comparable effects on fusion parameters (Fig. 3, c and d); a 35.8 ± 1.9% inhibition of the extent of fusion, an ~13 μM [Ca²⁺]free rightward shift in Ca²⁺ sensitivity (from EC₅₀ 30.2 ± 2.6 to 41.5 ± 2.1 μM [Ca²⁺]free), and ~34%/s inhibition of the initial fusion rate (from 76.8 ± 4.3%/s to 42.6 ± 1.6%/s). In contrast to the acute CV treatments (Fig. 3, c and d), which yielded selective inhibition of fusion kinetics and particularly potent effects with the VU compounds and QRC (the latter being the only compound to inhibit extent as well), the ex vivo egg treatments with the full diversity of PLD inhibitors yielded consistent and significant inhibition of the extent, Ca²⁺ sensitivity, and initial rate of CV fusion (Figs. 3, a–d, and 4).

With either 20 min or 20 h ex vivo incubations with the different PLD inhibitors, there were only select effects on the CV membrane lipid profile: a significant increase in the level of LPE from 8.6 ± 0.5 to ~12.5 amol/CV and a reduction of PE from 25.4 ± 1.5 amol/CV to ~18 amol/CV for FPII, HLP, QRC, and butanol and to ~19.5 amol/CV for VU compounds (Table 1). As with the CV from untreated eggs, there were no other lipid changes identified (Fig. 3e), and PA levels were below detection limits. To detect CV membrane PA and to determine the localization of PLD activity, we labeled both CSC and isolated CV with a fluorescent PLD substrate, NBD-PC. The isolated preparations readily incorporated the fluorescent lipid (Fig. 5); membrane uptake corresponded to 47.8 ± 3.8 nm and 38.7 ± 2.3 nm NBD-PC for CSC and CV suspensions, respectively (Table 2, Fig. 5). NBD-PC labeling followed by 30-min treatments with 2 μM FPII or 2 μM VU02 enhanced NBD-PC levels to ~52 nm in CSC and to ~44 nm in CV. There was pronounced generation of NBD-PA in CSC samples (476.6 ± 19.5 pm; Table 2; Fig. 5b) but a markedly lower production of fluorescent PA in the CV membranes (174.3 ± 8.6 pm; Table 2; Fig. 5a), indicating limited PLD activity associated with CV membranes but far greater activity localized to the inner monolayer of the PM, presumably closely associated with CV docking sites. Taking actual CV counts into consideration, these levels of NBD-PA corresponded to 583.0 ± 28.9 ymol/CV (Table 2) or 351 ± 11 NBD-PA molecules per CV. Inhibition of PLD activity by 2 μM FPII or 2 μM VU02 decreased NBD-PA levels to ~435 pm and ~150 pm in CSC and CV suspensions, respectively; for isolated CVs, this corresponded to ~500 ymol/CV or ~300 NBD-PA molecules/CV, equating to an ~9% (for CSC) and ~14% (for CV) inhibition of PA production. Results from an alternate PLD activity assay (Invitrogen Amplex Red PLD activity assay) were also consistent with the highest PLD activity being detected on CSC relative to the intact egg, cytosol, or the CV membrane. PLD activity associated with CSC membranes was 267.4 ± 10.0 milliunits/ml relative to a significantly lower activity associated with CV membranes (i.e. 79.2 ± 5.8; Table 3). Thus, subtraction of the CV-associated activity indicated ~188 milliunits/ml PLD activity associated with the PM, likely associated with the CV docking sites. All the inhibitors tested significantly inhibited PLD activity in CSC, whereas no significant changes were detected in CV samples (Table 3).

Thus, with a working hypothesis that the PLD activity measured was likely associated with CV docking sites, an assessment of the effects of the inhibitors on intermembrane attachment was undertaken; this membrane docking is routinely assessed using a well-established alternate settle version of the standard CV fusion assay (30, 32, 33, 39, 42) (Fig. 6, b and c). In contrast to the standard assay, after treatments with the different PLD inhibitors the CV settle fusion assays identified a consistent and marked inhibition of all fusion parameters (Figs. 6 and 7). All inhibitors significantly reduced the ability of vesicles to fuse (20–39% inhibition) and the initial fusion rate (inhibited by 22–38%/s) after both acute and ex vivo egg treatments. A more pronounced inhibition of Ca²⁺ sensitivity was observed for settle fusion assays of CV isolated from eggs treated with butanol, HLP, FPII, and QRC for 20 min (21–30 μM [Ca²⁺]free rightward shift) or 20 h (12–22 μM [Ca²⁺]free rightward shift; Figs. 6, c and e, and 7) relative to acutely treated CV (5–12 μM). The VU compounds showed a similar pattern of inhibition of fusion parameters, with slightly more potent effects after the ex vivo egg treatments (Figs. 6b, d and f, and 7).
extensive assessment of docking links the inhibition of PLD activity to an alteration in intermembrane attachment, indicating that locally generated PA functions upstream of the actual fusion step (i.e. membrane merger), promoting late steps of CV docking and associated priming reactions. Moreover, differences in the effects on standard fusion assays (pronounce inhibition of initial rates of fusion) versus settle fusion assays (inhibition of both the ability of vesicles to fuse and efficiency of fusion) would seem to indicate a contribution of PM-associated PLD to the docking steps.

Discussion

A detailed analysis of fusion parameters using a range of reagents to both block PA and inhibit PLD functions confirmed low level PLD activity associated with CV and a substantially higher activity associated with the PM. Overall, the data tend to dissociate PLD activity and PA from direct effects on fusion (i.e. membrane merger) but implicate late upstream roles in docking and associated priming reactions. Moreover, differences in the effects on standard fusion assays (pronounce inhibition of initial rates of fusion) versus settle fusion assays (inhibition of both the ability of vesicles to fuse and efficiency of fusion) would seem to indicate a contribution of PM-associated PLD to the docking steps.

PLD Bioinformatics—Conservation of critical PLD sequences, even in phylogenetically divergent species as bacteria and eukaryotes, is well documented (51). Notably, yeast, *Drosophila*, and nematode species have only one common evolutionary ancestor PLD1-like gene (26); PLD1 and PLD2 diverged from this ancestor gene relatively late in evolution. Critical conserved sequences include PLD-specific catalytic domains, active site sequences, and a pleckstrin homology domain. Mammalian PLDs have tandem pleckstrin homology and Phox domains, which correspond to the C2 domain in plant isoforms (52). An inhibitory role has been suggested for the pleckstrin homology domain, whereas the Phox domain has been implicated in targeting PLD to endocytic vesicles (53–55). All PLD homologs have a highly conserved, invariant, duplicated HxKxxxxD (HKD) catalytic motif, which undergoes intramolecular dimerization upon enzyme activation (26, 56). Both HKD catalytic motifs are associated with the unique transphosphatidylation reaction that produces PA and contain highly conserved histidine, lysine, and aspartate residues (6 His, 8 Lys, and 13 Asp), suggestive of crucial roles in lipase activity. Urchin and human PLD1 and PLD2 share 49 and 40% identity (65 and 55% similarity). The phosphodiesterase HKD active sites 1 and 2 of the urchin PLD have an even higher degree of conservation to HKD active sites 1 and 2 of human PLD1, 78 and 75% identity (85 and 82% similarity) and 63 and 75% identity (74 and 78% similarity), to human PLD2. As this study used an array of PLD-specific inhibitors, it is noteworthy that we have previously confirmed the effective use of targeted inhibitors in cases of such pronounced sequence conservation between sea urchin and mammalian orthologs (33).

PLD Localization—PLD activity has been reported in different cellular locations, including the PM and organelles, with
conflicting reports concerning the localization of isoforms. Studies on different cell types found PLD2 preferentially at the PM (53, 57) and PLD1 on endosomes, lysosomes, and secretory granules (58). The PM localization was reported to be consistent with high basal activity of the PLD2 isoform with no significant contribution from PLD1 (37). PLD1 was localized to the PM and intracellular vesicles in activated macrophages and PLD2 to the nucleus (59, 60). Studies in rat embryo fibroblasts and COS-7 cells localized PLD1 to Golgi, endoplasmic reticulum, and secretory vesicles (56, 58). PLD1 was also found to be associated with caveolae-enriched microdomains (61). Therefore, there is no general consensus concerning the localization of PLD isoforms (59). Due to detection limits in assaying endogenous PA in the CV membrane, here we localize PLD activities by labeling CSC and CV with NBD-PC. This enabled detection of pronounced generation of NBD-PA in CSC but very limited PLD activities on the CV membrane (Table 2; Fig. 5). Negligible generation of PA in fusion-ready CV suggested that PA was likely highly localized at specific sites of the CV membrane that are critical for the docking process. On the other hand, ~3-fold more NBD-PA, generated in CSC, demonstrated that the inner leaflet of the PM was the primary site of PLD activity (Table 2; Fig. 5). An alternate assay also confirmed substantial PLD activity in the PM but very little basal activity in CV membranes (Table 3). The results might be interpreted to suggest two different membrane-bound PLD isoforms at or near the docking/fusion site (i.e. associated with the PM and CV membrane, respectively). Alternatively, considering no pronounced differences in the effects of PLD1- or PLD2-specific inhibitors (i.e. VU01 versus VU02) and the strong evolutionary conservation of critical domains within PLD, the results are also consistent with a single PLD isoform subject to differentially localized regulatory influences as well as activity levels determined by the composition of local membrane microdomains.
**The Role of PLD-derived Phosphatidic Acid in Exocytosis**

**PLD in Regulated Exocytosis**—The role of PLD activity is mainly discussed in the context of its product PA, which serves as an intracellular messenger or a target for protein localization and function. PLD has been suggested to serve as a down-stream effector of ARF (ADP-ribosylation factor) in the regulated secretory pathway (62). The role of the resulting PA was postulated to involve changes in membrane surface charge that could also affect membrane curvature (26). Studies using siRNA-mediated down-regulation of individual PLD isoforms linked PLD and its product PA to regulated exocytosis (29, 63).

Understanding the involvement of PLD in the Ca^{2+}-triggered fusion pathway necessitates tight control of its activities via structurally and mechanistically diverse PLD inhibitors (Fig. 2). These inhibitors either display dual specificities for both PLD1 and PLD2 (*e.g.* HLP and FIPI) or selectivity for either...

**FIGURE 5. Detection of PA in CV and CSC membranes after 30 min of labeling with NBD-PC.** a, representative chromatograms of the CV membrane lipid profile detected with CuSO₄ staining and for fluorescent NBD-PA and NBD-PC (*n* = 3 separate experiments). PI, phosphatidylinositol. b, representative chromatograms of the CSC membrane lipid profile detected with CuSO₄ staining and for fluorescent NBD-PA and NBD-PC (*n* = 3 separate experiments).
PLD1 (e.g. VU01) or PLD2 (e.g. VU02); VU03 has only a slight preference for PLD1 (37). Furthermore, these inhibitors were designed to act directly on the catalytic HKD sequences (37, 38). Nonetheless, despite this selectivity, our first observations were most telling: none had any effect on fusion parameters even at the lowest SRM concentrations (Fig. 2, a and b). This was consistent with data concerning the PA-blocking reagent SRM (50), which had no effect on CV or CSC fusion, indicating that PA was not critical for the fusion step per se (Fig. 1, a and b). In contrast, docking assays revealed potent effects on all fusion parameters even at the lowest SRM concentrations tested (Fig. 1c). Furthermore, fusion was suppressed in the docking assays after either ex vivo egg treatments or in vitro CV/CSC treatments with PLD inhibitors (Fig. 6, a–f), whereas acute CV treatments (except for QRC) selectively suppressed only fusion kinetics (Fig. 2, c and d). Together, the data suggest an upstream modulatory role for PLD in regulated fusion (19, 30, 32, 33, 39) and thus indicate that PA generated by the local PLD activities promotes the efficacy of docking and associated late priming states. This is consistent with studies of neuroendocrine cells that found PLD1-derived PA at the membrane docking sites (29).

In addition to comparable Ca^{2+} activity curves, a well-established and rigorously tested mathematical model of CV-PM fusion also describes CV-CV fusion (42, 43, 46, 64). Considering that (i) full docking is critical to efficient fusion and (ii) the rigorous mathematical model was originally derived for fully docked, fusion-ready CV at the PM, the data here are consistent with previous conclusions that the CV membrane contains the minimal essential machinery for docking, Ca^{2+}-sensing, and fusion (reviewed in detail in Refs. 19, 45, and 65–67). Thus, although the docking/settle assay used here involves a modification of the CV-CV fusion assay, this strongly reflects native docking. This is not to say that there are not additional modulatory components on the PM, and it would seem that PA is likely among them (Fig. 5).

Notably, the pattern of inhibition was similar in standard fusion and docking assays for CV from ex vivo-treated eggs (Figs. 3, a–d, and 6, c–f). All inhibitors also had comparable, apparently saturating effects on the loss of Ca^{2+} sensitivity in docking assays after acute CV treatments, demonstrating limited PLD activity on the CV membrane (Fig. 5, Tables 2 and 3). As the inhibition of the initial rates in the standard CV fusion assay was not seen in CSC fusion (Fig. 2), we speculate that the priming steps of the fusion pathway are likely affected by the CV-associated PLD isofrom. Furthermore, the significant inhibition of Ca^{2+} sensitivity in CV from treated eggs relative to acutely treated CV (Figs. 2 and 3) could indicate the involvement of PM-linked PLD activity with Ca^{2+}-sensing/sensors. Thus the differences in inhibition seen with standard versus docking assays likely distinguish the contribution of PLD isoforms to priming/docking.

We do not exclude the possibility of some nonspecific effects associated with the hydrophobic nature of some of the inhibitors and, thus, potential intercalation into the membrane. However, as the lower inhibitor concentrations did not show any inhibition in standard fusion assays after acute CV and CSC treatments (Fig. 2), this tends to largely rule out issues of nonspecific effects. However, the same concentrations of the agents potently affected intermembrane attachment, indicating specific effects on docking and priming (Fig. 6). The slight effects of high SRM concentrations (100 nm–5 μm) in CSC fusion (Fig. 1a) likely reflected the added contribution of PA in the PM to the native fully docked state of CV. This could also reflect nonspecific binding to other anionic phospholipids like phosphatidylinerine (PS) (50); we recently established that PS has a role in late steps of the fusion pathway that may relate to a Ca^{2+}-sensing function (19).

Notably, the changes seen with QRC and butanol may reflect additional effects. For instance, small alcohols are known to affect exocytosis and membrane structure (51, 68) by diverting PA production to phosphatidyl alcohols (69); this might also explain the potent effect of acute butanol treatments on the docking assay (Fig. 6, a, c, e, and Fig. 7). QRC is also known to inhibit phosphatidylinositol 3-kinase (70, 71) involved in upstream steps of the fusion pathway (33) as well as PLA_2 (72) implicated in exocytosis (31, 73) and known to release lysophospholipids. However, the inhibition of PLA_2 would imply a decrease in CV LPE levels; our data, however, consistently showed increases in the CV LPE regardless of the inhibitor used for ex vivo treatments (Fig. 4e, Table 1). We previously identified links between (i) reduced PE and inhibition of the fusion extent and kinetics and (ii) increased LPE levels and reduced Ca^{2+} sensitivity of fusion (33). Here the data indicated more

**TABLE 2**

NBD-PC and NBD-PA levels in CV and CSC membranes after incubations in 50 μM NBD-PC for 30 min

| Treatments          | Per CV | CV fraction | NBD-PC | zmol ± S.E. | pm ± S.E. | NBD-PA, Generated in CV and CSC | Per CV | CV fraction | NBD-PA, Incorporated in CV and CSC | zmol ± S.E. | pm ± S.E. |
|---------------------|--------|-------------|--------|-------------|-----------|-----------------------------|--------|-------------|----------------------------------|-------------|-----------|
| NBD-PC 50 μM        | 583.0 ± 28.9 | 174.3 ± 86.6 | 476.6 ± 19.5 | 129.3 ± 92 | 38.7 ± 2.3 | 47.8 ± 3.8 |
| NBD-PC+ FIPI 2 μM   | 505.6 ± 28.0 | 151.2 ± 84  | 433.7 ± 19.6 | 146.7 ± 118 | 43.8 ± 8.7 | 52.7 ± 8.8 |
| NBD-PC+ VU02 2 μM   | 499.1 ± 35.7 | 149.2 ± 10.1 | 440.0 ± 14.9 | 149.9 ± 14.6 | 44.0 ± 3.9 | 52.3 ± 3.5 |
| NBD-PC+ QRC 100 μM  | 516.8 ± 25.1 | 154.5 ± 7.5  | 428.8 ± 17.4 | 159.0 ± 12.9 | 47.8 ± 3.7 | 60.5 ± 5.7 |

**TABLE 3**

PLD activities in CV and CSC treated with PLD inhibitors

| Treatments | CV, OD 0.4 | CSC, OD 0.3 |
|------------|------------|------------|
| Control    | 79.2 ± 5.8 | 267.4 ± 10.0 |
| VU1 2 μM   | 81.8 ± 2.9 | 187.6 ± 8.0 |
| VU2 2 μM   | 78.4 ± 2.5 | 184.7 ± 8.7 |
| VU3 2 μM   | 79.1 ± 2.9 | 185.1 ± 9.2 |
| HLP 3 μM   | 84.2 ± 7.3 | 201.1 ± 11.4 |
| FIPI 500 μM| 82.3 ± 5.7 | 200.9 ± 11.0 |
| QRC 100 μM | 80.9 ± 4.4 | 204.4 ± 9.8 |
| Butanol 2% | 84.6 ± 5.2 | 206.6 ± 11.5 |

* Significant differences from the relevant control (p < 0.05).
pronounced changes in the PE and LPE levels for *ex vivo* egg treatments relative to acute CV treatments. Only QRC and VU compounds had significant effects on CV membrane PE and LPE after acute treatments (35% and 23–26% inhibition of fusion extent, respectively, and 30–36%/s inhibition of kinetics). In contrast, blocking PA on the CV membrane with SRM caused 10–23% inhibition of fusion extent and 24%/s inhibition of kinetics. Thus, only about half the inhibition seen is likely linked directly to interfering with PA production. Overall, the changes in PE and LPE indicate one or more possibilities in...
terms of localized lipid changes at or near the docking/fusion site; (i) PE may also be a substrate of the local PLD; (ii) PE is selectively metabolized to compensate for alterations in local PC levels due to it being the preferred PLD substrate (although we might then have expected to detect some change in PC levels); (iii) there is also a linked local PLA$_2$ activity that maintains some critical balance of lysophospholipids (e.g. perhaps to maintain a local block to fusion until the arrival of the Ca$^{2+}$ trigger). Thus the data suggest a link between PLD and PLA$_2$ activities and support the idea of orchestrated actions between PLD and PLA$_2$ (74). Critical linkages between PE and PA in terms of membrane active effects have been identified (17).

**Conclusions**—Overall, the data clearly establish a role for PA in docking and late priming stages. A low basal PLD activity and thus limited PA generation in the CV membrane indicates that PA is highly localized at specific sites of the membrane that are critical for CV docking and fusion (e.g. membrane microdomains defining the physiological fusion machine) (18, 19, 33). On the other hand, a substantially higher level of PM PLD activity is perhaps not surprising considering the multiple functions now attributed to PA (1–6). The data suggest that the profound suppression of fusion extent and kinetics seen for ex vivo incubations with PLD inhibitors (i.e. >23% and >>24%/s, respectively) relative to acute SRM treatments can be attributed to the combined effects of reductions in PA and PE levels and increases in LPE. We have also shown that PA is unlikely to effectively contribute negative curvature to the fundamental fusion mechanism (30). Furthermore, although we have recently demonstrated that anionic lipids localized to physiological fusion machine microdomains could promote Ca$^{2+}$ sensitivity (19), the results with SRM indicate that PA does not contribute to local Ca$^{2+}$ binding. As PLD activities have been

**FIGURE 7.** Summary of settle assay fusion parameters of CV treated with PLD inhibitors for 30 min or CV from eggs treated with PLD inhibitors for 20 min or 20 h ($n = 3–9$). $a$, percent of maximal fusion. $b$, Ca$^{2+}$ sensitivity; $c$, initial fusion kinetics. *, †, and ‡ indicate significant differences from relevant controls ($p < 0.05$).
The Role of PLD-derived Phosphatidic Acid in Exocytosis

reported in specific microdomains (61, 75), it seems likely that anionic PA serves as a binding site or lipid cofactor for proteins that are critical to the tethering, docking, and priming steps of regulated exocytosis. Therefore, PA helps localize specific proteins to the physiological fusion machine and affects their functional conformations (3, 76). Thus, contrary to some untested assertions in the literature, the data here do not support a direct “fusogenic” role for PA in the late steps of exocytosis but are most consistent with critical upstream functions that influence the readiness and efficiency of the subsequent release reaction and thus modulate the resulting physiological mechanism or systems response in question. Again, use of different inhibitors and treatments coupled with quantitative functional and molecular analyses has provided further insight into the composition of the physiological fusion machine and associated fundamental fusion mechanism (i.e. the local membrane microdomains underlying docking and fast, Ca\(^{2+}\)-triggered fusion) and of the functional roles of specific components (18, 43, 77, 78). Only such an integrated approach encompassing both the fundamentally critical as well as key modular components of the release pathway will enable the safe and rational design of interventions necessary to target any number of diseases affecting the secretory pathway in different cell types.

Author Contributions—J. R. C. conceived the study and provided all resources and funding. J. R. C. and T. P. R. designed the study and experiments. T. P. R. performed the experiments and analyzed the data. T. P. R. and J. R. C. interpreted the data. T. P. R. wrote the manuscript and J. R. C. provided critical revision.

Acknowledgment—We are grateful to H. Lundbeck A/S (Copenhagen, Denmark) for graciously supporting this work by freely providing a sample of SRM.

References

1. Arisz, S. A., Testerink, C., and Munnik, T. (2009) Plant PA signaling via diacylglycerol kinase. Biochim. Biophys. Acta 1791, 869–875
2. Cazzolli, R., Shemen, A. N., Fang, M. Q., and Hughes, W. E. (2006) Phospholipid signalling through phospholipase D and phosphatidic acid. IUBMB Life 58, 457–461
3. Chasserot-Golaz, S., Coorssen, J. R., Meunier, F. A., and Vitale, N. (2010) Lipid dynamics in exocytosis. Cell. Mol. Neurobiol. 30, 1335–1342
4. McMahon, H. T., and Gallop, J. L. (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 438, 590–596
5. Reywar, B. J., Illya, G., Harmandaris, V. A., Müller, M. M., Ktrermer, K., and Deserno, M. (2007) Aggregation and vesiculation of membrane proteins by curvature-mediated interactions. Nature 447, 461–464
6. Vitale, N. (2010) Synthesis of fusogenic lipids through activation of phospholipase D1 by GTPases and the kinase RSK2 is required for calcium-regulated exocytosis in neuroendocrine cells. Biochem. Soc. Trans. 38, 167–171
7. Aas, V., Algerøy, S., Sand, K. L., and Iversen, J. G. (2001) Fibronection promotes calcium signaling by interferon-γamma in human neutrophils via G-protein and sphingosine kinase-dependent mechanisms. Cell Commun. Adhes. 8, 125–138
8. Delon, C., Maniava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S., and Krstakiis, N. T. (2004) Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. J. Biol. Chem. 279, 44763–44774
9. Itagaki, K., and Hauser, C. J. (2003) Sphingosine 1-phosphate, a diffusible calcium influx factor mediating store-operated calcium entry. J. Biol. Chem. 278, 27540–27547
10. Hornberger, T. A., Chu, W. K., Mak, Y. W., Hsiung, J. W., Huang, S. A., and Chien, S. (2006) The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc. Natl. Acad. Sci. U.S.A. 103, 4741–4746
11. Chowdary, T. K., Raman, B., Ramakrishna, T., and Rao, C. (2007) Interac- tion of mammalian Hsp22 with lipid membranes. Biochem. J. 401, 437–445
12. Andreisen, B. T., Rizzo, M. A., Shome, K., and Romero, G. (2002) The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. FEBS Lett. 531, 65–68
13. Kooijman, E. E., Chuvin, V., de Kruifff, B., and Burger, K. N. (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. Traffic 4, 162–174
14. Kooijman, E. E., Chuvin, V., Fuller, N. L., Kozlov, M. M., de Kruifff, B., Burger, K. N., and Rand, P. R. (2005) Spontaneous curvature of phosphatidic acid and lysophosphatidic acid. Biochemistry 44, 2097–2102
15. Wang, X., Devaias, S. P., Zhang, W., and Weili, R. (2006) Signaling func- tions of phosphatidic acid. Prog. Lipid Res. 45, 250–278
16. Wang, W., Anderson, N. A., Travesset, A., and Vaknin, D. (2012) Regulation of the electric charge in phosphatidic acid domains. J. Phys. Chem. B 116, 7213–7220
17. Kooijman, E. E., Carter, K. M., van Laar, E. G., Chupin, V., Burger, K. N., and de Kruifff, B. (2005) What makes the bioactive lipids phosphatidic acid and lysophosphatidic acid so special? Biochemistry 44, 17007–17015
18. Churchward, M. A., and Coorssen, J. R. (2009) Cholesterol, regulated exo- cytosis, and the physiological fusion. Biochem. J. 423, 1–14
19. Rogasevskaia, T. P., Churchward, M. A., and Coorssen, J. R. (2012) Ani- onic lipids in Ca\(^{2+}\)-triggered fusion. Cell Calcium 52, 259–269
20. Isenberg, G. (1991) Actin binding proteins: lipid interactions. J. Muscle Res. Cell Motil. 12, 136–144
21. Jensen, R. E., and Sesaki, H. (2006) Ahead of the curve: mitochondrial fusion and phospholipase D. Nat. Cell Biol. 8, 1215–1217
22. Young, B. P., Shin, J. I., Oriji, R., Chao, J. T., Li, S. C., Guan, X. L., Khong, A., Jan, E., Wenk, M. R., Prinz, W. A., Smits, G. J., and Loewen, C. J. (2010) Phosphatidic acid is a pH biosensor that links membrane biogenesis to metabolism. Science 329, 1085–1088
23. Bader, M. F., and Vitale, N. (2009) Phospholipase D in calcium-regulated exocytosis: lessons from chromaffin cells. Biochin. Biophys. Acta 1791, 936–941
24. Chernomordik, L. V., Zimmerberg, J., and Kozlov, M. M. (2006) Membranes of the world unite! J. Cell Biol. 175, 201–207
25. Siddhanta, A., Backer, J. M., and Shields, D. (2000) Inhibition of phosphatidic acid synthesis alters the structure of the Golgi apparatus and inhibits secretion in endocrine cells. J. Biol. Chem. 275, 12023–12031
26. McDermott, M., Wakelam, M. J., and Morris, A. J. (2004) Phospholipase D. Biochem. Cell Biol. 82, 225–253
27. 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
28. Humeau, Y., Vitale, N., Chasserot-Golaz, S., Dupont, J. L., Du, G., Froh- man, M. A., Bader, M. F., and Poulain, B. (2001) A role for phospholipase D1 in neurotransmitter release. Proc. Natl. Acad. Sci. U.S.A. 98, 15300–15305
29. Zeniou-Meyer, M., Zabari, N., Ashery, U., Chasserot-Golaz, S., Haebérlé, A. M., Memains, V., Bailly, T., Gottfried, I., Nakanishi, H., Neiman, A. M., Du, G., Frohman, M. A., Bader, M. F., and Vitale, N. (2007) Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. J. Biol. Chem. 282, 21746–21757
30. Churchward, M. A., Rogasevskaia, T., Brandman, D. M., Khosravan, H., Nava, P., Atkinson, J. K., and Coorssen, J. R. (2008) Specific lipids supply critical negative spontaneous curvature: an essential component of native Ca\(^{2+}\)-triggered membrane fusion. Biophys. J. 94, 3976–3986
31. Coorssen, J. R. (1996) Phospholipase activation and secretion: evidence that PLA2, PLC, and PLD are not essential to exocytosis. Am. J. Physiol. 270, C1153–C1163
32. Rogasevskaia, T., and Coorssen, J. R. (2006) Sphingomyelin-enriched mi- crodomains define the efficiency of native Ca\(^{2+}\)-triggered membrane fu- sion. J. Cell Sci. 119, 2688–2694
The Role of PLD-derived Phosphatidic Acid in Exocytosis

73. Fujiwara, S., Shimamoto, C., Nakanishi, Y., Katsu, K., Kato, M., and Nakahari, T. (2006) Enhancement of Ca^{2+}-regulated exocytosis by indomethacin in guinea-pig antral mucous cells: arachidonic acid accumulation. Exp. Physiol 91, 249–259

74. Wang, Z., Clarke, C. R., and Clinkenbeard, K. D. (1999) Role of phospholipase D in Pasteurella haemolytica leukotoxin-induced increase in phospholipase A2 activity in bovine neutrophils. Infect. Immun. 67, 3768–3772

75. 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

76. Stace, C. L., and Ktistakis, N. T. (2006) Phosphatidic acid- and phosphatidylserine-binding proteins. Biochim. Biophys. Acta 1761, 913–926

77. Ormerod, K. G., Rogasevskaja, T. P., Coorssen, J. R., and Mercier, A. J. (2012) Cholesterol-independent effects of methyl-β-cyclodextrin on chemical synapses. PLoS ONE 7, e36395

78. Rituper, B., Chowdhury, H. H., Jorgacevski, J., Coorssen, J. R., Kreft, M., and Zorec, R. (2013) Cholesterol-mediated membrane surface area dynamics in neuroendocrine cells. Biochim. Biophys. Acta 1831, 1228–1238