The role of PtdIns(4,5)P2 in exocytotic membrane fusion

T. F. J. Martin, K. M. Loyet, V. A. Barry and J. A. Kowalchyk
Department of Biochemistry, University of Wisconsin, 420 Henry Mall, Madison, WI 53706, U.S.A.

The physiological release of neurotransmitters and peptide hormones from neural and endocrine cells is regulated by a calcium-dependent membrane fusion apparatus that controls the exocytotic fusion of secretory vesicles with the plasma membrane. Many of the gene products that are required for regulated exocytosis have been identified, and some of the mechanisms responsible for membrane fusion have been characterized to a limited extent [1-4]. Several years ago an unanticipated role for phosphoinositides in this process was discovered [5-7]. The evidence that inositol phosphorylation is required for membrane fusion, and efforts to elucidate the role of PtdIns(4,5)P2 in regulated exocytosis will be discussed.

Background

A number of studies of regulated fusion have used permeable secretory cells to provide access to the exocytotic apparatus. Adrenal chromaffin cells and immortalized PC12 cells have advantages for these studies, which include the extensive background of biochemical studies of the catecholamine-storing chromaffin granule. Early studies discovered a resident PtdIns 4-kinase activity on chromaffin granules [8-10], a finding that has been generalized to many other secretory vesicles. In prescient studies, Holz and co-workers [5] discovered that calcium-dependent noradrenaline secretion in digitonin-permeabilized chromaffin cells was inhibited by treatment with a PtdIns-specific phospholipase C. Additional studies suggested to the authors that the ATP dependence of calcium-activated noradrenaline secretion might reside with the process of lipid phosphorylation and synthesis of PtdIns(4,5)P2.

With extensively permeabilized PC12 cells, we sought to determine the mechanisms underlying the ATP-dependence of regulated secretion. An ATP-dependent priming step was found to precede and to be required for the subsequent calcium-activated membrane fusion step [11]. ATP-dependent priming exhibited a requirement for PtdIns(4,5)P2.

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for three cytosolic factors that were resolved by gel-filtration chromatography. These were termed PEP1, -2 and -3 (in order of elution) for ‘priming in exocytosis proteins’. Following purification and sequencing, the smallest of these (PEP3) was revealed to be the \( \alpha \) isoform of PtdIns transfer protein [6]. Since this protein does not require ATP for \textit{in vitro} phospholipid transfer, it seemed likely that its function in regulated secretion was coupled to an ATP-dependent process. This was confirmed to be the case upon characterization of PEP1 as consisting, at least in part, of the brain type I isoform of PtdIns4P 5-kinase [7].

The identification of two of the PEP proteins as constituents in a pathway for PtdIns(4,5)P\(_2\) biosynthesis provided direct evidence for the suggestion of Eberhard et al. [5] that inositol phosphorylation played a role in the ATP-dependent priming of regulated exocytosis. Synergy between PEP1 and PEP3 further indicated their participation in a common pathway [7]. Recently, Wiedemann et al. [12] provided evidence that the chromaffin granule PtdIns 4-kinase is also essential for calcium-activated exocytosis. A number of studies indicate PtdIns(4,5)P\(_2\) is required for membrane fusion. Either hydrolysing the lipid with phospholipase C or binding it with specific monoclonal antibodies resulted in a very strong inhibition of calcium-activated membrane fusion in ATP-primed permeable PC12 cells [7]. The fact that Ca\(^{2+}\)-activated secretion is highly sensitive to neomycin or many other basic substances (e.g. Lys-rich peptides) may also be related to this requirement. Recently, genetic evidence that PtdIns transfer protein and PtdIns4P 5-kinase are essential for nervous system function was reported [13,14]. Membrane trafficking defects in yeasts harbouring PtdIns transfer protein (SEC14) or 5-kinase (FAB1) mutations have also been described [15,16].

Unlike the role of PtdIns(4,5)P\(_2\) in signal transduction, where it serves as a precursor for hydrolysis to lipid-derived metabolites, it is likely that the intact phosphoinoside is required for membrane fusion. PtdIns-specific phospholipase C [5] and a recombinant mammalian phospholipase C [7] inhibit rather than stimulate secretion, indicating that a metabolite derived from phospholipase C hydrolysis is not involved. A number of metabolites and related compounds have been tested in permeable cells for effects on secretion. At concentrations up to 100 \( \mu \)M, inositol polyphosphates, PtdIns, PtdIns4P, glycerol-phosphorylinositol bisphosphate, arachidonic acid and phosphatidic acid have minimal effects on secretion. The effects of diacylglycerol could not be interpreted since it caused granule lysis but not fusion. Interestingly, PtdIns(4,5)P\(_2\) is strongly inhibitory at low concentrations (Figure 1), whereas equal concentrations of PtdIns(3,4,5)P\(_3\) fail to inhibit (and may slightly stimulate) calcium-activated secretion. Presumably the inhibitory effects of PtdIns(4,5)P\(_2\) result from competition with endogenous phosphoinoside-binding proteins. Overall, the results indicate that the PtdIns(4,5)P\(_2\) synthesized during ATP-dependent priming is not employed as a precursor for the generation of lipid-derived metabolites that function in fusion.

Like the process of inositol phosphorylation, priming of exocytosis had been found to be fully reversible [11]. While the ATP-dependent priming process was dependent upon a PtdIns4P 5-kinase, the reversal of priming appears to involve a 5-phosphatase (Figure 2). This was suggested by the accelerated reversal of priming by cytosol and Mg\(^{2+}\). Although the cytosolic factor remains to be characterized, the provision of a recombinant type II inositol phosphate 5-phosphatase [17] was found to accelerate the reversal of priming in a manner similar to cyto-

**Figure 1**

PtdIns(4,5)P\(_2\) but not PtdIns(3,4,5)P\(_3\) inhibits Ca\(^{2+}\)-activated secretion in permeable PC12 cells

A two-stage secretion assay was conducted with \(^{1}H\)noradrenaline (NE)-labelled PC12 cells as previously described [11]. Lipids at the indicated concentrations were added to ATP-primed permeable cells on ice for 30 min before conducting 3-min Ca\(^{2+}\) triggering incubations at 30 °C.

![Figure 1](image-url)
The Cell Biology of Lipid Signalling

Figure 2

The primed state is reversible

ATP-dependent priming incubations were conducted with permeable PC12 cells as previously described [11]. Primed cells were washed and incubated at 30°C for the indicated times before determining Ca²⁺-triggered noradrenaline (NE) secretion in 3-min incubations at 30°C [11]. Left panel: Incubations were conducted without ATP in the presence of 2 mM Mg²⁺ and 1 mg/ml rat brain cytosol as indicated. Right panel: Incubations were conducted without ATP and with 2 mM Mg²⁺ in the presence of bacterial extracts from either non-expressing (control) cells or from cells expressing a type II 5-phosphatase [17].

sol. These preliminary studies were consistent with a cycle involving 5-kinase and 5-phosphatase as the basis for priming and its reversal. The recent characterization of a synaptic type II 5-phosphatase [18], suggested to play a role in synaptic vesicle endocytic retrieval, raises the possibility that regulation of phosphoinositide phosphorylation may control the fate of vesicle membrane toward exocytic or endocytic destinations.

Whereas the preceding evidence indicates a role for PtdIns(4,5)P₂ in regulated exocytosis, it was possible that this role was to serve as a precursor for formation of a 3-phosphorylated inositol. PtdIns or PtdIns(4,5)P₂ 3-kinases have been shown to play a role in the sorting of proteins to the vacuole in yeast [19], and in endosomal and lysosomal protein sorting in mammalian cells [20-22]. Much of the work in mammalian cells has employed PtdIns 3-kinase inhibitors such as wortmannin or LY294002. As shown in Figure 3, the ATP-dependent priming of calcium-activated exocytosis in permeable PC12 cells was largely insensitive to either wortmannin or LY294002. On this basis, it can be concluded that an inhibitor-sensitive lipid kinase is not required for priming the exocytotic apparatus.

PtdIns(4,5)P₂ synthesis on docked granules

An important step toward elucidating the mechanism for PtdIns(4,5)P₂ involvement in membrane fusion is to determine in which membrane(s) lipid phosphorylation occurs during ATP-dependent priming. This was recently accomplished in immunocytochemical studies with a PtdIns(4,5)P₂-specific monoclonal antibody (K. M. Loyet, F. Fukami, T. Takenarva and T. F. J. Martin, unpublished work). PC12 cells were mechanically permeabilized, incubated under conditions that prime or reverse priming, and tethered on to glass coverslips. Without fixation, the permeable cells were incubated with anti-PtdIns(4,5)P₂ antibodies and fluorescent secondary antibodies. At high dilutions of the primary antibody, fluorescence was restricted to a punctate array close to the plasma membrane. The fluorescence was dependent upon incubation with MgATP, and eliminated by treatment with phospholipase C. The punctate nature of the fluorescence, and its proximity to the plasma membrane, suggested that the PtdIns(4,5)P₂ was on secretory granules. This was confirmed by fluorescence co-localization studies with antibodies to chromogranin B, a granule-specific
constituent, and by immunogold electron microscopy. Calibration of the immunofluorescence with liposomes containing known amounts of PtdIns(4,5)P_2 showed that detection required PtdIns(4,5)P_2 concentrations in excess of 10 mol%.

**Effectors of PtdIns(4,5)P_2 in regulated exocytosis**

The high negative charge, strong degree of hydration and the positive curvature of the extremely high concentrations of PtdIns(4,5)P_2 formed on the secretory vesicle would probably inhibit membrane fusion based on any of the mechanisms that have been proposed for fusion [23]. Thus it seems likely that the PtdIns(4,5)P_2 would need to be sequestered away from active points of fusion, possibly by phosphoinositide-binding proteins. PtdIns(4,5)P_2-binding proteins have the capability of segregating this lipid into domains that contain the lipid and the binding protein and exclude other constituents in the membrane [24]. Recently, a large number of phosphoinositide-binding proteins have been identified in efforts to elucidate the effectors of PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns3P for growth regulation, cytoskeletal rearrangements and membrane trafficking [25,26]. Several distinct phosphoinositide-binding motifs have been found to mediate phosphoinositide–protein interactions, including PH domains, Lys/Arg-rich regions as well as many others. At the present time, the effectors for PtdIns(4,5)P_2 in membrane fusion remain to be identified. Synaptotagmin, a candidate calcium sensor for exocytosis, was reported to engage in calcium-stimulated interactions with PtdIns(4,5)P_2 [27], raising the possibility that this vesicle protein could act to sequester PtdIns(4,5)P_2 in preparation for fusion. Consistent with this suggestion, recent studies demonstrated that InsP_6, which competitively interferes with synaptotagmin C2B domain interactions with PtdIns(4,5)P_2, inhibits neurotransmitter release in a manner reversed by anti-(synaptotagmin C2B) antibodies [27,28]. Recent studies also document a highly specific interaction of PtdIns(4,5)P_2 with CAPS (K. M. Loyet, A. Chaudhary, G. D. Prestwich and T. F. J. Martin, unpublished work), the vertebrate homologue of *Caenorhabditis elegans* UNC-31 protein, which acts at a late post-priming step in regulated exocytosis [29,30]. Studies are in progress to test the involvement of PtdIns(4,5)P_2 binding in the essential role of CAPS for regulated membrane fusion as well as to identify additional potential effectors for PtdIns(4,5)P_2.

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**Regulation of the Saccharomyces cerevisiae Vps34p phosphatidylinositol 3-kinase**

D. B. DeWald* and S. D. Emr†

*Department of Biology, Utah State University, Logan UT 84322-5305, U.S.A., and †Division of Cellular and Molecular Medicine and Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, CA 92039-0668, U.S.A.

**Introduction**

The yeast protein Vps34p is a phosphatidylinositol-specific phosphoinositide PtdIns 3-kinase. This is the first identified member of one class of the general family of phosphoinositide 3-kinases termed PI 3-kinases in eukaryotic cells [1,2]. Multiple classes of PI 3-kinases have been isolated, and classification of these enzymes is made on the basis of their amino acid sequence.

 Abbreviations used: CPY, carboxypeptidase Y; PI 3-kinase, general phosphoinositide 3-kinase; PtdIns 3-kinase, specific phosphatidylinositol 3-kinase.

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