Lipids at exocytotic sites: How and why?

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To date, many proteins that catalyze the formation, targeting and fusion of secretory vesicles with the plasma membrane have been identified. However, the lipid composition of vesicles and their target membrane is also critical and lipid modifications may be required in several stages of the exocytotic pathway. Phospholipase D (PLD) generates phosphatidic acid (PA), a multifunctional lipid that can activate selected enzymes, serve as protein attachment site or alter membrane curvature. Expression of the catalytically inactive $PLD1_{K898R}$ mutant in PC12 cells or microinjection of $PLD1_{K898R}$ into chromaffin cells strongly inhibits secretion, supporting the notion that PLD1 plays a major role in the exocytotic pathway. Using deletion and mutagenesis analysis, we found that the PX and $PI4,5P_2$-binding domains are critical for the association of PLD1 to the plasma membrane in resting PC12 cells. However, in secretagogue-activated cells, PLD1 lacking palmitoylation falls into the cytoplasm and fails to activate exocytosis, suggesting that a translocation step into lipid rafts is required for PLD1 to function in exocytosis. We propose that exocytotic sites are determined by the local formation of lipid microdomains, which are potentially important to allow structural and spatial organization of the exocytotic machinery. Among them, PLD1 seems to play a decisive role in the late stages of exocytosis, most likely by adding specific lipid modifications that may be required to allow the membrane fusion machinery to function.

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Membrane fusion is the final step of all vesicular trafficking reactions in eukaryotic cells including exocytosis of hormones and neurotransmitters. In the case of biological membranes, fusion reactions require specific proteins that pull membranes close together to destabilize the lipid/water interface and initiate mixing of the lipids. Several models for protein-mediated fusion reactions have been proposed that differ in the interactions of the fusion proteins with membrane lipids and in the transition states of the lipid bilayers, but they share as common feature the participation of lipids in the late stages of the fusion reaction[1]. Hence, fusion pores in exocytosis have been described as aqueous pores of irregular size that open and close rapidly before irreversibly expanding, supporting the notion that even if fusion reactions are mediated by proteins, fusion pores themselves are essentially lipidic[2]. In other words, the lipid composition of vesicles and their target membrane is likely to be critical and lipid modifications at the site of fusion may be required to allow the membrane fusion machinery to function. We have concentrated our attention on PLD that brakes phosphatidylcholine to produce PA and choline. Two mammalian isoforms, PLD1 and PLD2, being differently regulated by small GTPases, have been identified. Here, we will review our results that led us to propose PLD1 as a key factor for the late stages of exocytosis and discuss the mechanisms that specifically recruit activated PLD1 to the granule docking sites in stimulated chromaffin cells.

RESULTS AND DISCUSSION

In the presence of a primary alcohol, PLD is able to catalyze a specific reaction, the transphophatidylation, to produce the corresponding phosphatidyl-alcohol instead of phosphatidic acid. This unique reaction has been used for many years to reveal the activation of PLD in cells and tissues. To probe the idea that PLD plays a role in the exocytotic reaction, we first compared the PLD activity in subcellular fractions prepared from resting and stimulated chromaffin cells. We found very little PLD activity in the fractions prepared from resting cells but detected a peak of PLD activity in the plasma membrane-containing fractions obtained from stimulated cells[3]. Thus, secretagogue-evoked stimulation and exocytosis is

accompanied by the activation of PLD at the plasma membrane. To establish more directly the role of PLD in exocytosis, we examined the effect of overexpressing in PC12 cells wild type PLD1 or PLD2 or the corresponding catalytically inactive mutants. Co-transfection with a plasmid encoding for growth hormone GH was used to monitor exocytosis in transfected cells[4].

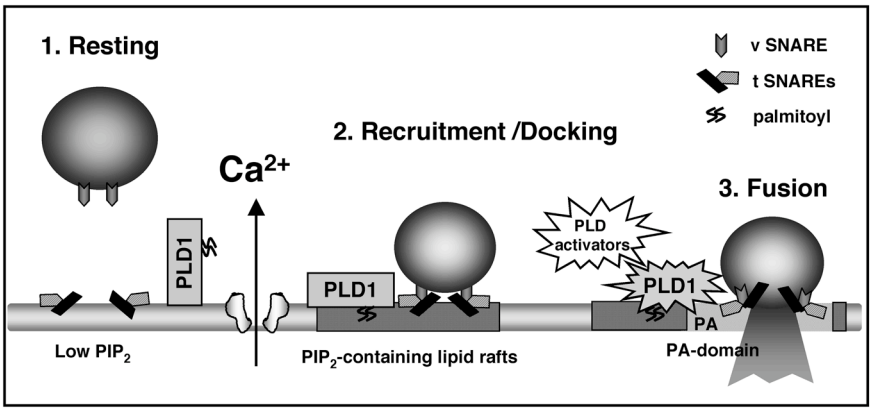

Figure 1. Model for the involvement of PLD1 in regulated exocytosis. In resting cells, PLD1 is associated to the plasma membrane through its N-terminal PX domain. Stimulation and calcium entry triggers the formation of PIP_2-containing micro-domains in the plasma membrane that cluster SNARE complexes and recruit PLD1 through its palmitoylated PH domain. Following granule docking, activated PLD1 locally elevates PA, which promotes the late fusion process.

We found that wild type PLD1 strongly enhanced the exocytotic response, whereas wild type PLD2 had no effect[3]. Moreover, the catalytically inactive $PLD1_{K898R}$ mutant abolished almost completely the exocytotic response evoked by high potassium, whereas the inactive PLD2 mutant had no effect[3]. In line with these results, microinjection of $PLD1_{K898R}$ into chromaffin cells strongly reduced the exocytotic response in a dose-dependent manner[3]. Thus, PLD1 but not PLD2 plays a role in the exocytotic pathway in chromaffin and PC12 cells.

Amperometry provides a number of additional information including the number of granules that have been released given by the number of spikes, the average content of the granules, and the

kinetics of the pore formation, expansion and closure reflected by the rising and decay phases of each spike. We investigated further the step of the exocytotic pathway that requires PLD1 by analyzing the residual spikes in cells microinjected with the inactive PLD1 mutant. $PLD1_{K898R}$ not only inhibited the extent of secretion but it also affected the characteristics of the individual spikes by significantly increasing the spike rise time[3]. Since the spike rise time is thought to reflect the kinetics of the fusion pore opening or expansion, we concluded from these results that PLD1 is probably not involved in the recruitment or movement of the granules to the exocytotic sites, but most likely in the very late fusion step of the granule membrane with the plasma membrane.

The possible involvement of PLD1 in the fusion process implies a tight temporal and spatial regulation of its enzymatic activity. In vitro, PLD1 has a low basal activity and requires activation in a synergistic manner by protein kinase C, Rho and ARF family members[5]. Hence, we found that ARF6 and PLD1 directly interact at sites of exocytosis in PC12 cells, and we could demonstrate that ARF6-stimulated PLD1 activation at the plasma membrane is critical for exocytosis[6]. PLD1 has many membrane-interacting domains including a PX domain at the N-terminus known to interact with various phosphoinositides, a PH domain containing two palmitoylated cysteines, and a central PIP_2 interacting domain[5]. We expressed in PC12 cells several PLD1 proteins mutated in these domains, namely a Δ-PX mutant lacking the PX domain, a protein mutated at the cysteine residues 240 and 241 that cannot be palmitoylated[7] and a PLD1 mutated at the PIP_2-binding site that lost its binding capacity to phosphoinositides[8]. We investigated in parallel the distribution of these proteins in resting and stimulated PC12 cells and their effect on GH secretion. In contrast to the wild type protein found at the plasma membrane in resting and stimulated cells, PLD1 mutated in the PIP_2-binding site was cytosolic both in resting and stimulated cells[9]. Conversely, this mutant was unable to stimulate secretion[9]. Δ-PX-PLD1 was also cytosolic in resting cells but this mutant was recruited to the plasma membrane upon cell stimulation[9]. Interestingly, Δ-PX-PLD1 stimulated secretion to a similar extent that the wild type PLD1[9]. This suggests that the PX

domain is required to maintain PLD1 at the plasma membrane in resting cells but it is not sufficient to recruit PLD1 to the granule docking sites for its functional involvement in the exocytotic machinery. In contrast, PLD1 that is not palmitoylated is still at the plasma membrane in resting cells, most likely through its PX domain, but it falls in the cytosol upon cell stimulation and it is unable to stimulate secretion[9]. Thus, the palmitoylated PH domain is necessary for the recruitment of PLD1 to the exocytotic sites.

Taken together, these observations suggest that the association of PLD1 to the plasma membrane occurs through distinct mechanism in resting and stimulated cells, implying to some extent that secretagogue-evoked stimulation induces changes in the lipid composition or organization in the plasma membrane to prepare the docking sites for granules (see Figure 1). Accordingly, the appearance of PIP_2-binding sites in the plasma membrane that are required for exocytosis have been described[10], and cholesterol- and PIP_2-enriched rafts seem to be critical to the clustering and function of SNARE proteins[11,12]. Once recruited at the granule docking site, how may PLD1 play a role in the late fusion step? PLD1 produces PA, a cone-shaped lipid that has intrinsic negative monolayer curvature propensity. Fusion of liposomes or viral fusion is facilitated by adding compounds that promote negative curvature. Thus, when granules are recruited and docked at the plasma membrane through the formation of the SNARE complexes, the local increase of PA might induce deformations at the point of contact between the two membranes, thereby favoring the formation or the expansion of the fusion pore. Alternatively, PA may recruit specific proteins involved in the late fusion step or be rapidly transformed into other signaling lipids. Additional experimental evidence is now required to prove or refute these possibilities.

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