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PI(4,5)P_2-binding effector proteins for vesicle exocytosis

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Abstract

PI(4,5)P_2 participates directly in priming and possibly fusion steps of Ca^{2+}-triggered vesicle exocytosis. High concentration nanodomains of PI(4,5)P_2 reside on the plasma membrane of neuroendocrine cells. A subset of vesicles that co-localize with PI(4,5)P_2 domains appear to undergo preferential exocytosis in stimulated cells. PI(4,5)P_2 directly regulates vesicle exocytosis by recruiting and activating PI(4,5)P_2-binding proteins that regulate SNARE protein function including CAPS, Munc13-1/2, synaptotagmin-1, and other C2 domain-containing proteins. These PI(4,5)P_2 effector proteins are coincidence detectors that engage in multiple interactions at vesicle exocytic sites. The SNARE protein syntaxin-1 also binds to PI(4,5)P_2, which promotes clustering, but an activating role for PI(4,5)P_2 in syntaxin-1 function remains to be fully characterized. Similar principles underlie polarized constitutive vesicle fusion mediated in part by the PI(4,5)P_2-binding subunits of the exocyst complex (Sec3, Exo70). Overall, focal vesicle exocytosis occurs at sites landmarked by PI(4,5)P_2, which serves to recruit and/or activate multifunctional PI(4,5)P_2-binding proteins.

Keywords

phosphatidylinositol(4, 5)bisphosphate; vesicle exocytosis; CAPS/CADPS; Munc13; synaptotagmin; SNARE proteins

1. Introduction

Early studies showed that Ca^{2+}-triggered vesicle exocytosis in permeable neuroendocrine cells requires MgATP for a priming step that precedes Ca^{2+}-triggered fusion [1, 2]. The MgATP-dependent priming step involves phosphoinositides [3] and requires cytosolic factors (phosphatidylinositol transfer protein and phosphatidylinositol(4)phosphate 5-kinase, PI(4)P 5-kinase)[4, 5], which indicates that PI(4)P^* phosphorylation to PI(4,5)P_2 is essential for maintaining regulated exocytosis. This was further shown with a high affinity PI(4,5)P_2-binding pleckstrin homology domain (PH) from PLCδ_1 [6] and by the enzyme-catalyzed hydrolysis of PI(4,5)P_2 [5, 7, 8], which inhibit evoked vesicle exocytosis in neuroendocrine

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cells. Evoked exocytosis was also shown to be inhibited by the small HIV-1 Tat protein, which directly enters cells and binds $PI(4,5)P_2$ with ~20-fold greater affinity than the $PLC\delta_1$ PH domain [9, 10]. Similarly, a mouse knockout for PI(4)P 5-kinase Iγ caused a reduction in the priming of neuronal dense-core vesicles [11]. Conversely, increased synthesis of $PI(4,5)P_2$ by PI(4)P 5-kinase activation increases sustained rates of evoked secretion [8, 12]. Phospholipase C-catalyzed inhibition of exocytosis in permeable cells [5] suggested there was no major role for $PI(3,4,5)P_3$ (but see below), which was reinforced by the lack of inhibition of ATP-dependent priming by LY294002, a PI 3-kinase inhibitor, [13]; however, inhibition of evoked exocytosis by LY294002 was reported in other studies [14] but this compound also inhibits type III PI 4-kinases [15]. Electrophysiological studies of evoked vesicle exocytosis in neuroendocrine cells showed that $PI(4,5)P_2$ is required for the generation of a primed pool of ready-releasable vesicles as well as for sustained secretion, which represents priming of recruited vesicles [8, 16]. These studies strongly indicate an essential role for $PI(4,5)P_2$ in priming reactions for vesicle exocytosis but they do not exclude additional roles for $PI(4,5)P_2$ at later steps following priming (e.g., fusion). Moreover, this work did not elucidate the precise roles for $PI(4,5)P_2$, which has been the major focus of more recent research.

$PI(4,5)P_2$ as a signaling molecule is abundant in the inner leaflet of the plasma membrane (2 mol%) but much sparser in intracellular membrane compartments. The intact phosphoinositide $PI(4,5)P_2$ plays a critical role in most if not all cellular events associated with the plasma membrane including regulated vesicle exocytosis [17], constitutive vesicle exocytosis [18], endocytosis [19], F-actin assembly [20], cell adhesion [21], phagocytosis [22], viral budding [23], enzyme activation [24], ion channel regulation [25] and cytokinesis [26]. $PI(4,5)P_2$ serves as a marker for plasma membrane identity and establishes a landmark for plasma membrane-associated cellular events [27]. The landmark role for $PI(4,5)P_2$ is interpreted through the interactions of $PI(4,5)P_2$ with proteins that are involved in each of the above cellular processes. Protein binding to $PI(4,5)P_2$ occurs either through structured domains that have basic charge regions such as PH or C2 domains, or through contiguous or non-contiguous basic charge clusters on proteins [28–30]. $PI(4,5)P_2$-binding proteins are the effectors for the biological roles of $PI(4,5)P_2$ where $PI(4,5)P_2$ either functions as a co-factor to activate membrane proteins or to recruit proteins to the plasma membrane for function. $PI(4,5)P_2$ effector proteins are commonly multi-domain coincidence detectors that exhibit interactions with $PI(4,5)P_2$ and with other membrane constituents. Membrane-binding energies sum from multiple low affinity interactions to drive high affinity membrane binding. Especially for Ca^{2+}-dependent membrane binding, there is a marked mutual synergy among interaction partners. For vesicle fusion at the plasma membrane, there are now several examples of $PI(4,5)P_2$-binding proteins that interact with SNARE proteins to promote their assembly for membrane fusion. Multivalent protein-lipid and protein-protein

*__Abbreviations used:__ PI, phosphosphatidylinositol; PI(4)P, phosphatidylinositol 4-monophosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C; TIRF, total internal reflectance fluorescence; PALM, photoactivated localization microscopy; STORM/dSTORM, stochastic optical reconstruction microscopy/direct; STED, stimulated emission depletion microscopy; FWHM, full width half maximum; MARCKS, myristoylated alanine-rich C kinase substrate; CAPS (aka CADPS), Ca^{2+}-dependent activator protein for secretion; Munc13, mammalian Unc13 protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP-2 (aka synaptobrevin-2), vesicle-associated membrane protein-2; GRP1, general receptor of phosphoinositides.

interactions result in higher affinity binding to the plasma membrane and provide orientation of the recruited protein relative to membrane sites landmarked by $PI(4,5)P_2$.

Recent work has characterized the unique distribution of $PI(4,5)P_2$ in the inner leaflet of the plasma membrane in neuroendocrine cells that contributes to establishing sites for vesicle exocytosis. Increasingly, the $PI(4,5)P_2$ effector proteins involved in vesicle exocytosis have been characterized for their recruitment to or activation at sites of exocytosis. TIRF (total internal reflectance fluorescence) and super-resolution microscopies have played an increasing role in establishing the lipid and protein distribution at sites of exocytosis. A number of recent reviews have summarized the proteins and lipids involved in vesicle exocytosis [31–40].

2. $PI(4,5)P_2$ localizes to sites of vesicle exocytosis

While a role for $PI(4,5)P_2$ in vesicle exocytosis has been established, it is important to determine whether regulation by $PI(4,5)P_2$ is local or distant, and whether $PI(4,5)P_2$ localizes to sites of exocytosis. Early studies [3, 5] indicated that $PI(4,5)P_2$ hydrolysis to diffusible products did not mediate the essential role of $PI(4,5)P_2$ in vesicle exocytosis suggesting that regulation was local. Current technology is limited for localizing and perturbing $PI(4,5)P_2$ although this is improving [41–43]. Several studies revealed a heterogeneous distribution of $PI(4,5)P_2$ in the plasma membrane of neuroendocrine and other cells. Caroni and co-workers reported that immunoreactive clusters of $PI(4,5)P_2$ were evident in fixed PC12 cells [44] using a $PI(4,5)P_2$ antibody [45]. Because fixation does not immobilize lipids, it was possible that bivalent antibodies induce $PI(4,5)P_2$ clustering, but a number of cellular conditions were found that alter the size of the clusters (e.g., MARCKS overexpression) suggesting the clusters were physiological. Several studies showed that diffraction-limited puncta of $PI(4,5)P_2$ could be imaged on plasma membrane sheets derived from PC12 or chromaffin cells [8, 46, 47]. These $PI(4,5)P_2$ puncta were similarly imaged either with $PI(4,5)P_2$ antibody [8, 46] or with a GFP fusion of the PH domain of $PLC\delta_1$ [46, 47]. Immuno-EM studies localized $PI(4,5)P_2$ close to chromaffin granules [48]. In the studies of Aoyagi et al., ~20% of the dense-core vesicles on membrane sheets co-localized with $PI(4,5)P_2$ with about half of these also co-localizing with syntaxin-1 [46]. In the studies of James et al., ~20% of the dense-core vesicles on membrane sheets co-localized with the priming factor CAPS/CADPS and with $PI(4,5)P_2$ [47]. These studies indicate that $PI(4,5)P_2$ is distributed in microdomains on the plasma membrane of neuroendocrine cells. A subset of $PI(4,5)P_2$ microdomains co-localize with vesicles and with proteins essential for vesicle exocytosis, which indicates that $PI(4,5)P_2$ is present at sites of vesicle exocytosis (as well as elsewhere) and likely exerts local regulation.

In the studies of James et al., the $PLC\delta_1$-PH-GFP probe was calibrated on supported bilayer membranes to assess membrane $PI(4,5)P_2$ concentrations [47]. $PI(4,5)P_2$ was present in domains at ≥ 6 mol% although this was an underestimate limited by lack of knowledge of the actual size of the diffraction-limited domain. A subsequent study [49] estimated the size of $PI(4,5)P_2$ domains using STED microscopy. The $PI(4,5)P_2$ domains had an average diameter (FWHM) of 73 ± 42nm (s.d.) enabling the authors to calculate that $PI(4,5)P_2$ concentrations at the peak of the domain may reach ~82 mol%. This may also be an

underestimate based on possible crowding of the PLCδ_1-PH domain interacting 1:1 with PI(4,5)P_2 headgroups. Nonetheless, the nanodomains of PI(4,5)P_2 imaged in this study [49] appear to consist of very high concentrations of PI(4,5)P_2 approaching 100 mol%. A study utilizing dSTORM with directly-labeled monoclonal antibodies to PI(4,5)P_2 in PC12 cells confirmed the small size of PI(4,5)P_2 nanodomains (~65 nm). From the high signal-to-noise ratio of dSTORM, the authors concluded that the majority of plasma membrane inner leaflet PI(4,5)P_2 was detected in nanodomain clusters [50].

As noted, PI(4,5)P_2 domains co-localize with only a subset of docked vesicles (~20% in membrane sheets; but see below); however, these could represent a primed subset of vesicles. This was suggested by studies in which the co-localization of PI(4,5)P_2 with vesicles was reduced by briefly evoking exocytosis with Ca^{2+} influx [46]. Whether evoked vesicle fusion occurs preferentially at PI(4,5)P_2-rich plasma membrane sites was addressed in a recent study [51]. Kabachinski et al. used a lower affinity PI(4,5)P_2-binding PH domain probe derived from PLCδ_4 rather than PLCδ_1 to image PI(4,5)P_2 microdomains in live PC12 cells by TIRF microscopy. As was the case for membrane lawn studies, there were a much larger number of PI(4,5)P_2 microdomains than membrane-proximal vesicles, which is consistent with a role for PI(4,5)P_2 in many membrane-linked events. However, ~50% of the dense-core vesicles in the TIRF field of live cells co-localized with the PLCδ_4-PH-GFP probe. Ca^{2+}-induced vesicle exocytosis was found to occur at membrane sites enriched for PI(4,5)P_2 based on the PLCδ_4-PH-GFP fluorescence. A PKC-C1-GFP probe detected no PI(4,5)P_2 hydrolysis to DAG at sites of vesicle exocytosis under Ca^{2+} influx conditions optimal for exocytosis. Greater Ca^{2+} influx did generate DAG (see below). As anticipated for the lower affinity of the PLCδ_4-PH domain for PI(4,5)P_2 [15] as compared to a PLCδ_1-PH domain probe, the PLCδ_4-PH domain probe exhibited reduced partitioning onto the plasma membrane and only partially inhibited vesicle exocytosis [51]. These studies indicate that vesicle exocytosis can occur at membrane sites highly enriched for PI(4,5)P_2. It will be of interest to use super-resolution microscopy to determine whether PI(4,5)P_2 nanodomains disperse or merge with the vesicle membrane at sites of fusion. Diffusion of PI(4,5)P_2 onto the vesicle membrane during fusion, as shown to occur in Xenopus egg cortical granules [52], would promote re-organization of the actin cytoskeleton that could alter vesicle fusion modes or vesicle retrieval in endocytosis.

3. Basis for PI(4,5)P_2 cluster formation

An important but unresolved question is the basis for PI(4,5)P_2 micro/nanodomain formation. Early studies suggested that cellular PI(4,5)P_2 co-purifies with cholesterol-rich, detergent-resistant membrane domains [44, 53] but other studies provided some evidence against this [46, 49]. Recent super-resolution microscopy studies provide support for the concept that PI(4,5)P_2 clusters in the cytoplasmic leaflet align with cholesterol- and sphingomyelin(SM)-rich regions in the ectoplasmic leaflet at least at some plasma membrane sites. Kobayashi and co-workers expressed a fluorescent PLCδ_1-PH domain probe to label the cytoplasmic leaflet, and used an SM-binding protein to label the ectoplasmic leaflet in PALM/dSTORM studies. They detected aligned PI(4,5)P_2/SM clusters that were on average ~250 nm [26]. SM clustering appeared to be required for

$PI(4,5)P_2$ domain formation [26]. These studies provide evidence that cytoplasmic leaflet $PI(4,5)P_2$ domains may correspond in part to cholesterol/SM-rich raft domains.

Substantial experimental work suggests that $PI(4,5)P_2$ in synthetic membranes can self-associate in microdomains via H-bonding interactions especially when electrostatic repulsive interactions are neutralized with cations such as Ca^{2+} [54, 55]. $PI(4,5)P_2$ clustering can also be achieved by electrostatic interactions between proteins with basic charge regions and highly anionic $PI(4,5)P_2$ (−4 charge at pH 7.0) [56]. The charge cluster on MARCKS peptide (KKKKKRFSFKKSFKLSGFSFKKNKK) can sequester three $PI(4,5)P_2$ molecules whereas the much larger PH domains bind one $PI(4,5)P_2$ molecule. The overexpression of proteins with highly basic charge domains such as MARCKS or GAP43 was shown to increase the size of $PI(4,5)P_2$ clusters [44]. These proteins are also acylated and may play a role in raft localization. $PI(4,5)P_2$ also binds to the basic membrane-proximal domain of the SNARE protein syntaxin-1 (see below). However, not all $PI(4,5)P_2$ microdomains localize with syntaxin-1 clusters [46, 49] indicating that $PI(4,5)P_2$ interactions with syntaxin-1 in neuroendocrine cells cannot account for $PI(4,5)P_2$ clustering. However, there are many other proteins involved in exocytosis that interact with $PI(4,5)P_2$ (see below) and these could play a role in clustering. In addition, most intrinsic membrane proteins exhibit basic regions at the cytoplasmic face of their membrane-spanning domains [57]. Thus, $PI(4,5)P_2$ clustering may be mediated by a variety of proteins to form $PI(4,5)P_2$ microdomains that exhibit functions characteristic of the proteins involved. The association of proteins with SM and cholesterol could localize the $PI(4,5)P_2$ into lipid raft domains [58].

PI(4)P 5-kinase, when overexpressed in cells or applied to membrane lawns, increases the intensity of $PI(4,5)P_2$ microdomains, and the enzyme attains a punctate distribution on the membrane [8, 12, 46, 47]. This indicates that PI(4)P phosphorylation by PI(4)P 5-kinases provides a local source of $PI(4,5)P_2$ for microdomain formation but little is understood about the PI(4)P substrate or enzyme targeting [59]. The membrane association of PI(4)P 5-kinases is under active investigation [60, 61] but the basis for PI(4)P 5-kinase targeting to sites of exocytosis is currently unknown. PI(4)P 5-kinases interact with anionic phospholipids including PI(4)P and $PI(4,5)P_2$, which could be the basis for recruitment [62]. ARF6 may also play a role in the recruitment and activation of PI(4)P 5-kinase at exocytic sites [12] as promoted by ARF nucleotide binding site opener (ARNO) [63]. Type Iγ isoforms of PI(4)P 5-kinase can interact with proteins that are themselves $PI(4,5)P_2$ effectors [60]. The type Iγ isoform of PI(4)P 5-kinase is highly enriched in brain synapses and co-localizes with proteins involved in synaptic vesicle exocytosis and endocytosis [64]. Interactions between PI(4)P 5-kinase Iγ and talin and AP2 stimulate $PI(4,5)P_2$ synthesis for actin cytoskeletal regulation and endocytosis [65–68]. PI(4)P 5-kinase Iγ has also been shown to interact with Exo70, a $PI(4,5)P_2$-binding subunit of the octomeric exocyst complex required for cell polarity and constitutive vesicle exocytosis [69]. A PI(4)P 5-kinase β isoform co-localized with $PI(4,5)P_2$/SM raft domains possibly through associations with RhoA [26]. While it is clear that $PI(4,5)P_2$ concentrations in microdomains can be modulated by kinases, phosphatases, and PLCs [8, 47, 51], additional studies are needed to identify turnover rates and the basis for enzyme access into high concentration clusters of $PI(4,5)P_2$.

4. Protein effectors of PI(4,5)P_2 utilized in the secretory pathway

PI(4,5)P_2 in membrane domains at high concentrations is expected to have a pronounced effect on membrane phase behavior, the occurrence of hydrophobic defects, and local membrane curvature [17, 54, 70]. However the impact of direct lipid effects on vesicle exocytosis has been difficult to assess. Most progress has been made in efforts to understand the role of PI(4,5)P_2-binding effector proteins in exocytosis as discussed in the following sections (Figure 1).

4.1. PI(4,5)P_2 interactions with SNARE proteins

Syntaxin-1 plays a central role in the regulated exocytosis of vesicles as a partner for SNAP-25 on the plasma membrane that assembles into a four helix bundle with VAMP-2 (aka synaptobrevin-2) on the vesicle to mediate bilayer fusion (Figure 1). Evidence that syntaxin-1 interacts with PI(4,5)P_2 (see below) prompted studies to determine whether PI(4,5)P_2 regulates syntaxin-1 function in exocytosis. Syntaxin-1 as well as SNAP-25 are present in the plasma membrane mainly distributed in high copy number clusters in equilibrium with smaller pools of monomers [71–82]. Initial studies on PC12 cell membrane preparations suggested that there was some overlap between syntaxin-1 and SNAP-25 clusters as well as some degree of colocalization (30–40%) of syntaxin-1 clusters with docked vesicles [71]. Ca^{2+}-triggered dense-core vesicle exocytosis appeared to occur near syntaxin-1 clusters in these membrane preparations suggesting a role for syntaxin-1 clusters in vesicle docking and fusion [71].

Studies in synthetic bilayer membranes showed that PI(4,5)P_2 reduces the mobility of syntaxin-1/SNAP-25 heterodimers possibly indicating a role for PI(4,5)P_2 in SNARE protein clustering [83]. It was subsequently suggested that fusion-competent vesicles in PC12 cells localize preferentially to plasma membrane sites that contain either PI(4,5)P_2 domains or PI(4,5)P_2 domains co-localized with syntaxin-1 clusters [46]. Studies in live PC12 cells revealed that a subset of docked vesicles are indeed present at PI(4,5)P_2-enriched domains where exocytosis occurs without hydrolysis of PI(4,5)P_2 under optimal Ca^{2+} influx conditions [51]. Other studies in PC12 cells showed that syntaxin-1 clusters are present at sites of vesicle docking but the syntaxin-1 clusters disappear with exocytosis [75]. PI(4,5)P_2 domains co-localize with detected syntaxin-1 clusters to only a very limited extent (5–30%), and co-localizing PI(4,5)P_2 and syntaxin-1 clusters occupy only a small fraction of vesicle docking sites [46, 47, 49]. Such results could indicate that sites for vesicle exocytosis are highly specialized in consisting of PI(4,5)P_2 domains and syntaxin-1 clusters. However, the strongest colocalization is with vesicles and PI(4,5)P_2 domains (30–50%) rather than with syntaxin-1 clusters. Additional live cell studies monitoring PI(4,5)P_2, syntaxin-1 and vesicle exocytosis are needed to assess this especially since there are reports that secretory vesicles localize to sites of low SNARE density [84]. The exact function of syntaxin-1 clusters in vesicle exocytosis is currently unclear [85].

Syntaxin-1 clusters consist of 50–90 copies of syntaxin-1 occupying 50–90 nm diameter regions of the plasma membrane [73, 75, 79]. Syntaxin-1 clusters may partially or fully overlap with SNAP-25 clusters with some indication for the presence of syntaxin-1/SNAP-25 heterodimers as well as Munc18-1 [71, 74, 76, 82, 86]. Syntaxin-1 clusters may

exhibit a gradient of protein density with possible protein or phospholipid interactions and monomer exchange at the periphery of the cluster [79]. Clustering is largely maintained by homophilic interactions by the SNARE domain of syntaxin-1 [72] and interactions with cholesterol [49, 87, 88]. Recent studies indicate that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ promote syntaxin-1 clustering [49, 81]. Syntaxin-1 interactions with phosphoinositides are mediated by electrostatic interactions with a membrane-proximal sequence of basic residues K^{260}ARRKK^{265} [47, 89]. Giant unilamellar vesicles containing cholesterol and 1.5 mol% (but not 5 mol%) $PI(4,5)P_2$ or 1.5 mol% $PI(3,4,5)P_3$ formed large (micron-sized) clusters of syntaxin-1 but cluster formation was abrogated by mutation in the basic cluster (to KARRAA) [49, 81]. Importantly, treatment of PC12 cell membranes with the 5-phosphatase synaptojanin-1 eliminated syntaxin-1 clusters [49]. Because the coincidence of high concentration $PI(4,5)P_2$ microdomains and syntaxin-1 clusters is low (see above), these results indicate that syntaxin-1 clusters are promoted by the moderate (1–2 mol%) concentrations of $PI(4,5)P_2$ found in membrane regions between high concentration $PI(4,5)P_2$ microdomains [47]. The high concentrations of $PI(4,5)P_2$ found in the microdomains at sites of vesicle exocytosis would not be required for syntaxin-1 clustering but may be important for other functions (see below).

Several studies indicate that $PI(4,5)P_2$ or $PI(3,4,5)P_3$ interactions with syntaxin-1 significantly affect syntaxin-1 function in membrane fusion. $PI(4,5)P_2$ at 1–10 mol% inhibits SNARE-dependent liposome fusion in a manner similar to lysophosphatidylcholine, an inverted cone-shaped phospholipid that alters membrane curvature [47]. K264A, K265A and K252A, K253A mutations in syntaxin-1 markedly increased the inhibition, which led to the suggestion that syntaxin-1 sequesters $PI(4,5)P_2$ to enable membrane curvature favorable for fusion [47]. A K260Q, K265L syntaxin-1 mutant was found to partially inhibit CAPS stimulation of fusion suggesting that CAPS interacts with syntaxin-1 near its $PI(4,5)P_2$ binding site [90]. In cellular studies, the replacement of wild-type syntaxin-1 with K^{260}AAAKK or A^{260}AAAAA mutants reduced evoked vesicle fusion consistent with an overall positive action in fusion for syntaxin interactions with acidic phospholipids [89]. Syntaxin-1 generally binds acidic phospholipids such as PA and $PI(4,5)P_2$ so that the actual lipid bound in cells is difficult to determine [89]. Lam et al. [89] showed that PLD1 overexpression compensated for the inhibitory effect of syntaxin-1 mutants in evoked exocytosis, which suggested that PA may regulate syntaxin-1. By contrast, $PI(3,4,5)P_3$ interactions with syntaxin-1 were inferred to be important for neurotransmitter secretion at the *Drosophila* neuromuscular junction by replacing syntaxin-1 with the KARRAA mutant, which reduced synaptic syntaxin-1 clustering and evoked transmitter release [81].

The membrane-proximal basic charge region of syntaxin-1 inserts deeply into the phospholipid headgroup layer [91]. Such interactions might alter the conformation of syntaxin-1 in the bilayer possibly opening its SNARE domain for self-interactions (for clustering) or for interactions with other SNARE proteins. Charge neutralization mutants in the juxtamembrane domain syntaxin-1 could affect its conformation and protein interactions. The current work indicates that $PI(4,5)P_2$ and/or $PI(3,4,5)P_3$ (or PA) regulates syntaxin-1, but it will be important to establish whether it is clustering or another property (conformation) of syntaxin-1 required for regulated exocytosis that is affected by acidic

phospholipids. This may be a highly-conserved property of syntaxins as indicated by the conservation of membrane-proximal charge clusters and the essential nature of the juxtamembrane domain of the yeast syntaxin proteins Sso1/2p [92].

4.2. PI(3,4,5)P_3 as a regulator of synaptic vesicle exocytosis

As noted in the preceding section, studies at the *Drosophila* neuromuscular junction implicated PI(3,4,5)P_2 as a regulator of syntaxin-1 and neurotransmitter release [81]. These studies, which used RNAi to PI 3-kinase to reduce pre-synaptic PI(3,4,5)P_3 levels and a targeted GRP1 PH domain to block PI(3,4,5)P_3 function, found that neurotransmitter release was moderately (~50%) reduced whereas endocytosis was not affected [81]. The results indicated that PI(3,4,5)P_3 rather than PI(4,5)P_2 plays an important regulatory role in synaptic vesicle exocytosis at *Drosophila* neuromuscular junctions. Studies are needed to determine if this result is specific to invertebrates or to synapses, and whether it extrapolates to regulated dense-core vesicle exocytosis in vertebrate neuroendocrine cells. Previous studies in vertebrate neuroendocrine cells had assessed a role for PI(3,4,5)P_3 in evoked vesicle exocytosis using LY294002, a broad spectrum inhibitor of PI 3-kinase, resulting in no effect [13] or an inhibitory effect at high concentrations [14, 93]. More recent studies revealed that LY294002 as well as IC87114, an isoform-specific inhibitor of PI 3-kinaseδ, actually stimulated vesicle exocytosis because of a transient enhancement of PI(4,5)P_2 levels [8, 40, 94]. While some of the previous studies perturbing PI(4,5)P_2 in vertebrate neuroendocrine cells (e.g., 5-phosphatase overexpression) can be interpreted as also affecting PI(3,4,5)P_3, other studies (inhibition by PLCδ$_1$-PH domain and PLC overexpression) are more difficult to re-interpret as a role for PI(3,4,5)P_3 unless functional pools of PI(3,4,5)P_3 are in rapid exchange with PI(4,5)P_2. High concentration ~100 nm domains of PI(3,4,5)P_3 and ~60 nm domains of PI(4,5)P_2 appear to be clearly segregated on the plasma membrane of PC12 cells [50]. Because PI(4,5)P_2 domains have been shown to activate and recruit proteins in the regulation of dense-core vesicle exocytosis [46, 51], it will be of interest to localize PI(3,4,5)P_3 and PI(4,5)P_2 domains with exocytosis in both vertebrate neuroendocrine cells and synapses. Most PI(4,5)P_2-binding effector proteins for regulated vesicle exocytosis utilize mainly electrostatic interactions with little selectivity that may exhibit higher affinity binding to PI(3,4,5)P_3. A central role for PI(3,4,5)P_3 in synaptic vesicle exocytosis could represent a synaptic specialization that increases the efficiency of synaptic transmission. It could also provide a means to independently regulate exocytosis and endocytosis in the synapse.

4.3. CAPS as an effector for PI(4,5)P_2

CAPS (Calcium-dependent activator protein for secretion, aka CADPS) was originally identified as a required factor for Ca^{2+}-triggered vesicle exocytosis that was essential for a priming step following PI(4)P phosphorylation [2, 95, 96]. It was named for the Ca^{2+}-dependent process it functions in but, in spite of very low affinity Ca^{2+} binding [97], does not have a known Ca^{2+}-dependent activity. CAPS does interact with phosphoinositides and its role as a regulator of vesicle exocytosis is PI(4,5)P_2-dependent (Figure 1). The central PH domain in CAPS interacts with PI(4,5)P_2 and to a lesser extent with PI(4)P [7, 47, 98, 99]. However, dependent on the assay, the CAPS PH domain also interacts with other highly-charged inositides [47]. PH domain mutants of CAPS abrogated for PI(4,5)P_2 binding fail to

function in regulated exocytosis in permeable [98] or intact cells [51] indicating that $PI(4,5)P_2$ is an essential co-factor for activating CAPS via binding to its PH domain. Because of low affinity interactions, it is likely that CAPS is activated by $PI(4,5)P_2$, the dominant phosphoinositide in the plasma membrane. CAPS was also characterized as a SNARE-binding protein that interacts with each of the three neuronal SNARE proteins, and with syntaxin-1 at its membrane-proximal, $PI(4,5)P_2$-binding domain [90]. As a SNARE- and $PI(4,5)P_2$-binding protein, CAPS was found to accelerate SNARE-dependent liposome fusion in a $PI(4,5)P_2$-dependent manner [47, 100]. CAPS with PH domain mutations was strongly impaired in its activation of SNARE-dependent liposome fusion [47, 100].

Recent studies show that CAPS is distributed in the cytoplasm but is also bound to dense-core vesicles in neuroendocrine cells [51, 98]. C-terminal truncations of CAPS fail to associate with vesicles and fail to rescue evoked exocytosis in cells depleted for CAPS, which indicates that CAPS likely functions from the vesicle, although it is unclear what mediates CAPS binding to dense-core vesicles. CAPS may promote SNARE complex assembly when vesicles are near the plasma membrane to enable CAPS interactions with $PI(4,5)P_2$ domains in trans. Because the PH domain of CAPS binds $PI(4,5)P_2$ with low affinity, close proximity of vesicles to high concentration domains of $PI(4,5)P_2$ may be essential for CAPS activation [47]. Previous studies showed that $PI(4,5)P_2$ binding to purified CAPS alters its protease sensitivity in vitro [99]. It will be important to determine the $PI(4,5)P_2$-dependent molecular transitions in CAPS responsible for altered protease sensitivity and how they relate to CAPS protein activation for SNARE complex assembly. CAPS may function as a co-incidence detector to signal vesicle arrival at $PI(4,5)P_2$ domains in proximity to plasma membrane SNARE proteins (Figure 1).

Recent studies showed that the hydrolysis of $PI(4,5)P_2$ at Ca^{2+} levels that activate PLCη2 attenuated CAPS function in evoked vesicle exocytosis [51]. This was interpreted to indicate that local $PI(4,5)P_2$ concentrations in microdomains were reduced to below a threshold required for CAPS activation. By contrast, the DAG generated by PLCη2-catalyzed hydrolysis activated Munc13 [51]. These studies indicated that PLCη2 is a Ca^{2+}-dependent regulator of vesicle exocytosis that can shift the function of $PI(4,5)P_2$-dependent effectors.

4.4. Munc13-1/2 as effectors for $PI(4,5)P_2$

Recent studies indicate that Munc13 proteins are effectors for $PI(4,5)P_2$ in evoked vesicle exocytosis (Figure 1). Munc13-1 and ubMunc13-2 contain three distinct C2 domains but only one of these, the central or C2B domain, exhibits canonical Ca^{2+}-binding sequences and Ca^{2+}-dependent phospholipid binding [39]. The crystal structure of C2B revealed a short amphipathic alpha helix in loop 3 that was proposed to bind phospholipids [101]. Phospholipid binding studies showed that the C2B domain of Munc13-1 or ubMunc13-2 exhibited Ca^{2+}-dependent binding to PI(4)P and $PI(4,5)P_2$. Asp to Asn mutations (DN) in the Ca^{2+}-binding sites of C2B were shown to affect the properties of ubMunc13-2 in supporting evoked synaptic vesicle exocytosis [101]. DN mutations in ubMunc13-2 did not affect vesicle exocytosis in response to single action potentials, but markedly reduced synaptic facilitation in response to multiple action potentials. Synaptic facilitation results from accumulated synaptic Ca^{2+} in response to trains of action potentials. Previous studies

indicated that Munc13 activation at its C1 domain by DAG [102] and at a more N-terminal site by Ca^{2+}-calmodulin [103] contribute to short-term synaptic plasticity. Thus, the Ca^{2+}-dependent activation at C2B represents a third mechanism for facilitation. If there was also increased PI(4)P and $PI(4,5)P_2$ synthesis promoted by elevated Ca^{2+} [104, 105], this would further enable Munc13 binding to the plasma membrane via its C2B domain [101]. However, Munc13 proteins localize to the cytomatrix of the active zone [106, 107] and are not obviously translocated to the presynaptic membrane in response to Ca^{2+} elevations [108]. Ca^{2+}-dependent $PI(4,5)P_2$ binding by Munc13 may instead alter molecular interactions (e.g., SNARE complex assembly)[109] that promote synaptic vesicle exocytosis. A somewhat different mechanism involving the translocation of Munc13 to $PI(4,5)P_2$ domains was recently described for neuroendocrine cells [51].

In neuroendocrine PC12 and chromaffin cells, Munc13-1/2 proteins are cytosolic but function at plasma membrane sites of docked vesicles to regulate SNARE complex formation [102, 110]. Recent studies revealed that Ca^{2+} influx promotes the translocation of Munc13-1 to the plasma membrane in PC12 cells [51, 111]. The translocation of Munc13-1-GFP into punctate domains on the plasma membrane was extremely rapid (<10s) in response to depolarization and Ca^{2+} influx [51, 111]. Neutralization of the Ca^{2+}-binding sites of the C2B domain was found to inhibit evoked vesicle exocytosis and to prevent the Ca^{2+}-dependent translocation of Munc13-1, which indicated that $PI(4,5)P_2$ binding by C2B may mediate translocation [51]. Consistent with this, overexpression of the high affinity $PI(4,5)P_2$-binding PH domain of $PLC\delta_1$ blocked translocation of Munc13-1-GFP to microdomains [51]. Mutation in the C1 domain to compromise DAG binding had little effect on the initial Ca^{2+}-induced translocation of Munc13-1 [51]. However, the overexpression of PLCη2, a Ca^{2+}-activated PLC, enhanced and stabilized the translocation of Munc13-1 due to increased DAG generation at $PI(4,5)P_2$ microdomains (G. Kabachinski, M. Yamaga, T. Martin, unpublished). The translocation of Munc13-1 was very similar to the activation mechanism for PKC with initial Ca^{2+}-dependent interactions with $PI(4,5)P_2$ mediated by the C2 domain and subsequent membrane stabilization by DAG interactions with the C1 domain [112]. Interestingly, Ashery and co-workers found that Ca^{2+}-dependent Munc13-1 translocation was markedly enhanced by or dependent upon the overexpression of Doc2b [111], which interacts with Munc13-1 [113]. The C2 domains of Doc2b also exhibit Ca^{2+}-dependent interactions with $PI(4,5)P_2$ [114] so Doc2b and Munc13-1 may be co-recruited to $PI(4,5)P_2$ domains as a complex. Munc13-1 dissociated more slowly than Doc2b from the membrane upon Ca^{2+} level decreases consistent with a distinct mechanism for Munc13-1 stabilization at the membrane [111]. Munc13-1 is proposed to function at the membrane by promoting SNARE protein complex assembly by binding to syntaxin-1 [39, 102, 109, 110]. Multiple ligands ($PI(4,5)P_2$, syntaxin-1, Doc2b, RIM) for Munc13-1 recruitment would provide a high density of interactions for coincidence detection at sites of vesicle exocytosis.

4.5. C2 domain proteins as effectors for $PI(4,5)P_2$

C2 domain-containing proteins are enriched at membrane trafficking nodes. These include Munc13, CAPS, synaptotagmin, rabphilin, synaptotagmin-like proteins (e.g., Slp4a/granuphilin), extended synaptotagmins, double C2 domain (DOC2) proteins, piccolo/

aczonin, Rab11 FIP, ferlins, phospholipases, protein kinases, lipid kinases, and Rho GEF and GAP proteins [35, 115–118]. Synaptotagmin-1 has been extensively studied because of its role as a Ca^{2+}-sensor for regulated vesicle exocytosis [32, 119]. As a vesicle-localized type I membrane protein, synaptotagmin-1 consists of an N-terminal luminal domain, a transmembrane region, a linker region, and two tandem (C2A and C2B) Ca^{2+}-binding C2 domains (Figure 1). Both C2 domains exhibit Ca^{2+}-dependent interactions with phosphatidylserine but the C2B domain also interacts with $PI(4,5)P_2$ via a lysine-rich polybasic domain within the concave surface of the β3-β4 region [36, 80, 118, 120-124]. $PI(4,5)P_2$ binding to this basic patch enhances the Ca^{2+}-sensitivity of synaptotagmin for membrane interactions [123, 125, 126]. Because $PI(4,5)P_2$ is a dominant phospholipid in the cytoplasmic leaflet at vesicle exocytic sites [46, 47, 51], C2B interactions with $PI(4,5)P_2$ could mediate vectorial interactions between vesicle-tethered synaptotagmin-1 and plasma membrane sites for exocytosis [80, 121]. Mutations (K326A, K327A) in the polybasic site have been shown to impair synaptic vesicle exocytosis [127] and to impair SNARE-dependent liposome fusion employing reconstituted synaptotagmin/VAMP2 donor liposomes with $PI(4,5)P_2$/syntaxin-1/SNAP-25 acceptor liposomes [128].

At elevated Ca^{2+} levels, synaptotagmin-1 promotes close membrane apposition by bridging the membranes although the detailed mechanism for bridging remains uncertain [124, 129–132]. Membrane bridging would promote proximity of vesicle and plasma membrane SNARE proteins for complex assembly for fusion. In addition, synaptotagmin-1 interacts directly with SNARE protein complexes potentially promoting C-terminal zippering of SNARE complexes [133–138]. Some studies indicate that the polybasic region in C2B mediates SNARE binding by synaptotagmin-1 [139, 140] but it appears that SNARE binding and $PI(4,5)P_2$ associations can occur simultaneously [80, 114, 119]. Dual interactions of synaptotagmin-1 with $PI(4,5)P_2$ and plasma membrane SNARE proteins is an example of coincidence detection at the plasma membrane. Synaptotagmin-1-mediated bridging of vesicle and plasma membrane would confer vectorial properties on vesicle fusion.

Affinity chromatography of chromaffin granule membrane extracts on biotinylated $PI(4,5)P_2$ bound to avidin-conjugated beads identified several $PI(4,5)P_2$-binding proteins including synaptotagmin-1, synaptotagmin-7 and Slp4a/granuphilin, which are C2 domain-containing proteins [141]. There are a large number of diverse C2 domain proteins involved in the regulation of vesicle exocytosis. These are either soluble (e.g., DOC2, Munc13-1), tethered on vesicles through Rab interactions (e.g., Munc13-4, rabphilin, Slp4a/granuphilin) [35, 117, 142], or vesicle transmembrane proteins (e.g., synaptotagmins-1, -7 and -9). These proteins possess polybasic regions in their C2 domains with several demonstrated to interact with $PI(4,5)P_2$ as well as with SNARE proteins [114, 120, 143, 144]. It will be of considerable interest to determine whether these mechanistically similar proteins function collectively, redundantly, or antagonistically to regulate vesicle exocytosis.

4.6. Protein effectors of $PI(4,5)P_2$ utilized in constitutive secretory pathways

Analysis of late Sec genes in the secretory pathway of *Saccharomyces cerevisae* revealed the exocyst, an octomeric complex of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and

Exo84 subunits, which serves as a major hub for the actin-dependent, polarized exocytosis of post-Golgi vesicles at the bud site of yeasts [145, 146]. Homologous proteins are expressed in vertebrate cells [147] where the exocyst plays roles in polarized membrane growth and vesicle trafficking [148]. For example, exocyst is required for the insulin-stimulated trafficking of glucose transporter Glut4 vesicles to the plasma membrane [149]. Exocyst is proposed to mediate vesicle localization and tethering to target membranes and to enable assembly of SNARE complexes for fusion. A large number of protein-protein and protein-lipid interactions have been identified for the subunits of this oligomeric complex including interactions with phosphoinositides, SNAREs and GTPases [148]. Live cell imaging studies suggest that a vesicle-associated subcomplex of 6 subunits associates with plasma membrane-associated Sec3 and Exo70 subunits to complete a tethering complex [150]. The Sec6 subunit of the complex regulates SNARE complex formation in association with a member of the Sec1/Munc18 family of SNARE regulators [151]. Exocyst is a multi-protein effector for a vesicle Rab(Sec4) that communicates with $PI(4,5)P_2$ and SNAREs at plasma membrane sites, and is functionally similar to multisubunit tethering complexes found at other membrane fusion sites [152, 153]. Several exocyst subunits (Sec15, Exo70, Exo84, Sec6) share structural similarity of tandem helical bundles also found in other tethering complexes (GARP, COG, Dsl1) [152, 154, 155] as well as in CAPS and Munc13 proteins [33, 156] suggesting an ancestral relationship.

The plasma membrane-associated Sec3 and Exo70 subunits serve as landmarks for determining sites of vesicle docking and exocytosis at the plasma membrane [150]. Each of these exocyst subunits binds $PI(4,5)P_2$ and small GTPases [157, 158]. Sec3 contains a novel $PI(4,5)P_2$-binding PH domain that also interacts with Rho1 and Cdc42, which serves as a coincidence detector for plasma membrane associations [159, 160]. Exo70 utilizes a surface patch of basic residues at its C terminus to mediate interactions with $PI(4,5)P_2$ [161]. Studies with a mutant yeast PI(4)P 5-kinase (Mss4) to deplete plasma membrane $PI(4,5)P_2$ showed that polarized secretion was impaired [18, 162]. It should be noted that polarized secretion in yeast is highly dependent on the organization of the actin cytoskeleton where several $PI(4,5)P_2$-binding proteins regulate Rho, Rac and Cdc42 function [18]. In mammalian cells, the exocytosis of post-Golgi vesicles at the plasma membrane was blocked by replacing endogenous Exo70 with a mutant lacking $PI(4,5)P_2$ binding [161]. Recent studies also indicate that Exo70 functions to form $PI(4,5)P_2$-dependent scaffolds that promote plasma membrane deformation for cell migration [163, 164]. Exocyst subunits appear to be important $PI(4,5)P_2$ effectors in yeast and mammalian cells for polarity establishment and polarized vesicle exocytosis.

5. Summary

$PI(4,5)P_2$ is a landmark for plasma membrane-associated cellular events. A substantial fraction of $PI(4,5)P_2$ in neuroendocrine cells segregates into numerous high concentration ~70 nm domains. Some of the $PI(4,5)P_2$ domains localize with dense-core vesicles and with proteins essential for regulated vesicle exocytosis possibly representing preferential sites for vesicle exocytosis. CAPS on vesicles requires high concentrations of $PI(4,5)P_2$ for its activation. Vesicle synaptotagmins utilize $PI(4,5)P_2$ to orient to plasma membrane sites. Munc13 proteins undergo Ca^{2+}-dependent recruitment to $PI(4,5)P_2$ domains. These proteins

function as coincidence detectors by interacting with SNARE proteins to regulate membrane fusion. A key SNARE protein, syntaxin-1, also interacts acidic phospholipids including $PI(4,5)P_2$ to enable its clustering. Similar principles are at work in constitutive vesicle fusion relying in part on $PI(4,5)P_2$-binding by the exocyst complex for plasma membrane localization. Live cell studies employing super-resolution microscopy with better fluorescent probes for $PI(4,5)P_2$ are needed to clarify spatial and temporal aspects of $PI(4,5)P_2$ function in recruiting and activating proteins for vesicle exocytosis.

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Highlights

- $PI(4,5)P_2$ is clustered into high concentration nanodomains on the plasma membrane.
- Vesicle exocytosis occurs at $PI(4,5)P_2$-rich membrane domains.
- $PI(4,5)P_2$ activates/recruits SNARE-binding proteins (CAPS, Munc13, synaptotagmin).
- $PI(4,5)P_2$ regulates the SNARE protein syntaxin-1.
- $PI(4,5)P_2$- and SNARE-binding exocyst complex mediates polarized vesicle exocytosis.

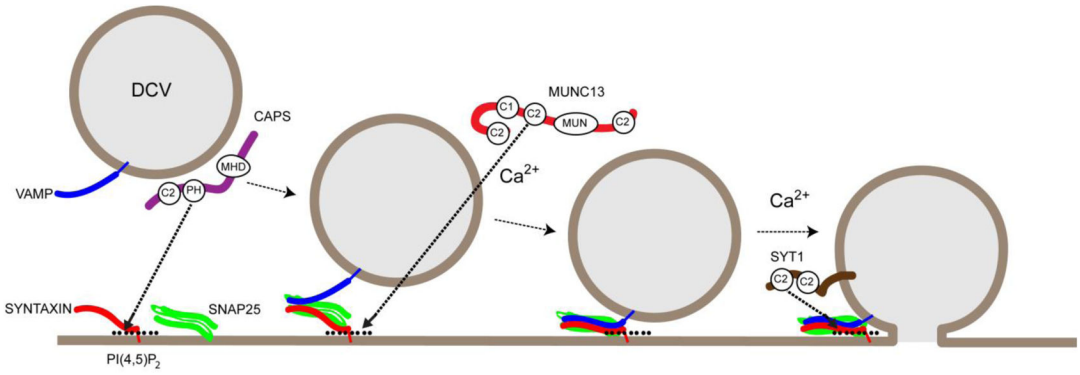

Figure 1.

Schematic of proposed roles for PI(4,5)P_2 (black circles) in regulated vesicle exocytosis. Dense-core vesicles (DCV) are depicted in a linear scheme undergoing docking, priming and fusion. Three PI(4,5)P_2- and SNARE-binding proteins are shown: CAPS (purple) as a DCV-bound protein that undergoes activation at PI(4,5)P_2 domains via a central PH domain; Munc13-1 (or ubMunc13-2, red) as a cytosolic protein recruited to PI(4,5)P_2 domains via a central C2 domain; and synaptotagmin-1 (brown) as a DCV protein directed to fusion sites by PI(4,5)P_2 via its membrane-distal C2 domain. The SNARE proteins (syntaxin-1, red; SNAP-25, green; VAMP-2/synaptobrevin-2, blue) are shown progressively assembling into complexes with syntaxin-1 shown interacting with PI(4,5)P_2.