Phospholipase D1 Production of Phosphatidic Acid at the Plasma Membrane Promotes Exocytosis of Large Dense-core Granules at a Late Stage

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Substantial efforts have recently been made to demonstrate the importance of lipids and lipid-modifying enzymes in various membrane trafficking processes, including calcium-regulated exocytosis of hormones and neurotransmitters. Among bioactive lipids, phosphatidic acid (PA) is an attractive candidate to promote membrane fusion through its ability to change membrane topology. To date, however, the bio-synthetic pathway, the dynamic location, and actual function of PA in secretory cells remain unknown. Using a short interference RNA strategy on chromaffin and PC12 cells, we demonstrate here that phospholipase D1 is activated in secretagogue-stimulated cells and that it produces PA at the plasma membrane at the secretory granule docking sites. We show that phospholipase D1 activation and PA production represent key events in the exocytotic progression. Membrane capacitance measurements indicate that reduction of endogenous PA impairs the formation of fusion-competent granules. Finally, we show that the PLD1 short interference RNA-mediated inhibition of exocytosis can be rescued by exogenous provision of a lipid that favors the transition of opposed bi-layer membranes to hemifused membranes having the outer leaflets fused. Our findings demonstrate that PA synthesis is required during exocytosis to facilitate a late event in the granule fusion pathway. We propose that the underlying mechanism is related to the ability of PA to alter membrane curvature and promote hemi-fusion.

Phosphatidic acid (PA) is a pleiotropic bioactive lipid that has been proposed to activate selected enzymes (1), recruit proteins to membrane surfaces (2), and serve as a substrate for the formation of other signaling lipids (3). Most intriguingly, PA has also been shown to promote negative curvature in bi-layer membranes due to its small polar head-group in combination with two fatty-acyl side chains (4). The bulk of cellular PA is synthesized via two different acylation pathways, the glycerol 3-phosphate pathway and the dihydroxy acetone phosphate pathway, which are named according to their respective precursors. However, PA is also produced via hydrolysis of phosphatidylcholine by phospholipase D (PLD) (5) on a much faster time scale, and this latter source is thought to underlie the dynamic regulation of PA that allows it to function as a signaling lipid in agonist-stimulated cell biological responses such as secretion and changes in cellular morphology.

In mammals, the classic PLD family is composed of a pair of membrane-associated proteins, PLD1 and PLD2. Both PLD isoforms require phosphatidylinositol 4,5-bisphosphate for their enzymatic activity. However, whereas PLD2 exhibits relatively high basal activity in isolation, full activation of PLD1 requires its stimulation by small GTPases of the ADP-ribosylation factor (ARF), Rho and Ral families, and protein kinase C (3, 6). PLD enzymes have been proposed to be involved in a number of cellular processes, including cell growth and survival, cell differentiation, and vesicular trafficking (3). There is also increasing evidence for a PLD role in calcium-regulated exocytosis, the process by which specialized secretory cells release peptides, hormones, and neurotransmitters. For example, PLD1 and PLD2 have been shown to regulate different phases of exocytosis in mast cells (7). PLD1 has also been implicated in insulin secretion from pancreatic β cells (8, 9) and in neurotransmitter release (10). In neuroendocrine adrenal chromaffin cells and their tumor derivative, the PC12 cell line, we have previously

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2 The abbreviations used are: PA, phosphatidic acid; PLD, phospholipase D; ARF, ADP-ribosylation factor; GFP, green fluorescent protein; siRNA, short interference RNA; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; LPC, lysophosphatidylcholine; GH, growth hormone; EGFP, enhanced GFP; SPM, subplasmalemmal shell; TIRF, total internal reflection microscopy; PABD, PA-binding domain; wt, wild type; Mut, mutated; F, femtofarad(s).

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described that PLD is activated by the stimuli that trigger exocytosis (11) and that this correlated in timing and calcium dependence with the exocytotic response (11). Moreover, inhibition of PA production by primary alcohols and ceramides or expression of a catalytically inactive mutant of PLD1 strongly inhibits exocytosis (12). Taken together, these observations implicate PLD1 in exocytosis, although none of these results directly demonstrates the functional importance of the endogenous enzyme in this process. Finally, the dynamic distribution of PA and its function in the exocytotic machinery have remained unsolved key issues.

We describe here a variety of direct means to study the functional role of PLD1-derived PA in regulated exocytosis from chromaffin and PC12 cells. First, we show that endogenous PLD1 produces PA at the plasma membrane in cells stimulated for exocytosis. Second, we demonstrate that PA synthesis is a prerequisite to normal exocytotic function, and, using capacitance recordings, we show that PA produced by PLD1 is required for the formation of fusion-competent granules in chromaffin cells. Finally, we provide evidence to support the hypothesis that the requirement for PA synthesis during the exocytotic process ensues from changes in effects in the biophysical properties of the plasma membrane lipid bi-layer that act to facilitate membrane fusion at a late step in the process.

**MATERIALS AND METHODS**

**Reagents, Antibodies, and Plasmids**—Palmitoyllyso-phatidylcholine was from Avanti Polar Lipids (Alabaster, AL). The affinity-purified rabbit anti-PLD1 C-terminal antibody was described in Zhang et al. (13). Anti-human GH, anti-SNAP25 antibodies, and secondary goat antibodies coupled to Alexa conjugates (555 or 647) have been described previously (14). Mouse monoclonal anti-GFP antibody was from Roche Applied Science, and goat anti-mouse fluoronanogold Alexa 594-conjugated secondary antibody was from Nanoprobes.

For siRNA targeting, human PLD1 cDNA fragments encoding the 19-nucleotide siRNA sequence CTGGAAGATTACTTGACAA derived from the target transcript and separated from its reverse 19-nucleotide complement by a short spacer, were annealed and cloned in the BglII and HindIII sites in front of the H1 promoter of either the pEGFP-N2-RNAi plasmid or a modified pXGH5 plasmid encoding for GH as described previously (15). Modified pXGH5 vector with no siRNA sequence was named pGHsuper. The PLD1 siRNA sequence was identical in human, rat, and bovine. PLD2 siRNA sequence (mouse nucleotides 1145–1164; identical in mouse, rat, and bovine), validated previously (16), was introduced in the modified pXGH5 vector as described for PLD1. Cells were also transfected with a pCGN plasmid tagged with hemagglutinin containing mutated rescue PLD constructs resistant to siRNA degradation (17). Yeast soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein Spo20p PA-binding domain (wtPA) in a pEGFP-C1 vector (Clontech) was employed (18). A mutated domain (MutPA) was obtained by site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene) to introduce a leucine-to-proline mutation at residue 67.

**Cell Culture**—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glucose (4500 mg/liter) and containing 30 mM NaHCO₃, 5% fetal bovine serum, 10% horse serum, and 100 units/ml penicillin/streptomycin. Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture as described previously (11).

**Growth Hormone Release from PC12 and Chromaffin Cells**—PC12 cells (24-well plates, 80% confluent) were transfected with the various constructs (0.5 μg/well of each plasmid) using GenePorter (Gene Therapy Systems). 72 h after transfection, cells were washed four times with Locke’s solution and then incubated for 10 min in calcium-free Locke’s solution (basal release) or stimulated for 10 min with a depolarizing concentration of K⁺ (Locke’s solution containing 59 mM KCl and 85 mM NaCl). When indicated, 1 μM of lysophosphatidylincholine (LPC) was added during the last wash and the 10-min incubation in resting conditions (calcium-free Locke’s solution) or in stimulated conditions (elevated K⁺ solution). Supernatants were collected, and cells were broken by three freeze and thaw cycles. The amounts of GH secreted into the medium or retained within the cells were measured using an enzyme-linked immunosorbent assay kit (Roche Applied Science). GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

GH release was also measured from adrenal chromaffin cells expressing PLD1 or PLD2 siRNAs. In this case, cells were transfected using the Amaza Nucleofector system (Amaza Biosystems, program X-01). GH release from the cells was measured 72 h after transfection as described above for PC12 cells.

**Determination of PLD Activity**—72 h after transfection, PC12 cells were washed four times with Locke’s solution and then incubated for 10 min in calcium-free Locke’s solution (basal PLD activity) or stimulated in Locke’s solution containing a depolarizing concentration of K⁺. Medium was then replaced by 100 μl of an ice-cold Tris 50 mM, pH 8.0, solution, and the cells were broken by three freeze and thaw cycles. Samples were collected, mixed with an equal amount of the Amplex Red reaction buffer (Amplex Red Phospholipase D assay kit, Molecular Probes), and the PLD activity was estimated after 1-h incubation at 37 °C with a Mithras (Berthold) fluorometer. A standard curve was performed with purified PLD from Streptomyces chromofuscus (Sigma). In the figure, data are given as the mean of six determinations performed on three different cell preparations ± S.E.

**Western Blot Analysis**—The control vectors or the vectors driving the expression of PLD1 siRNAs were introduced in PC12 cells (10⁷ cells/reaction) by electroporation (290 V and 1200 microfarads) or in chromaffin cells using the Amaza Nucleofector system. 72 h following transfection, cell protein extracts were prepared as described previously (15). Proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated in the presence of anti-PLD1 (1/500), anti-actin (1/10 000), anti-GH (1/300), and anti-GFP (1/250) antibodies. Detection was performed by chemiluminescence using the Super Signal West Dura Extended Duration Substrate (Pierce). Transfection efficiency, assessed in parallel by immunocytochemistry (see below) and
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A

SNAP25  wtPABD

Resting

Stimulated 5 min

Stimulated 10 min

Stimulated 10 min + Resting

B

SNAP25  MutPABD  Mask

Resting

Stimulated 10 min

C

Time of stimulation (min)

0 5 10 10+R10

C

F

TIRF intensity (%)

0 50 100 150 200

184.5 ± 3.9 nm. The percentage of granule was counted in the SPM region and non-SPM internal cytoplasm of the cells to estimate the current secretory activity of the stimulated cells compared with resting cells, which did not display any granule in SPM shell. The density of immunogold particles in the SPM region and in the internal cytoplasm was determined as the number particles/μm² of area measured using the image analysis software Axiovision AC Rel. 4.5. The percentages of plasma membrane- and granule-bound immunogold particles in SPM were calculated relative to total immunogold particles counted in SPM. Finally, to estimate the particular accumulation of PA at the plasma membrane involved in granule docking, we compared the numbers of immunogold particles/μm of plasma membrane at docking sites versus non-docking sites of the plasma membrane (t test). The lengths of plasma membrane involved in granule docking were measured according to the schematic in Fig. 3B.

counting of GFP- or GH-expressing cells, ranged from 50 to 65%.

Immunocytochemistry—Transfected PC12 and chromaffin cells were washed twice with Locke’s solution and then incubated for 10 min either in calcium-free Locke’s solution (resting conditions) or in Locke’s solution containing a depolarizing concentration of potassium (stimulation) before the fixation step and further processed for immunofluorescence as described previously (12, 19). The primary antibodies were used at the following dilutions: anti-GH antibody (1/150) and anti-SNAP25 antibody (1/200). Fluorochrome-conjugated secondary antibodies were used at a dilution of 1/1000. Stained cells were visualized using a Zeiss confocal microscope LSM 510. The percentage of the EGFP-PABD binding probes (wild type or mutated) co-localizing at the plasma membrane with SNAP25 was determined using the Zeiss CLSM instrument software 3.2 (15).

Pre-embedding Immunoelectron Microscopy—Chromaffin cells transfected with the plasmid encoding for the wtPABD coupled to EGFP were kept under resting conditions in Locke’s solution or stimulated for 3 min in a solution containing 2 mM BaCl₂, 150 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4. The cells were then fixed for 30 min with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mM sodium phosphate, pH 7.3. After permeabilization in the presence of 0.05% Triton X-100 for 15 min or 0.1% saponin for 30 min, immunostaining was performed using anti-GFP antibodies (1/50) and a fluoronanogold secondary antibody (1/60) according to the silver-intensified immunogold method described by Yi et al. (20).

Morphometric Analysis—Quantitative analysis of the ultrastructural distribution of the granules and of the immunogold particles bound to the wtPABD-EGFP probe was performed in 21 transfected chromaffin cells after stimulation. Granules as well as gold particles were considered to be associated with cellular structures (plasma membrane, sub-plasmalemmal shell (SPM), and granule membrane) when separated by <50 nm. In the culture and fixation conditions used in the present study, the thickness of SPM was 184.5 ± 3.9 nm. The percentage of granule was counted in the SPM region and non-SPM internal cytoplasm of the cells to estimate the current secretory activity of the stimulated cells compared with resting cells, which did not display any granule in SPM shell. The density of immunogold particles in the SPM region and in the internal cytoplasm was determined as the number particles/μm² of area measured using the image analysis software Axiovision AC Rel. 4.5. The percentages of plasma membrane- and granule-bound immunogold particles in SPM were calculated relative to total immunogold particles counted in SPM. Finally, to estimate the particular accumulation of PA at the plasma membrane involved in granule docking, we compared the numbers of immunogold particles/μm of plasma membrane at docking sites versus non-docking sites of the plasma membrane (t test). The lengths of plasma membrane involved in granule docking were measured according to the schematic in Fig. 3B.

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FIGURE 1. Stimulation of exocytosis results in PA production at the plasma membrane. A, confocal immunofluorescence images showing the sub-cellular distribution of a wild-type PA-binding probe coupled to EGFP (wtPABD) in PC12 cells. Cells were maintained in Locke’s solution under resting conditions, or stimulated for 5 or 10 min with a depolarizing concentration of potassium, or stimulated for 10 min and then returned to Locke’s solution for rest for 10 min. The plasma membrane-bound protein SNAP25 was visualized using anti-SNAP25 antibody. In the mask images, the black staining indicates the areas of co-localization of wtPABD with SNAP25 as illustrated by displaying the double-labeled pixels. Bar, 5 μm. B, sub-cellular localization of a mutated, PA non-binding sensor (MutPABD) in resting or stimulated PC12 cells. Bar, 5 μm. C, histogram presenting a semi-quantitative analysis of the percentage of wtPABD and MutPABD that co-localize with SNAP25 at the plasma membrane in resting and stimulated PC12 cells. Data are represented as mean values ± S.E. n = 25 cells for each experimental condition. D, plasma membrane-associated wtPABD was analyzed in PC12 cells by TIRF microscopy. Average changes in TIRF intensity following depolarization of control PC12 cells expressing EGFP (n = 10) or PC12 cells expressing wtPABD (n = 8). Cells were stimulated with a depolarizing solution containing high K⁺ (black line). Data were calculated as the percentage of initial intensity and presented as mean ± S.E. E, catecholamine release in response to a local application of high K⁺ (black line) was recorded for 4 min with a carbon fiber electrode. A typical amperometric profile is shown. F, the amperometric signal measured in panel E was integrated over time to assess the time course of secretion from an individual chromaffin cell.
Amperometry, Membrane Capacitance, and \( \text{Ca}^{2+} \) Measurements—Electrochemical measurement (amperometry) of catecholamine secretion from single chromaffin cells was performed as described previously (21). Capacitance measurements were performed 72 h after electroporation at \( 30-32°C \). Conventional whole cell recordings and capacitance measurements were performed as described (21, 22) on control cells (expressing GFP) and cells expressing the PLD1 siRNA and GFP and analyzed with Igor Pro (WaveMetrics, Lake Oswego, OR). Flashes of UV light were generated by a flash lamp (TILL Photonics, Planegg, Germany), and fluorescence excitation light was generated by a monochromator (TILL Photonics). [\( \text{Ca}^{2+} \)], was calculated from the fluorescence ratio after calibration as described by Voets (23). Fluorescent excitation light was used not only to measure [\( \text{Ca}^{2+} \)], but also to adjust [\( \text{Ca}^{2+} \)], before and after the flash. The calcium concentration before the flash was 300–500 nM. Statistical analysis was done using the Mann-Whitney test. Given values represent mean \( \pm \) S.E. The analysis and comparison were always performed from pairs of control and PLD1-siRNA cells from the same batch of cells.

Total Internal Reflection Microscopy—An inverted Olympus IX-70 microscope equipped with a TIRF condenser (TILL Photonics) was used to view the cells under TIRF illumination. Excitation light at 473 nm was provided by a solid-state laser (Laser Quantum, Stockport, United Kingdom) coupled into a single fiber optic cable that was connected to the TIRF condenser. The laser was focused into the back focal plane of a high numerical aperture lens (\( \times 60 \), numerical aperture = 1.45) designated for TIRF imaging (Olympus). The resulting depth of illumination under these conditions was 200–300 nm from the coverslip. Fluorescence excited by this illumination was passed through a 500 nm dichroic mirror. Time lapse live imaging was captured using an Andor camera (iXon) controlled by MetaMorph software (Universal Imaging, Downingtown, PA), and images were taken every 5 s. Data were analyzed using MetaMorph.

RESULTS

PA Is Produced at the Plasma Membrane When Exocytosis Is Stimulated—Several reports have recently proposed PLD1 as a component of the molecular machinery underlying calcium-regulated exocytosis. If so, it would be reasonable to expect that PLD1 should be activated and PA produced at the site of exocytosis, which would be at the plasma membrane and/or on the membrane of arriving secretory granules. To assess this possibility, we attempted to visualize the intracellular location of PA production during the process of secretion in neuroendocrine cells, using a GFP sensor that specifically binds to PA (supplemental Fig. S1). PC12 cells were transfected with a chimeric protein consisting of EGFP fused to the PA-binding domain (PABD) of Spo20p, a yeast homolog of SNAP25 (18). This probe has previously been shown to be a sensitive and specific sensor of PA in yeast (18). The distribution of the PA sensor was examined in resting PC12 cells and in cells stimulated with a depolarizing concentration of potassium, which is known to trigger exocytosis. As illustrated in Fig. 1A, the PA binding probe (wtPABD) was found to accumulate in the cell nucleus in resting cells. Stimulation of exocytosis triggered the recruitment of a fraction of the PA sensor to the plasma membrane within 5 min, where it co-localized with the SNARE complex protein SNAP25 (Fig. 1A). Similar recruitment of the PA binding probe was observed by time-lapse video microscopy (supplemental movie S1). Accumulation of the PA sensor at the cell periphery was transient, as it rapidly became undetectable when the cells returned to the resting conditions.
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FIGURE 3. Morphometric analysis of the distribution of PA in stimulated cells. A, distribution of gold particles in a 170 nm-deep SPM. The number of gold particles bound to the plasma membrane (PM) and to the secretory granule membrane are expressed relative to the total number of particles detected in the subplasmalemmal region (n = 21 stimulated cells) ± S.E. B, schematic representing the method used to define the length in microns of the granule (G) docking sites at the plasma membrane (PM). The length of the plasma membrane docking sites (DS) and non-docking sites (NDS) was measured from 21 stimulated cells. C, distribution of the plasma membrane-bound gold particles. The number of gold particles was counted at the docking and non-docking sites of the plasma membrane and expressed relative to the length of each zone ± S.E. (t test, p < 0.0005).

To further define the cellular distribution of PA in resting and stimulated chromaffin cells, we used anti-GFP antibodies to perform immunoelectron microscopy on cells expressing wtPABD-GFP. Morphometric analysis revealed that in secretagogue-stimulated chromaffin cells 25.6 ± 2.3% (n = 21 cells) of the granules were found close to the plasma membrane in a ~180 nm wide SPM. This region was virtually devoid of granule in resting cells. Immunogold particles were observed to distribute uniformly in the cytoplasm in resting chromaffin cells (Fig. 2, A and C). Based on Fig. 1A, a strong signal would be expected to be observed in the nucleus. However, this was not the case, most likely due to restricted accessibility of the immunoreagents to the nucleus as a consequence of the mild permeabilization conditions used here to preserve membrane structures. Density of the gold particles in secretagogue-stimulated cells was 10.3 ± 1.2 particles/μm² in the cytoplasmic region and 44.6 ± 6.8 in SPM region (n = 21 cells, p < 0.0001), reflecting a dramatic increase in PABD staining at the periphery of stimulated cells. In the SPM region, numerous gold particles were found to be particularly concentrated at regions of the plasma membrane approached by peripheral secretory granules (Fig. 2, B, D–G, arrowheads), although some immunogold particles were also found on regions of the plasma membrane seemingly free of granules (Fig. 2E, arrows). Note that some peripheral granules were surrounded with gold particles (Fig. 2, E–G), indicating that PA is present not only in the plasma membrane, but also in the granule membrane. This was observed on granules that appeared to be docked (Fig. 2, E–G) as well as on granules deeper in the cytoplasm that did not appear to be in contact with the plasma membrane (Fig. 2, B and G). A detailed morphometric analysis allowed us to estimate that in the SPM region almost half of the gold particles are directly associated with the plasma membrane and ~20% appeared to be granule-bound (Fig. 3A). Moreover, within the plasma membrane gold particles tend to concentrate in the areas facing granules that appeared morphologically docked (Fig. 3, B and C). Altogether these findings support the idea that, in response to chromaffin cell stimulation, PA synthesis occurs at the plasma membrane and preferentially at the granule-docking site.

PLD1 Is Responsible for the Plasma Membrane PA Production Observed during Exocytosis—Since we previously described the presence of PLD1 at the plasma membrane in chromaffin and PC12 cells (11, 12), we hypothesized that PLD1 could be the signaling enzyme primarily responsible for the dynamic production of PA shown above to occur in cells stimulated for exocytosis. We tested this by using an siRNA approach to knock down endogenous PLD1 expression in chromaffin and PC12 cells. To accomplish this and simultaneously enable quantitative assessment of exocytosis, we engineered a...
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plasmid that expresses both full-length human GH and an siRNA-targeted against the sequence of PLD. This plasmid allows also the identification of the subpopulation of cells that transiently express the siRNAs through the immunostaining of GH. In transfected PC12 cells, GH is stored in secretory granules and released by exocytosis in response to cell stimulation (14, 24). Western blot analysis revealed that transient expression of PLD1 siRNA reduced the level of PLD1 but did not affect the level of actin or GH (Fig. 4A). Densitometry scans from independent experiments indicated that the level of PLD1 was reduced by \( \sim 40\% - 50\% \). When normalized to the transfection efficiency, the level of PLD1 in cells expressing the siRNAs was reduced by \( \sim 85\% \) in both chromaffin and PC12 cells (Fig. 4B). To determine whether the reduction of endogenous PLD1 expression affected the secretagogue-evoked PLD activation, we measured PLD activity in homogenates from resting and 

![Image](https://example.com/image.png)

**FIGURE 4.** siRNA-mediated PLD1 knockdown reduces secretagogue-evoked PLD activation in chromaffin and PC12 cells. A, bovine adrenal chromaffin cells or PC12 cells were transfected with either the pGHsuper vector (Control) or the pGHsuper-PLD1 siRNA vector (PLD1 siRNA). 72 h after transfection, proteins were extracted and analyzed by Western blot using anti-PLD1, anti-GH, and anti-actin antibodies. B, semi-quantitative analysis of the actin, GH, and PLD1 levels detected in chromaffin and PC12 cells transfected with the PLD1 siRNA and control vectors. Quantification was performed using scanning densitometry analysis of the Western blots and is presented as mean values ± S.E. of three independent experiments normalized to the transfection efficiency. C, PC12 cells were transfected for 72 h with pGHsuper (Control), pGHsuper-PLD1 siRNA, or a pGHsuper-PLD2 siRNA vector, and then incubated for 10 min in calcium-free Locke’s solution or stimulated for 10 min with 59 mM K \( ^{+} \), collected, and assayed for PLD activity. K \( ^{+} \)-evoked PLD activity is presented after subtracting the PLD activity detected in cells maintained in calcium-free Locke’s solution (control: 10.52 ± 0.43, siRNA PLD1: 9.85 ± 0.81, and siRNA PLD2: 6.21 ± 0.54) from the PLD activity measured in the K \( ^{+} \)-stimulated cells and normalizing for transfection efficiency. The transfection efficiency was 55 ± 5% for the pGHsuper, 52 ± 4% for the pGHsuper-PLD1 siRNA, and 51 ± 6% for the pGHsuper-PLD2 siRNA. Data are given as the mean values ± S.E. obtained from different cell preparations (n = 3). Inset shows that PLD2 siRNA prevents overexpression of PLD2 in PC12 cells co-transfected with pCGN-PLD2 and either the pGHsuper vector (Control) or the pGHsuper-PLD2 siRNA vector (PLD1 siRNA). 72 h after transfection, proteins were extracted and analyzed by Western blot using anti-PLD2 and anti-actin antibodies. PLD1-induced PA Plays an Essential Role in Calcium-regulated Exocytosis in Chromaffin and PC12 Cells—We previously reported that overexpression of wild-type PLD1 stimulates
secretion from chromaffin and PC12 cells, whereas a constitutively inactive mutant inhibits it (12). These findings indicated that PLD1 is able to influence the exocytic activity but did not prove that endogenous levels of PLD1 function as a genuine component of the basic exocytotic machinery. To address this question, we examined exocytotic activity in cells with reduced levels of endogenous PLD1, using the plasmid that expresses both GH and the PLD1-targeted siRNA. As stated above, expression of PLD1 siRNA did not modify the expression level of GH (Fig. 4, A and B), nor did it affect the distribution of GH-positive secretory granules as assessed by immunocytochemistry with anti-GH antibodies (Fig. 5, A and B). However, reduction of endogenous PLD1 by siRNA expression significantly inhibited the secretion of GH from nicotine-stimulated chromaffin cells (Fig. 6A). In line with our previous finding that catalytically inactive PLD2 does not affect catecholamine secretion (12), PLD2 siRNA expression did not modify nicotine-evoked GH release (Fig. 6A). Expression of PLD1 siRNAs also inhibited GH release from PC12 cells stimulated with elevated K⁺ (Fig. 6B). In PC12 (Fig. 6B) and chromaffin cells (data not shown), secretion could be rescued by co-expression of a PLD1 protein mutated at wobble codons within the siRNA targeted sequence (Fig. 6B), indicating that the siRNA-mediated phenotype ensued specifically from the reduction of PLD1 expression. Thus, endogenous PLD1 plays an essential function in dense-core granule exocytosis.

Reduction of Endogenous PLD1 Inhibits Exocytosis by Reducing the Number of Fusion Competent Granules—To further define the role of PLD1/PA in exocytosis, we examined the effect of PLD1 knockdown on the amounts and kinetics of secretion from chromaffin cells. To accomplish this, we placed the PLD1 siRNA expression cassette into a GFP expression vector, which allowed us to perform single cell membrane capacitance recordings on the subpopulation of cells transiently expressing the siRNAs (and GFP). Endogenous PLD1 levels were reduced by ~84% in these cells as determined by Western blot analysis (data not shown). GFP-positive cells were stimulated by a step-like increase in intracellular calcium caused by photolysis of the calcium cage NP-EGTA with a flash of UV light. In response to elevation of intracellular calcium ([Ca²⁺]ₐ), control cells displayed a typical biphasic increase in membrane capacitance in which an exocytotic burst was followed by a sustained phase of secretion (Fig. 7A). The exocytotic burst results from the fusion of a pool of release-competent granules, whereas the sustained phase represents granules that are mobilized to undergo priming during the calcium pulse and subsequent fusion (22, 25). Exocytosis from chromaffin cells expressing PLD1 siRNAs was significantly attenuated compared with control cells: both the exocytotic burst (Fig. 7B) and the sustained components (Fig. 7C) were reduced by almost 2-fold compared with the control cells. The decrease in the burst component indicates that the number of fusion-competent granules was reduced in cells with low PLD1 levels, whereas the smaller sustained component suggests that fewer granules matured and subsequently fused during the stimulatory period (Fig. 7A, lower trace). During the sustained component vesicles are mobilized to the primed pool and reach a fusion-competent state; thus, the absence of PLD attenuates the formation of fusion competent vesicles and therefore the whole component is attenuated. Average cytosolic calcium ([Ca²⁺]ₐ) during the flash stimulation in cells expressing PLD1 siRNAs was slightly higher than in control cells, excluding the possibility that the reduced secretion resulted from lower ([Ca²⁺]ₐ) (Fig. 7A, upper trace). Previous work has shown that the exocytotic burst can be further resolved into a fast component with a time constant of ~30 ms (readily releasable pool) and a slow component with a time constant of ~200 ms (slowly releasable pool). To better define the effect of PLD1 knockdown on the kinetics of exocytosis, we analyzed the distinct phases of the exocytotic burst. Our results indicate that PLD1 knockdown reduced the amplitudes of both the fast and slow burst components, but the

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**FIGURE 5.** PLD1 is responsible for the PA production at the plasma membrane during exocytosis. PC12 cells transfected with the pGHsuper vector (A) or the pGHsuper-PLD1 siRNA vector (B) were plated on 4-well plates. 48 h later, the cells were transfected with the wtPABD-EGFP plasmid. 24 h after the second transfection, the cells were incubated for 10 min in calcium-free Locke’s solution (Resting) or stimulated for 10 min with 50 mM K⁺ (Stimulated). The intracellular localization of GH (used to identify transfected cells) and SNAP25 was determined by confocal microscopy using anti-GH and anti-SNAP25 antibodies. Masks representing the regions of wtPABD/SNAP25 co-localization are illustrated through presentation of the double-labeled pixels. Bars, 5 μm. C, histogram representing a semi-quantitative analysis of the percentage of wtPABD co-localizing with SNAP25 in resting or stimulated PC12 cells expressing either the wtPABD alone, the wtPABD and GH, or the wtPABD, GH, and PLD1 siRNAs. Data are given as mean values ± S.E. n = 20 cells from at least three different cell cultures for each experimental condition.
release kinetics of the two releasable pools of granules remained unchanged (Table 1).

In control cells, the first flash stimulation depletes the releasable pools of granules and these pools can be refilled within 1–2 min (22). To determine if the reduction of endogenous PLD1 affects also the granule pool refilling, we applied a second flash stimulation 2 min after the first one. The second flash stimulation elicited a reduced response in cells expressing PLD1 siRNAs (Fig. 7, D–F). These results indicate that, within the short interval between the two flash stimulations, PLD1 is required to enhance granule maturation and its absence reduces the ability of the cells to replenish the pool of fusion-competent granules.

As a complementary set of experiments, we found that overexpression of wild-type PLD1 enhanced both the exocytotic burst and the sustained phase of secretion from chromaffin cells (not shown). Thus, PLD1 has a physiological role in the exocytosis of large dense core granules, possibly by controlling the number of fusogenic sites on the plasma membrane and thus affecting the number of fusion-competent granules.

**Exocytosis in Cells Depleted of Endogenous PLD1 Can Be Rescued by Providing Inverted Cone-shaped Lipids to the Outer Leaflet of the Plasma Membrane**—Lipids have been proposed to play a decisive role in the late post-docking stages of exocytosis. For instance, once a granule has become juxtaposed to the plasma membrane through the formation of SNARE complexes, it has been suggested that there is a progressive formation of a granule/plasma membrane stalk and a lipid zipping process that generates a hemi-fusion intermediate, which proceeds to pore formation and expansion (26, 27). Cone-shaped lipids like PA, when concentrated on the juxtaposed membrane leaflets, lessen the energy requirements of the curvature process and promote the formation of the hemi-fusion intermediates (Fig. 8A). Conversely, inverted cone-shaped lipids present on the outside of the cell should bend the outer membrane leaflet inwards and similarly promote hemi-fusion (Fig. 8A). To probe the idea that PA might function in the late post-docking stages via such a biophysical mechanism, we attempted to rescue the secretory activity of PLD1-depleted cells, by challenging them with external application of the inverted cone-shaped lipid LPC. Control PC12 cells or cells expressing PLD1 siRNA were incubated in the presence of 1 μM LPC and then stimulated with high K⁺. In our experimental conditions, the LPC did not modify basal or K⁺-stimulated GH release from control cells (Fig. 8, B and C). However, addition of LPC almost completely rescued K⁺-evoked GH secretion for the cells expressing PLD1 siRNA (Fig. 8B), indicating that exogenous LPC was able to compensate for the decreased production of PA on the inner leaflet of the plasma membrane due to the knockdown of endogenous PLD1. In contrast, exogenous LPC was unable to restore normal levels of exocytosis in cells expressing the light chain of *Clostridium botulinum* toxin C, which inhibits the granule priming process by cleaving the SNARE complex proteins SNAP25 and syntaxin (Fig. 8C). Thus, the step hindered by the absence of PA at the plasma membrane is sufficiently late in the exocytic pathway that an external agent that promotes membrane bending is able to compensate for the inhibition of secretion resulting from PLD1 deficiency.

**DISCUSSION**

PLD has emerged in recent years as a major actor in a varied set of cellular processes that have in common vesicular trafficking. These include insulin-stimulated fusion of GLUT4-containing vesicles with the plasma membrane in adipocytes (17), phagosome formation and maturation during phagocytosis in macrophages (28), and exocytosis from a number of specialized secretory cell types (9, 29). Based on the microinjection or expression of catalytically inactive PLD mutants, we previously reported that PLD1 represents a component of the fast calcium-regulated exocytotic machinery active in neurons and neuroendocrine cells (10, 12). However, the extent to which PLD1 is required for exocytosis, the location of the PLD1-produced...
PA in the course of exocytosis, and the actual role of PA in the sequential stages driving secretory granules to membrane fusion remained unknown. Using a silencing RNA strategy, we now demonstrate that PLD1 produces PA at the granule docking sites on the plasma membrane and provide evidence that PLD1-derived PA is essential to complete late stages of exocytosis.

The details of the involvement of PLD1 in calcium-regulated exocytosis imply a tight spatial and temporal regulation of its enzymatic activity. With this in mind, we previously investigated the upstream activators of PLD1 in chromaffin and PC12 cells and found that the secretory granule-bound GTPase ARF6 makes a major contribution to the pathway leading to PLD1 activation (30). Interestingly, the activation/inactivation cycle of ARF6 seemed itself intimately linked to the exocytotic reaction as the guanine nucleotide exchange factor ARNO promotes ARF6 activation at the plasma membrane in chromaffin and PC12 cells during exocytosis (30, 31). These results led us to propose that ARF6 activation, and subsequently PLD1 activation, occurs after the recruitment and docking of granules to the plasma membrane. We show here that PLD1 is activated in secretagogue-stimulated cells, resulting in the production of PA at the plasma membrane. Using immunogold electron microscopy, we observed PA in-between peripheral granules and the plasma membrane in stimulated cells, suggesting that PA is generated at the granule docking sites. This finding correlates well with the idea that the activation of PLD1 is under the control of the granule-associated ARF6, which requires

TABLE 1
Effect of PLD siRNA on the different kinetic components of the exocytotic burst

| Component       | Fast Component | Slow Component |
|-----------------|----------------|----------------|
| PLD1 siRNA      |                |                |
| Fast β          | 202.9 ± 26.3 (n = 10) | 37.8 ± 7.5 (n = 10) |
| Fast τ          | 80.8 ± 27.2 (n = 12)* | 29.3 ± 6.1 (n = 12) |
| Slow β          | 334.06 ± 54.6 (n = 10) | 171.26 ± 27.6 (n = 12)* |
| Slow τ          | 326.5 ± 34.8 (n = 10) | 265.4 ± 77.9 (n = 11) |

*p < 0.0005.
docking to become activated. Surprisingly, peripheral, presumably docked, granules were often surrounded with gold particles revealing the presence of PA not only in the cytosolic leaflet of the plasma membrane but also in the outer leaflet of the granule membrane. PLD1 is not associated with secretory granules in resting or in stimulated chromaffin or PC12 cells (12). Thus, an intriguing possibility is that the PA present in the granule membrane is formed in the plasma membrane by PLD1 and diffuses via lipid mixing to the granule membrane at the docking site, implying that granules are to some extent in a “stable” hemi-fused state prior to complete fusion. Alternatively, we cannot exclude the possibility that some PA might be produced in the granule membrane through a biosynthetic pathway that does not involve PLD1. Interestingly, in a recent study using two-photon excitation imaging, Kishimoto et al. (32) observed that the SNARE protein SNAP25 rapidly migrates from the plasma membrane to the membrane of fused vesicles in stimulated chromaffin cells. They suggested that the fusion machinery for compound exocytosis, in which primary readily releasable granules fuse and then become targets for exocytosis of a second granule population, might be supplied via lateral diffusion from the plasma membrane to the membrane of the primary granules after their fusion. Similarly, PA may be part of the fusion machinery that assembles on the membrane of fused granules to allow sequential compound exocytosis of granules present in the deeper layers of the cytoplasm.

Another important aspect of this study is that the PA produced by PLD1 near or at the sites where granules fuse with the plasma membrane seems to be absolutely required for exocytosis and secretion. Regulated exocytosis is a multistep process involving recruitment of secretory granules from a reserve pool, docking of the granules with the plasma membrane, priming to render the docked granules fusion competent, and the final membrane fusion process. To determine which of these steps requires PA, we performed capacitance measurements on
For instance, in vitro studies on reconstituted SNARE-dependent membrane fusion using vesicles carrying t-SNAREs or v-SNAREs revealed that PA added to syntaxin4/SNAP23 vesicles markedly enhances the rate of fusion, whereas PA added to VAMP2 vesicles inhibits it, indicating an unexpected dependence of SNARE complex-mediated fusion on asymmetrically distributed PA (33). PA has been also implicated specifically in vesicle fusion during sporulation in yeast (34). Moreover, mitochondrial fusion, which is not mediated by SNAREs, requires the generation of PA by mitochondrial PLD (35), indicating that fusion reactions are based on a common specific modification of the lipid environment despite the lack of a conserved protein machinery.

How can PA determine fusion sites? We show here that provision of an inverted-cone shaped lipid, such as LPC, at the outer leaflet of the plasma membrane, is able to compensate for the absence of PA on the inner leaflet and rescues exocytosis in cells expressing low PLD1 levels. These data support the idea that PA might promote fusion via a biophysical mechanism. In this regard, it should be noted that a recent study by Rigoni et al. (36) found that the biological effects of snake phospholipase A2 neurotoxins, which induce massive exocytosis of neurotransmitters and depletion of synaptic vesicles, can be mimicked by the incubation of nerve terminals with an equimolar mixture of lysophospholipids and fatty acids. They concluded that local lipid changes such as the accumulation of positive curvature-promoting lipids (such as LPC) in the outer leaflet of the plasma membrane or negative curvature-promoting lipids (such as PA) in the inner leaflet of the plasma membrane might be of functional significance in synaptic vesicle release. Our current study supports this proposal. SNARE-induced membrane bi-layer fusion has been described to proceed through a hemi-fused state, in which the outer membrane leaflets fuse first, followed by full fusion upon formation of the inner membrane leaflets and opening of a fusion pore (37–39). Formation of the hemi-fused state is characterized by significant physical constraints, because the outer leaflet needs to bend in a tight negative curve to preserve membrane integrity. Hence, hæmi-fusion seems to be highly dependent on membrane lipid composition. For example, hæmi-fusion with low concentrations of yeast SNARES was observed only when phosphatidylyethanolamine was included in the composition of the liposomes (40). Similarly, addition of LPC to a cell-free model of fusion of yeast vacuoles prevents formation of hæmi-fusion intermediates (41), whereas lipids such as PA, phosphatidylyethanolamine, and diacylglycerol favor and stabilize hæmi-fused membranes (26, 42). In other words, the presence of cone-shaped lipids in the outer leaflet facilitates hæmi-fusion, most likely because they lessen the energy requirements of the negative curvature process. Thus, an interesting possibility is that the local accumulation of PA by PLD1 on the plasma membrane cytoplasmic leaflet favors the formation of hæmi-fusion diaphragms between docked granules and the plasma membrane. In line with this idea, it was shown recently that formation of helical bundles by SNAREs precedes hæmi-fusion (39). This would also explain how PA might control the number of fusion-competent granules without affecting the kinetics of fusion as seen here by capacitance measurements in PLD1-depleted cells. Thus, production of PA at the site of SNARE complex assembly might drive docked granules into a hæmi-fused configuration, whereas fusion of the distal lipid leaflets and opening of the pore might require an additional trigger. Alternatively, PA-induced negative curvature of the cytoplasmic leaflet of the plasma membrane might contribute to the conformational transition states of the trans-SNARE complexes, which occur prior to membrane fusion (43). This latter hypothesis is supported by the in vitro observations indicating that SNARE-mediated membrane fusion is facilitated by the presence of PA in the acceptor membrane but inhibited by PA in the donor membrane (33). Our current data do not favor one of the possibilities over the others. New experiments and designs are now required to reveal the roles and the stage directions followed by the varied actors playing out the final scenes of exocytosis.

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REFERENCES
1. Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) Cell 99, 521–532
2. Ktistakis, N. T., Delon, C., Manifava, M., Wood, E., Ganley, I., and Sugars, J. M. (2003) Biochem. Soc. Trans. 31, 94–97
3. Jenkins, G. M., and Frohman, M. A. (2005) Cell Mol. Life Sci. 62, 2305–2316
4. Kooijman, E. E., and Frohman, M. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15300–15305
5. Athenstaedt, K., and Daum, G. (1999) Eur. J. Biochem. 266, 1–16
6. McDermott, M., Wakelam, M. J., and Morris, A. J. (2004) Biochem. Cell Biol. 82, 225–253
7. Choi, W. S., Kim, Y. M., Combs, C., Frohman, M. A., and Beaven, M. A. (2002) J. Immunol. 168, 5682–5689
8. Hughes, W. E., Elgundi, Z., Huang, P., Frohman, M. A., and Biden, T. J. (2004) J. Biol. Chem. 279, 27534–27541
9. Wassef, L., Gerona, R. R., Vitale, N., Martin, T. F., Bader, M. F., and Regazzi, R. (2005) Mol. Endocrinol. 19, 3097–3106
10. Humeau, Y., Vitale, N., Chasserot-Golaz, S., Dupont, J. L., Du, G., Frohman, M. A., Bader, M. F., and Poulan, B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15300–15305
11. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) J. Biol. Chem. 273, 1373–1379
12. Vitale, N., Caumont, A. S., Chasserot-Golaz, S., Du, G., Wu, S., Sciornia, V. A., Morris, A. J., Frohman, M. A., and Bader, M. F. (2001) EMBO J. 20, 2424–2434
13. Zhang, Y., Huang, P., Du, G., Kanaho, Y., Frohman, M. A., and Tsirka, S. E. (2004) Glia 46, 74–83
14. Meyer, M. Z., Deliot, N., Chasserot-Golaz, S., Premont, R. T., Bader, M. F., and Vitale, N. (2006) J. Biol. Chem. 281, 7919–7926
15. de Barry, J., Janoshazi, A., Dupont, J. L., Procksch, O., Chasserot-Golaz, S., Jeromin, A., and Vitale, N. (2006) *J. Biol. Chem.* **281**, 18098–18111
16. Du, G., Huang, P., Liang, B. T., and Frohman, M. A. (2004) *Mol. Biol. Cell* **15**, 1024–1030
17. Huang, P., Altschuller, Y. M., Hou, J. C., Pessin, J. E., and Frohman, M. A. (2005) *Mol. Biol. Cell* **16**, 2614–2623
18. Nakanishi, H., de los Santos, P., and Neiman, A. M. (2004) *Mol. Biol. Cell* **15**, 1802–1815
19. Chasserot-Golaz, S., Vitale, N., Sagot, I., Delouche, B., Dirrig, S., Pradel, L. A., Henry, J. P., Aunis, D., and Bader, M. F. (1996) *J. Cell Biol.* **133**, 1217–1236
20. Yi, H., Leunissen, J., Shi, G., Gutekunst, C., and Hersch, S. (2001) *J. Histochem. Cytochem.* **49**, 279–284
21. Yizhar, O., Matti, U., Melamed, R., Hagalili, Y., Bruns, D., Rettig, J., and Ashery, U. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2578–2583
22. Nili, U., de Wit, H., Gulyas-Kovacs, A., Toonen, R. F., Sorensen, J. B., Verhage, M., and Ashery, U. (2006) *Neuroscience* **143**, 487–500
23. Voets, T. (2000) *Neuron* **28**, 537–545
24. Chasserot-Golaz, S., Vitale, N., Umbrecht-Jenck, E., Knight, D., Gerke, V., and Bader, M. F. (2005) *Mol. Biol. Cell* **16**, 1108–1119
25. Voets, T., Neher, E., and Moser, T. (1999) *Neuron* **23**, 607–615
26. Chernomordik, L. V., and Kozlov, M. M. (2003) *Annu. Rev. Biochem.* **72**, 175–207
27. Jahn, R., Lang, T., and Sudhof, T. C. (2003) *Cell* **112**, 519–533
28. Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N. T., Frohman, M. A., Vitale, N., Bader, M. F., and Grant, N. J. (2006) *Traffic* **7**, 365–377
29. Peng, Z., and Beaven, M. A. (2005) *J. Immunol.* **174**, 5201–5208
30. Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M. A., and Bader, M. F. (2002) *J. Cell Biol.* **159**, 79–89
31. Caumont, A. S., Vitale, N., Gense, M., Galas, M. C., Casanova, J. E., and Bader, M. F. (2000) *J. Biol. Chem.* **275**, 15637–15644
32. Kishimoto, T., Kimura, R., Liu, T. T., Nemoto, T., Takahashi, N., and Kasai, H. (2006) *EMBO J.* **25**, 673–682
33. Vicogne, J., Vollenweider, D., Smith, J. R., Huang, P., Frohman, M. A., and Pessin, J. E. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 14761–14766
34. Nakanishi, H., Morishita, M., Schwartz, C. L., Coluccio, A., Engebretch, J., and Neiman, A. M. (2006) *J. Cell Sci.* **119**, 1406–1415
35. Choi, S. Y., Huang, P., Jenkins, G. M., Chan, D. C., Schiller, J., and Frohman, M. A. (2006) *Nat. Cell Biol.* **8**, 1255–1262
36. Rigoni, M., Caccin, P., Gschmeissner, S., Koster, G., Postle, A. D., Rossetto, O., Schiavo, G., and Montecucco, C. (2005) *Science* **310**, 1678–1680
37. Giraudo, C. G., Hu, C., You, D., Slovic, A. M., Mosharov, E. V., Sulzer, D., Melia, T. I., and Rothman, J. E. (2005) *J. Cell Biol.* **170**, 249–260
38. Lu, X., Zhang, F., McNew, J. A., and Shin, Y. K. (2005) *J. Biol. Chem.* **280**, 30538–30541
39. Reese, C., Heise, F., and Mayer, A. (2005) *Nature* **436**, 410–414
40. Xu, Y., Zhang, F., Su, Z., McNew, J. A., and Shin, Y. K. (2005) *Nat. Struct. Mol. Biol.* **12**, 417–422
41. Reese, C., and Mayer, A. (2005) *J. Cell Biol.* **171**, 981–990
42. Chernomordik, L. V., Zimmerberg, J., and Kozlov, M. M. (2006) *J. Cell Biol.* **175**, 201–207
43. Jahn, R., and Scheller, R. H. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 631–643