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Regulation of Intracellular Membrane Interactions: Recent Progress in the Field of Neurotransmitter Release

Max M. Burger* **and Theo Schäfer**

Friedrich Miescher-Institute, CH-4002 Basel, Switzerland

Abstract Maintenance of compartmental independence and diversity is part of the blueprint of the eukaryotic cell. The molecular composition of every organelle membrane is custom tailored to fulfill its unique tasks. It is retained by strict sorting and directional transport of newly synthesized cellular components by the use of specific transport vesicles. Temporally and spatially controlled membrane fission and fusion steps thus represent the basic process for delivery of both, membrane-bound and soluble components to their appropriate destination. This process is fundamental to cell growth, organelle inheritance during cell division, uptake and intracellular transport of membrane-bound and soluble molecules, and neuronal communication. The latter process has become one of the best studied examples in terms of regulatory mechanisms of membrane interactions. It has been dissected into the stages of transmitter vesicle docking, priming, and fusion: Specificity of membrane interactions depends on interactions between sets of organelle-specific membrane proteins. Priming of the secretory apparatus is an ATP-dependent process involving proteins and membrane phospholipids. Release of vesicle content is triggered by a rise in intracellular free Ca^{2+} levels that relieves a block previously established between the membranes poised to fuse. Neurotransmitter release is a paradigm of highly regulated intracellular membrane interaction and molecular mechanisms for this phenomenon begin to be delineated. J. Cell. Biochem. Suppls. 30/31:103–110, 1998. © 1998 Wiley-Liss, Inc.

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Membranes of organelles are endowed with distinct and unique molecular composition. This specific composition never has to be generated de novo because cells originate from cells and hence, in principle, inherit preformed organelles. This is not true for the Golgi and the endoplasmic reticulum that have to be fragmented in mitosis to allow for equal distribution to the daughter cells. Corresponding organelle membrane fragments have to be reassembled by homotypic fusion to conserve cell structure. After mitosis, there is constant turnover of protein and lipid components by intercompartmental membrane traffic. Homogenization of the various membranes has to be counteracted by mechanisms that guarantee for dynamical integrity of the organelles and correct final localization of all cellular components. Synthesis in the endoplasmic reticulum of both, organelle membrane and soluble organelle content components has to be followed by highly efficient sorting and directional transport steps in order to maintain specific composition of the organelles. What are the mechanisms that sort all the components produced in the endoplasmic reticulum but destined to end up in the various distinct organelles? What are the mechanisms that regulate directional transport and specificity of vesicle–target membrane fusion? This review focuses on recent findings in the field of neurotransmitter release, on findings that have greatly advanced our understanding of the mechanisms that contribute to the control of spatial and temporal aspects of directional membrane fusion (Fig. 1).

THE SNARE HYPOTHESIS AND SPECIFICITY OF MEMBRANE INTERACTIONS

Principles of vesicular transport were studied for more than a decade by genetic approaches using yeast mutants that are defective for various steps within the secretory process; by molecular approaches aimed at the cloning of vesicle membrane proteins functionally involved in secretion; and by biochemical analysis of components essential for reconstitution of vesicle fusion in cell-free systems. In

*Correspondence to: Max M. Burger, Friedrich Miescher-Institute, PO Box 2543, CH-4002 Basel, Switzerland.
E-mail: burger@fmi.ch

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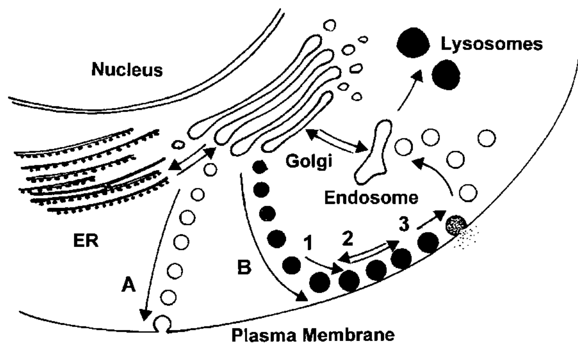

Fig. 1. Schematic representation of membrane traffic steps in eukaryotic cells. Pathways are not complete and organelles not drawn to scale. **A:** Constitutive exocytosis of Golgi-derived vesicles. **B:** Regulated pathway of Golgi-derived vesicles. In neurons, transmitter release in nerve endings is based on regulated exocytosis of endosome-derived transmitter vesicles (not shown). Regulated secretory pathways are characterized by **(1)** accumulation, i.e., docking of vesicles beneath the plasma membrane; **(2)** transfer of a subset of docked vesicles to a release-ready pool, i.e., priming; and **(3)** actual fusion of vesicle and plasma membrane triggered by an extracellular signal delivered by an intracellular second messenger, e.g., Ca^{2+} ions. Specificity and regulation of membrane interactions as exemplified by stimulated neurotransmitter release are discussed in the text.

1993, these seemingly disparate research topics converged on a common set of components and a concept termed the SNARE hypothesis [Söllner et al., 1993b]. Its central point was the postulation of a conserved membrane fusion machinery centered around two soluble proteins. The first one was an ATPase, the N-ethylmaleimide-sensitive fusion protein (NSF) previously identified as a factor required for vesicle fusion in vitro, and the second one was another essential protein that binds NSF, the soluble NSF-attachment protein (SNAP). When searching for membrane-localized SNAP receptors (SNAREs) in brain membrane extracts, two classes of proteins were identified, v-SNAREs present on vesicles, and t-SNAREs present on the target membrane: on the neurotransmitter vesicle synaptobrevin, on the synaptic plasma membrane syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa, unrelated to SNAPs!) (Fig. 2A). They were immediately recognized as homologues of proteins known to be essential for the secretory process in yeast [Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994]. An independent proof for a crucial role in neurotransmission of these synaptic v- and t-SNAREs was obtained by the identification of all three proteins as substrates for lethal clostridial neurotoxins [Niemann et al., 1994]. Most important for elucidation of their role was the observation that the three receptor proteins from opposing membranes could spontaneously form a very stable ternary complex in the absence of their common ligand SNAP/NSF [Hayashi et al., 1994]. The stable, low-energy complex bridging the membranes was assumed to be the acceptor for the soluble SNAP/NSF, which was found to dissociate the complex at the expense of ATP [Söllner et al., 1993a]. The hypothesis therefore seemed to explain how cognate vesicle proteins would specifically bind to cognate target membrane proteins, and how this receptor–receptor interaction itself would confer specificity upon the membrane interaction (Fig. 2A). Even more, dissociation of the SNARE complex by its ligand SNAP/NSF was postulated to drive exocytotic membrane fusion.

However, ATP-stimulated membrane fusion was at odds with previous findings of Ca^{2+}-triggered but ATP-independent neurotransmitter release [Eberhard et al., 1990; Hay and Martin, 1992; Morgan and Burgoyne, 1995]. In addition, the identification of the t-SNAREs syntaxin and SNAP-25 in functional complexes on synaptic vesicles [Otto et al., 1997] suggested that correct pairing of v- and t-SNAREs cannot be sufficient to guarantee specificity of vesicle–synaptic membrane interaction. But concurrently, members of the Rab family of small GTPases emerged as regulators of SNARE pairing [Sogaard et al., 1994; Mayer and Wickner, 1997] and as prime candidates for regulators of intracellular membrane traffic. Since complexity and compartmentalization of the Rab proteins is similar to those of the SNARE families, transient interactions between respective members may indeed control the complex array of interactions making up the whole of the intracellular membrane traffic [Novick and Zerial, 1997].

Recent investigations of structural organization of complexed v- and t-SNAREs have demonstrated that both proteins align in parallel [Hanson et al., 1997; Lin and Scheller, 1997], which contrasts the original assumption of an antiparallel arrangement. Moreover, reconstitution of synaptobrevin on one hand, and syntaxin combined with SNAP-25 on the other hand in separate lipid vesicles, has permitted observation of in vitro fusion of vesicles, provided v- and t-SNAREs were present on different vesicles [Weber et al., 1998]. This result clearly demon-

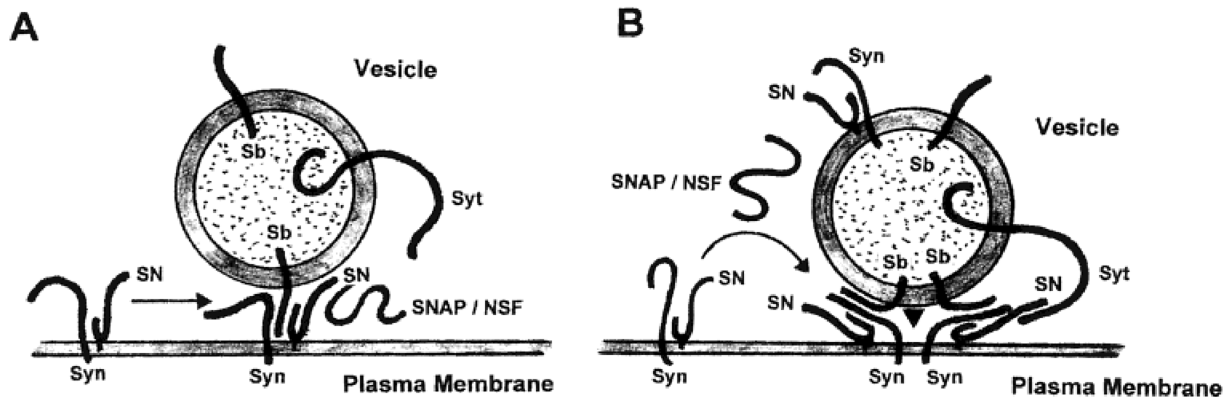

Fig. 2. Hypothetical role of SNAREs in the process of stimulated transmitter release. **A:** The original SNARE hypothesis [Söllner et al., 1993] postulated that synaptobrevin (Syb) was a vesicle membrane-specific marker protein determining specific interaction of the vesicle with the synaptic membrane-localized syntaxin (Syn) and SNAP-25 (SN). Ternary complex formation involving anti-parallel alignment of the v-SNARE and both t-SNAREs was thought to be the basis for docking of the vesicles and the generation of the membrane receptor for a soluble SNAP/NSF fusion machinery. ATP-dependent dissociation of the ternary complex by SNAP/NSF was assumed to trigger fusion of the membranes. **B:** Recent data have led to a modification of the hypothesis. SNAREs form a stable ternary complex by parallel alignment of synaptobrevin (Syb), syntaxin (Syn), and SNAP-25 (SN). The energy released during complex formation may be harnessed to bring membranes into close contact. The vesicle membrane protein synaptotagmin (Syt) is thought to act as a Ca^{2+}-sensitive inhibitor of membrane fusion. It may act by blocking the C-terminus of SNAP-25, thereby halting complex formation at the latest step before membrane fusion. Presence of functional t-SNARE syntaxin and SNAP-25 on the vesicles precludes an exclusive role of SNAREs in defining specificity of membrane interactions. SNAP/NSF action is seen as a prerequisite for complex formation (that delivers energy) and a means to dissociate the stable, low-energy state complexes after membrane fusion, allowing for the next round of vesicle exocytosis.

strates the validity of the SNARE hypothesis in terms of ternary SNARE complexes representing the minimal machinery, necessary and sufficient to induce phospholipid membrane fusion independent of SNAPs and NSF.

In essence, the SNARE hypothesis thus far provides us with the recognition that cognate v- and t-SNAREs may represent the core of the intracellular fusion machinery and that most, if not all, membrane fusion processes may be variations of a common theme—in cells as different as yeast and neurons [Bennett, Scheller, 1993; Ferro-Novick, Jahn, 1994]. The discovery that three individual SNAREs can form an intermolecular low-energy complex [Hayashi et al., 1994; Hanson et al., 1997; Lin and Scheller, 1997; Weber et al., 1998] reminiscent of the one found within single viral fusion proteins [Chan et al., 1997; Weissenhorn et al., 1997] may retrospectively explain the failure of identifying cellular fusion proteins: Viral envelope–cell membrane fusion seems to be promoted by dynamic rearrangements within the viral fusion protein that result in the formation of a hairpin-like structure merging viral and cell membrane. A similar structure, however, composed of two or more v- and t-SNARE domains, presumably is formed upon interaction of vesicle and target membrane during or after vesicle docking at the synaptic membrane. The domains responsible for the formation of both, viral hairpin and cellular SNAREpin [Weber et al., 1998] structure, are α-helical heptad repeats that are prone to generate coiled coils [Lupas, 1996], and that do not necessarily bear any sequence homologies. The common theme thus is the formation of a similar, thermodynamically stable structure. The energy released in the course of its formation may be harnessed to displace water molecules from the hydrophilic surfaces of the two membranes and thus to promote membrane fusion (Fig. 2B).

If NSF is not a fusion protein, what then is its role? From a series of elegant in vitro assays based on the reconstitution of homotypic yeast vacuole fusion, it became clear that NSF is not the fusion protein. Membrane fusion in those assays depended on the presence of yeast SNARE homologues on both interacting partners and on active NSF, but the latter did not have to be present during docking and fusion itself [Mayer et al., 1996]. Rather, NSF seemed to “prime” the SNAREs in an ATP-dependent action that energized the vacuoles for subsequent fusion, with this state being reversible, i.e., decaying within minutes. It has previously

been speculated that NSF in the secretory process, too, does not act as a fusion protein but as a chaperone for SNAREs that causes conformational changes putatively essential for a step preceding actual synaptic vesicle fusion [Morgan and Burgoyne, 1995]. Regulated fusion of secretory vesicles with the cell membrane thus seemed to comprise at least two steps, a preparatory or priming step that precedes the actual merging of the membranes—two steps that could be separated in cellular model systems and thus got accessible to separate experimental observation.

PRIMING AS A REGULATORY PROCESS PREPARATORY TO MEMBRANE FUSION

Exocytotic membrane fusion in endocrine cells and neurons by now represents one of the best studied processes of membrane interaction. It involves the core fusion machinery of v- and t-SNAREs and, since this process is unique by its stimulation through Ca^{2+} ions, additional components that relate the Ca^{2+} signal to the membrane fusion process. This then distinguishes it from other intracellular membrane trafficking events not controlled by Ca^{2+}.

A well-known trait of stimulated secretion is the accumulation or docking of transmitter-containing vesicles beneath the plasma membrane, where they seem to be blocked until fusion is induced by an intracellular signaling pathway. Although the mechanism of docking and the molecules involved in this process are far from being known, docked vesicles have always been assumed to represent the basis for the extremely rapid response to external stimuli, which is the characteristic feature of all tasks performed by the nervous system.

Docked vesicles are thought to be in close proximity to the plasma membrane which enables molecular interactions between the membranes without any additional transport steps (Fig. 1). Because potential differences in biochemical state could thus far not be assigned to docked and not yet docked vesicles, docking should rather be used as a morphological term to describe their location. Furthermore, recent experiments have shown that only a minority of docked vesicles can be triggered to fuse with optimal Ca^{2+} signals, a subpopulation of docked vesicles defined as release ready or fusion competent [Rosenmund and Stevens, 1996; Gillis et al., 1996; Plattner et al., 1997]. Transition to fusion competence, or priming for fusion now is recognized as a mechanism that regulates the number of vesicles that potentially fuse upon nerve stimulation and therefore determines the amount of transmitter released at a given nerve terminal [Gillis et al., 1996].

What are the molecular correlates of priming? A first experimental definition of a putative mechanism underlying priming was obtained in neuroendocrine cells, the catecholamine-secreting chromaffin cells of the adrenal medulla [Holz et al., 1989]. Analysis of the cellular phospholipid composition showed that the level of phosphoinositides seemed to govern the secretory response to the Ca^{2+} stimulus [Eberhard et al., 1990].

Phosphoinositides are a class of phospholipids characterized by their inositol (Ins) head group that itself is phosphorylated in several of six possible positions. They represent a very small proportion of the cellular phospholipids and had been assumed to act selectively as the precursors for phospholipase C-mediated production of the second messengers diacylglycerol and inositoltrisphosphate. New results suggested a function in an ATP-dependent process preceding Ca^{2+} action not connected to phospholipase C-controlled intracellular signaling [Eberhard et al., 1990]. This suggestion was later substantiated in an elegant experimental system based on the reconstitution of the secretory process in semi-intact cells [Hay and Martin, 1992]. Catecholamine-secreting PC12 cells could be cracked open in a controlled manner that allowed to separately investigate priming and fusion. Priming indeed was found to be dependent on ATP and soluble cytosolic proteins, whereas fusion was triggered by Ca^{2+}, which by itself was ATP independent [Hay and Martin, 1992]. When the cytosolic components were characterized, they turned out to be composed of two soluble proteins that are essential and that are involved in phosphoinositide metabolism, namely a phosphatidylinositol transfer protein [Hay and Martin, 1993] and a phosphatidylinositol(4)phosphate 5-kinase (PtdIns(4)P 5-kinase) [Hay et al., 1995]. In our own laboratory, we were able to demonstrate that neurotransmitter secretion depended in addition on the activity of a PtdIns 4-kinase residing on the transmitter vesicles [Wiedemann et al., 1998] (Fig. 3). Moreover, by reversible pharmacological inhibition of the PtdIns 4-kinase in chromaffin cells, we succeeded in determining that the primed stage decayed

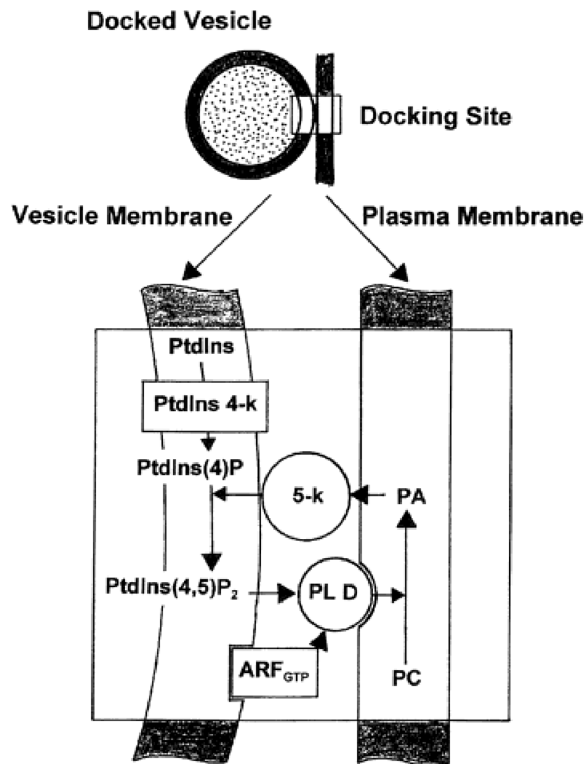

Fig. 3. Putative role of phosphoinositides in the priming of the release machinery. Fusion competence of membranes may locally be enhanced by production of fusogenic phosphatidic acid (PA) at the sites of membrane interaction [according to Liscovitch et al., 1994]. PA is formed within the membranes from phosphatidylcholine (PC) by phospholipase D (PL D) upon docking of a vesicle that carries GTP-activated ADP-ribosylation factors (ARF_{GTP}). Locally produced PA stimulates soluble phosphatidylinositol(4)phosphate 5-kinase (5-k) activity. Vesicle membranes contain phosphatidylinositol (PtdIns) and phosphatidylinositol(4)phosphate (PtdIns(4)P) formed by a vesicle-associated phosphatidylinositol 4-kinase (PtdIns 4-k). Active 5-kinase phosphorylates PtdIns(4)P and the resulting PtdIns(4,5)P_2 acts as a cofactor of PL D, thereby starting a positive feedback loop that produces high levels of PA and PtdIns(4,5)P_2. PA promotes fusion competence of membranes, whereas PtdIns(4,5)P_2 is rather inhibitory, and thus has to be inactivated upon stimulation (see text discussion).

within 15 min, and that secretion recovered upon removal of the kinase inhibitor within 10 min, provided that ATP was present [Wiedemann et al., 1996]. In conclusion, priming is an ATP-dependent process that, in addition to the NSF-catalyzed action on the SNAREs, involves at least three more proteins responsible for the production of PtdIns(4,5)P_2. Because priming is a reversible process, cells have to constantly produce phosphoinositides at the expense of ATP, a situation best described by analogy with bow and arrow: The potential to quickly react to a stimulus, the shooting of the arrow at any given moment, is made possible at the expense of energy necessary to bend the bow and keep it bent! Still, this of course does not yet suggest what the role of the phosphoinositides may be.

PHOSPHOINOSITIDES AND FUSION COMPETENCE OF MEMBRANE MICRODOMAINS

In recent years, phosphoinositides have been found to interact with a variety of proteins. They can act as cofactors or regulators of soluble enzymes and as membrane-bound receptors for structural components such as cytoskeletal proteins. Because of their rapid turnover, they are destined to act as regulators of dynamic processes. In the course of neurotransmitter release, two different aspects of phosphoinositide action have emerged. The first concerns a putative role in the generation of enhanced fusion competence of local microdomains of opposing membranes, and the second one points at a direct role in Ca^{2+} control of stimulus-secretion coupling.

As described above, the presence of v- and t-SNAREs in separate phospholipid vesicles proved to be sufficient to fuse those vesicles, albeit at a speed of several orders of magnitude too small to account for neuronal kinetics [Weber et al., 1998]. A potential cellular solution for increasing speed and probability of membrane fusion is based on fusion-facilitating modification of membrane phospholipid composition. Facilitation may be attained by inclusion or production within the membrane of fusogenic phospholipids, such as phosphatidic acid [Koter et al., 1978]. Regulated secretion requires mechanisms that provide a high probability of fusion upon stimulation, while keeping the probability of spontaneous fusion events low. A recent hypothesis intriguingly describes how cells may have evolved a process for concomitant locally restricted production of high concentrations of both, fusion-inhibitory PtdIns(4,5)P_2 and fusion-promoting phosphatidic acid (Fig. 3). This hypothesis can place a whole set of previously unrelated findings into a common frame [Liscovitch et al., 1994].

ADP-ribosylation factors are a family of small GTPases (different from the Rab family mentioned above). A member of the family, localized to the catecholamine-containing granules of chromaffin cells, has been shown to activate plasma membrane-associated phospholipase D, an enzyme that catalyzes the formation of phos-

phatidic acid from phospholipids [Caumont et al., 1998]. Interestingly, phospholipase D activity is also stimulated by PtdIns(4,5)P_2, whereas its product, phosphatidic acid has a stimulatory effect on the soluble enzyme PtdIns(4)P5-kinase [Jenkins et al., 1994]. Transmitter vesicle membranes contain PtdIns [Hay and Martin, 1993] and PtdIns(4)P produced by a constitutively active PtdIns 4-kinase (Wiedemann et al., 1996, 1998], as well as a GTP-activated ADP-ribosylation factor. Upon docking at the plasma membrane, the latter activates phospholipase D, which attracts the cytosolic PtdIns(4)P5-kinase [Hay et al., 1995] to the docking site. The result is a positive feedback loop (Fig. 3) that selectively creates microdomains of elevated phosphatidic acid and PtdIns(4,5)P_2 levels where the two membranes are in close contact. The former increases fusion-competence of the membranes, whereas the latter might rather inhibit fusion in a Ca^{2+}-reversible manner. Owing to lateral diffusion within the membrane and metabolic degradation outside the microdomains, spatially segregated, ATP-consuming production of both components has to be kept up to counteract depriming.

In addition to this signaling role in the production of phosphatidic acid, and therefore in priming, phosphoinositides may play an additional role in Ca^{2+} regulation of membrane fusion. The current view of stimulus-secretion coupling at the molecular level focuses on the synaptic vesicle protein synaptotagmin. This membrane protein has properties compatible with a putative role as an intracellular Ca^{2+} sensor acting as an inhibitor of membrane fusion at basal Ca^{2+} levels, but the mechanism of inhibition has not yet been elucidated. Most significantly, it binds Ca^{2+} with secretion-relevant affinity, as well as acidic phospholipids and several synaptic proteins, and it has been found to be indispensable for stimulated secretion in several species [summarized in Südhof and Rizo, 1996]. Conspicuous features of synaptotagmin within the current hypothesis are the Ca^{2+} dependence of binding to syntaxin and to PtdIns(4,5)P_2, and the Ca^{2+}-independent binding to SNAP-25. We postulate that synaptotagmin performs its inhibitory role by interfering with the formation of stable, low-energy SNARE complexes (see above). It can accomplish this function by binding to and therefore blocking the C-terminal domain of SNAP-25 (our recent observation). Because of selective binding of the C-terminal domain, the initial steps of complex formation involving the N-terminal domains of SNAP-25 may not be affected. On the contrary, synaptotagmin-binding to SNAP-25 during the course of complex formation may even increase local membrane tension. By halting complex formation at an intermediate state, it may thus be able to keep the membranes in an energized state close to fusion [Xu et al., 1998]. Ca^{2+} influx induces binding of syntaxin and of PtdIns(4,5)P_2, both of which may provoke the release of SNAP-25 from synaptotagmin. Consequently, the SNARE complex formation can proceed to completion, providing additional energy for membrane fusion. At the same time, fusion-inhibitory PtdIns(4,5)P_2 is neutralized by at least a partial binding to synaptotagmin and by chelation with Ca^{2+} ions. Neutralization of PtdIns(4,5)P_2 in turn enhances the fusion-promoting influence of the previously generated phosphatidic acid. This hypothesis is not only compatible with the postulated function of synaptotagmin in blocking exocytotic membrane fusion at the latest step possible, but also with observed effects of experimental removal of synaptotagmin: Secretion is not abolished but the reliability of stimulus-secretion coupling or stimulated secretion itself is greatly diminished [Südhof and Rizo, 1996].

In summary, current hypotheses suggest that docking of vesicles induces locally restricted and reversible generation of fusion-competent membrane domains. Interaction of the vesicle-localized v-SNAREs with the t-SNAREs of the synaptic membrane leads to formation of complexes of low-energy state, with the energy released being exploited to bring membranes into very close contact. Actual fusion, however, is inhibited by synaptotagmin interfering with complex formation at a late step. The probability of a fusion event is additionally kept low by high levels of PtdIns(4,5)P_2 within respective membrane domains. Ca^{2+} influx steeply increases the probability of fusion by concomitant removal of the synaptotagmin-block and neutralization of the phosphoinositides.

FUTURE GOALS AND BENEFITS

Even though Ca^{2+}-controlled synaptic vesicle exocytosis represents a highly specialized form of membrane interaction, elucidation of the regulatory mechanisms may shed light on fundamental principles conserved between differ-

ent vesicular pathways in all eukaryotic cells. Understanding of the molecular basis of specificity of interaction, priming of the fusion machinery, or fusion competence of membranes will surely help to solve questions in different areas of cell biology. This will also have immediate consequences for related medical issues that are manifested as diseases. Diabetes caused by lack of transfer of glucose transporters to the plasma membrane is just one such example on an extensive list.

In the field of neurological disorders, further elucidation of the components and mechanisms involved in neurotransmitter release may uncover novel possibilities for pharmacological interference. A number of severe and prevalent central nervous system disorders are currently thought to be related to defects in neurotransmitter release. Because of the lack of knowledge about the intracellular mechanisms involved in this process, potential medication is restricted to extracellular aspects of neurotransmitter action, including manipulation of receptors, ion channels, and transporters. In addition, the characterization of components involved in transmitter release has helped explain the genetic and biochemical basis of diseases known. A recent example of this list is the oculocerebrorenal, or Lowe, syndrome. The product of the mutated gene has been identified as a phosphatase involved in the degradation of PtdIns(4,5)P_2 [Zhang et al., 1995]. Within the context of the results discussed above, a defect in this enzyme may thus affect the priming or the membrane fusion process. With increasing knowledge of the mechanisms of transmitter release, it will not only become possible to find explanations, but also to devise and attempt cures for neurological diseases. It is hoped that research started by investigation of the molecular basis of yeast defective for the secretory process will provide us with approaches to tackle human ailments.

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