CHAPTER TWO

How to Make a Stable Exocytotic Fusion Pore, Incompetent of Neurotransmitter and Hormone Release from the Vesicle Lumen?

Jernej Jorgačevski,[1,2] Boštjan Rituper,[1] Miha Fošnarič,[3] Ajda Flašker,[1] Nina Vardjan,[1,2] Matjaž Stenovec,[1,2] Maja Potokar,[1,2] Marko Kreft,[1,2] Veronika Kralj-iglič,[4] Aleš Iglič,[3] *and* Robert Zorec[1,2,*]

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* Corresponding author. Tel.: +386 1 543 70 20; Fax: +386 1 543 70 36.
E-mail address: robert.zorec@mf.uni-lj.si

[1] Laboratory of Neuroendocrinology–Molecular Cell Physiology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[2] Celica, Biomedical Center, Tehnološki park 24, Ljubljana, Slovenia
[3] Laboratory of Biophysics, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia
[4] Laboratory of Clinical Biophysics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

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Abstract

In multicellular organisms, signaling is a necessity and an important mode of communication between cells is mediated by neurotransmitters, hormones, and other chemical messengers that are stored in secretory vesicles. In stimulated conditions secretory vesicles, which are trafficked to be docked at the plasma membrane, enter exocytosis, characterized by vesicle and plasma membrane merger. Due to repulsive forces of negatively charged membrane surfaces, it was long believed that the fusion pore is merely a short lived intermediate state leading irreversibly to a complete merger of both membranes. However, recent results show that the fusion pore is a rather stable structure, which can reversibly reopen to subnanometer diameters; dimensions too narrow to permit the exit of the cargo into the extracellular space. The aim of this chapter is to first review how can such a structure attain stability and compare two models in which membrane constituents are either isotropic or anisotropic in nature. Then we address the molecular nature of such a stable, release-unproductive fusion pore. We conclude that membrane constituents of the stable fusion pore membrane, being made of proteins and/or lipids, very likely consist of architectural elements that exhibit anisotropicity. The dynamics of fusion pore diameter is then determined by the density and architectural properties of these membrane constituents at fusion pore locales.

1. Introduction

When eukaryotic cells evolved from a prokaryotic precursor 1000 to 2000 million years ago, this was associated with a significant cell volume increase. This prompted a new structural organization to evolve, since diffusion-based signaling, efficient at nanometer distances, is inadequate to support the function of single nucleated cells, and consequently all multicellular organisms, which emerged once nucleated cells evolved. Besides the nucleus and other membrane-bound organelles, a key structural invention of eukaryotic cells is the secretory vesicle. This subcellular organelle has a complicated structure; its membrane consists of lipids and proteins. It is playing an essential role in the function of animals, including humans, where chemical signals, such as neurotransmitters and hormones, are stored. The highly concentrated chemical signals in the secretory vesicle are released into the extracellular medium following a stimulus delivery, which is thought to mediate the fusion of the secretory vesicle membrane with the plasma membrane. This latter event is part of the process of exocytosis.

Exocytosis involves the formation of the fusion pore-an aqueous channel between the vesicle and the plasma membranes. Despite intense investigation of the regulatory mechanisms of exocytosis in the last decades, the nature of the fusion pore remains obscure [1]. The main obstacle has been an inability to directly monitor the cargo release through single fusion

pores. Over the past decade, methodologies have been developed to monitor neurotransmitter release during single exocytotic events by detecting the amperometric oxidation current of the released transmitter [2–4]. However, neurotransmitter release proceeds in at least two main stages [5]. First, the vesicle establishes a stable fusion pore and then the pore suddenly enlarges (Fig. 1). In support of this view, amperometric spike-like currents are preceded by a small pedestal, called the "foot" of the spike [2]. Combined patch-clamp and amperometric studies have indicated that the foot represents neurotransmitter release through the initially formed fusion pore [6], although recent results have failed to confirm this [7]. On the other hand, the neurotransmitter released during the foot and during the main spike is the same [3], demonstrating the existence of a dynamic fusion pore. However, from the amperometric approach one can not conclude about the nature of dynamism of the fusion pore directly. In particular, it is not clear whether the relatively small release of transmitter during the initially formed fusion pore is due to a narrow fusion pore diameter and/or due to restrained kinetics-short effective fusion pore dwell-time. Moreover, the rather small signal associated with the foot versus the spike-like current and the transient and short-lived nature of the fusion pore preclude a detailed experimental investigation of the early fusion pore stages, which may not necessarily lead to the subsequent increased neurotransmitter/hormone flux rate. Furthermore, to learn more about the nature of the fusion pore in its initial stages, it would be ideal to have a preparation in which the initial fusion pore exhibits robust appearance and rarely proceeds to the full fusion stage (Fig. 1), thus permitting the study of the fusion pore properties more directly by electrophysiological means. A preparation that meets these criteria is the pituitary peptidergic vesicle [8].

The mechanism(s), by which the initial fusion pore attains stability, is poorly understood. Once formed, the fusion pore either fully widens, leading to the complete merger of vesicle membrane with the plasma

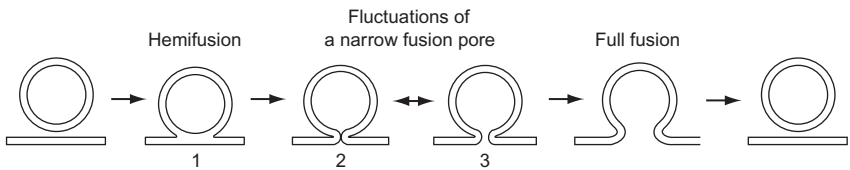

Figure 1 Stages a secretory vesicle has to undergo in exocytosis. Stages a secretory vesicle has to undergo to attain vesicle membrane merger with the plasma membrane via the hemifusion stage (1), the fusion pore formation (2), which exhibits stability and the fusion pore diameter may fluctuate to a wider stage (3) before full fusion, that is, full integration of the vesicle membrane into the plasma membrane. The integrated vesicle membrane may return to the cytosol via the process of endocytosis as depicted by the last stage on the right in the scheme.

membrane (full fusion exocytosis; [9]) or can reversibly close (kiss-and-run exocytosis; [10]). The patch-clamp membrane capacitance measurements [11] revealed that the fusion pore can also fluctuate between an open and a closed state in the subsecond time domain before full fusion [12] or can retain the transient nature of opening and closing for several tens of minutes [8,13,14]. Reopening of the same fusion pore indicates a remarkable stability, a property not observed previously. This and the fact that single exocytotic events can be observed with fluorescence microscopy in real time with styryl dyes [15], and by electrophysiological methods [16] render pituitary cells convenient preparations to study elementary properties of fusion events. Therefore, these cells were used to develop a mathematical model describing the observed energetic stability of the transient fusion pore [17].

2. Narrow Fusion Pores Are Stable Due to the Accumulation of Anisotropically Shaped Molecules in the Highly Curved Membrane Regions

One can consider that highly curved membrane domains consist of specially shaped membrane constituents. These can be classified as isotropic and anisotropic as is defined in Fig. 2 [see 18–20]. The appropriate ordering of such specially shaped membrane constituents in the highly curved membrane regions can likely increase the stability of the membrane region. Therefore, the model describing the fusion pore stability of peptidergic vesicles [17] sources on these considerations, and is based on the elasticity of the membrane layers which includes orientational ordering of membrane constituents that are anisotropic with respect to the axis perpendicular to the membrane [21]. Membrane constituents can be single molecules or small complexes of molecules, which have high negative intrinsic (spontaneous) curvatures [22]. Figure 3 summarizes the results of the model (taken with permission from [17]), showing that the correct ordering of membrane constituents result in the stable fusion pore. Figure 3A captures three states of a vesicle connected to the plasma membrane with fusion pores of different diameters. Top three diagrams show that the relative area density of anisotropic membrane constituents is increased in the membrane region of the fusion pore, which connects the vesicle with the plasma membrane. Note that the relative density of anisotropic membrane constituents in the pore region is increased as the fusion pore diameter is narrowed. Figure 3B reports the calculated membrane free energy (ΔF) as a function of the fusion pore diameter, for different inner diameters of the fusion pore Δ. It can be observed in Fig. 3B that for highly anisotropic membrane constituents a minimum of ΔF at certain Δ is predicted, as if the fusion pore can attain

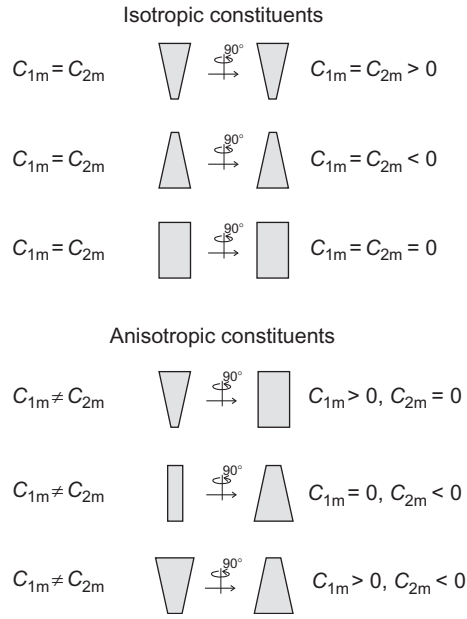

Figure 2 Isotropic and anisotropic shapes of membrane in highly curved membrane locales. Different possible intrinsic shapes (isotropic and anisotropic) of small membrane constituents in relation to intrinsic principal curvatures C_{1m} and C_{2m} [18–20].

distinct stable fusion pore diameters. Experimental evidence in support of discrete diameter states of a stable fusion pore was provided recently by studying the role of Munc18-1 proteins in fusion pore physiology [24]. A similar, although indirect, conclusion was reported previously [25]. Figure 3C shows that the stable equilibrium fusion pore diameter is narrower, if the membrane constituents have higher anisotropy. Moreover, Fig. 3D depicts the narrowness of such stable fusion pores in relation to the membrane thickness. Furthermore, it is important to note that such a model includes properties related to the observed relationship between the vesicle size and fusion pore properties [4,17].

3. Isotropic Membrane Constituents Are Unable to Generate Narrow Stable Fusion Pores?

A logical question is whether isotropic constituents (as defined in Fig. 2, with $C_{1m} = C_{2m} < 0$) can replace the anisotropic ones? The results on Fig. 4 show that this is not the case. Isotropic membrane constituents

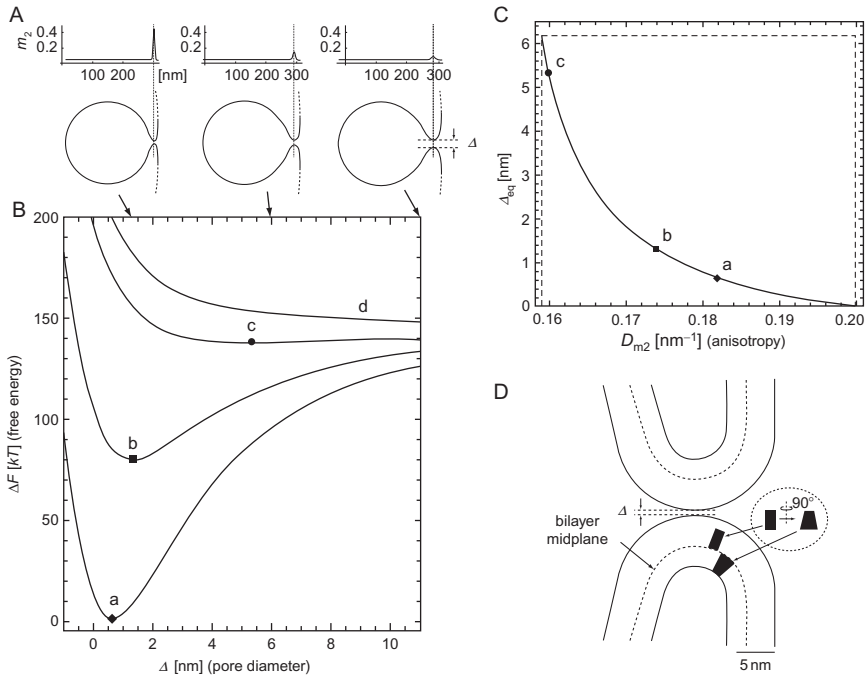

Figure 3 Stable configuration of the fusion pore (vesicle fused to the plasma membrane) as a function of the fusion pore diameter and intrinsic anisotropy of the membrane constituents. (A) The sum of the relative area densities of the anisotropic (type 2) membrane constituents ($m_2 = m_{2,1} + m_{2,2}$) in both membrane monolayers, shown for three different vesicle shapes (see panel below) of 300 nm in diameter, fused to the inner membrane surface. Anisotropic membrane constituents have $C_{1m,2} \approx 0$ and $C_{2m,2} = -1/3$ nm^{-1} which corresponds to $H_{m,2} = -D_{m,2} = -1/6$ nm^{-1}. (B) Free energy of the two component bilayer membrane (ΔF) as a function of the fusion pore diameter (Δ) calculated for different values of the intrinsic curvature deviator of the anisotropic (type 2) membrane constituents in the membrane bilayer $H_{m,2} = -D_{m,2}$: $-1/5.75$ nm^{-1} (a), $-1/6$ nm^{-1} (b), $-1/6.2$ nm^{-1} (c), $-1/6.5$ nm^{-1} (d). Arrows show the values of Δ(nm) corresponding to the shapes presented in panel A. (C) The calculated equilibrium fusion / pore diameter (Δ_{eq}), corresponding to the minimum of ΔF (see panel B) as a function of the anisotropy $D_{m,2} = |H_{m,2}|$ (see Appendix A and Materials and Methods in [17]). Note that on the left side of the vertical dotted line, the local minimum of ΔF does not exist (see curve (d) in panel B). Values of the model parameters are: $H_{m,1} = D_{m,1} = 0$, $K_1 = 10$ kT nm^2 [21,23], $K_2 = 100$ kT nm, $K_2 = -70$ kT nm^2, $w = -0.25$, $z_2 = 6$, $\bar{m}_{2,in} = \bar{m}_{2,out} = 0.02$, $m_0 = 1.67$ nm^{-2} and $R_0 = 1000$ nm, taken with permission from [17]. (D) Schematic representation of the fusion pore with anisotropic constituents in both membrane layers and a fusion pore diameter (Δ) of $\sim$0.6 nm. The thickness of the membrane layer containing glycolipids ($\sim$4 nm) is larger than the thickness of the membrane layer without them ($\sim$2.5 nm).

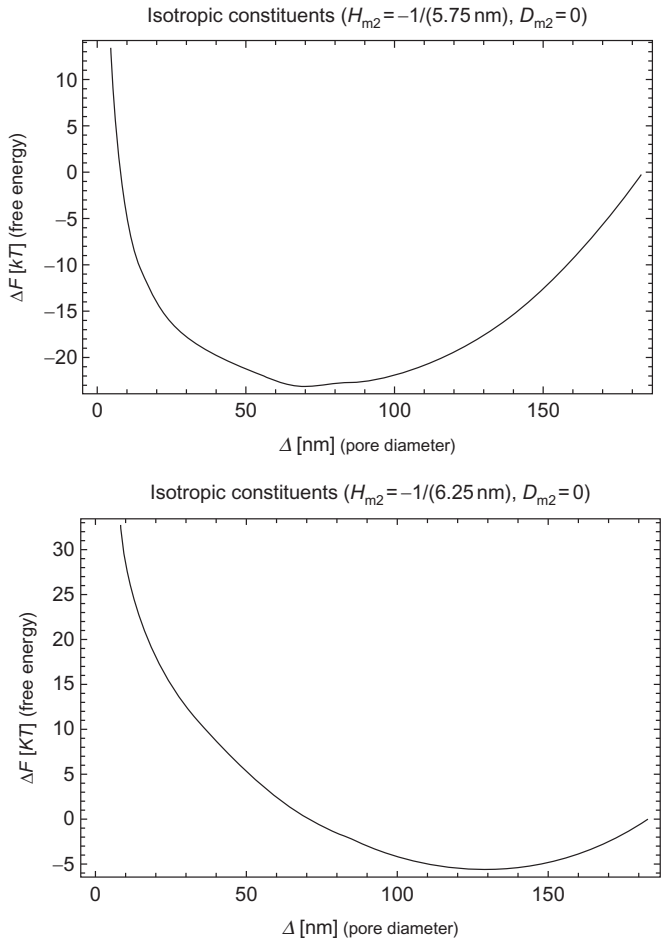

Figure 4 Membrane free energy as a function of the stable pore diameter with isotropic membrane constituents. Isotropic membrane constituents have intrinsic curvatures as marked in the figure. Values of other model parameters are given in the caption to Fig. 3.

only weakly accumulate in the inner and outer membrane layers in the vicinity of the saddle-like fusion pore and only form less stable fusion pores with diameters that are at least two orders of magnitude wider than those obtained by the anisotropic membrane constituents (with $C_{2m} < 0$ at $C_{1m} \approx 0$, compare the ordinates and abscissas with Fig. 3B). While the effects of isotropic inverted conical constituents on the membrane elasticity due to the mismatch of the intrinsic mean curvatures and the actual mean curvature of both layers in the two membrane layers partly cancel each other

due to opposing signs of the principal curvature in the two membrane layers, the effect of the average orientational ordering of the anisotropic constituents from both layers is summed up (since the average orientation of the anisotropic membrane constituents is different in both membrane layers of the fusion pore). This is one of the main reasons for the most striking difference between the effect of anisotropic membrane constituents and the isotropic inverted conical membrane constituents. Namely, the anisotropic membrane constituents (with $C_{2m} < 0$ at $C_{1m} \approx 0$) can stabilize the fusion pore geometry (as shown in Fig. 3B), while isotropic membrane constituents (with $C_{1m} = C_{2m} < 0$) cannot.

Thus, the answer is that a narrow stable fusion pore is likely made of anisotropic, rather than by isotropic membrane constituents. The nature of these, however, is not clear yet.

4. Protein and Lipids Affect Exocytosis and Fusion Pore Properties

Figure 3D shows the cross section of the fusion pore with a stable narrow diameter. It is remarkable that fusion pores with a diameter of about one-tenth of the membrane thickness can be formed. The question is, how and with which molecules such a structure can be built. It is clear that such a narrow pore is physiologically release unproductive (unable to release the vesicle cargo-neurotransmitters and hormones), since the pore is narrower than the size of even the smallest chemical messengers such as glutamate and acetylcholine [14,17,26]. Once such narrow pores are formed, they can enter into a release competent mode by stimulation [8,14,26], that is, the pore diameter widens or even generates the full fusion vesicle state (see Fig. 1).

In the next section, we discuss some of the key candidate molecules that may regulate the fusion pore formation and properties (Fig. 5). Although physiological results are still incomplete of how proteins and lipids affect the stages of exocytosis defined in Fig. 1, mainly biochemical and genetic studies (reviewed in [27]) place the SNARE proteins as prominent players in these processes, as it is shown in Fig. 5.

5. SNARE Zippering and SM-Proteins

The formation of the fusion pore may be driven by a conformational change of the SNARE complex (N-ethylmaleimide-sensitive fusion (NSF)-attachment protein receptor) [28]. Together with other interacting proteins (i.e., SM-proteins, for Sec1/Munc-18 like proteins), this complex is considered important in membrane merger [1], that is, in the overcoming

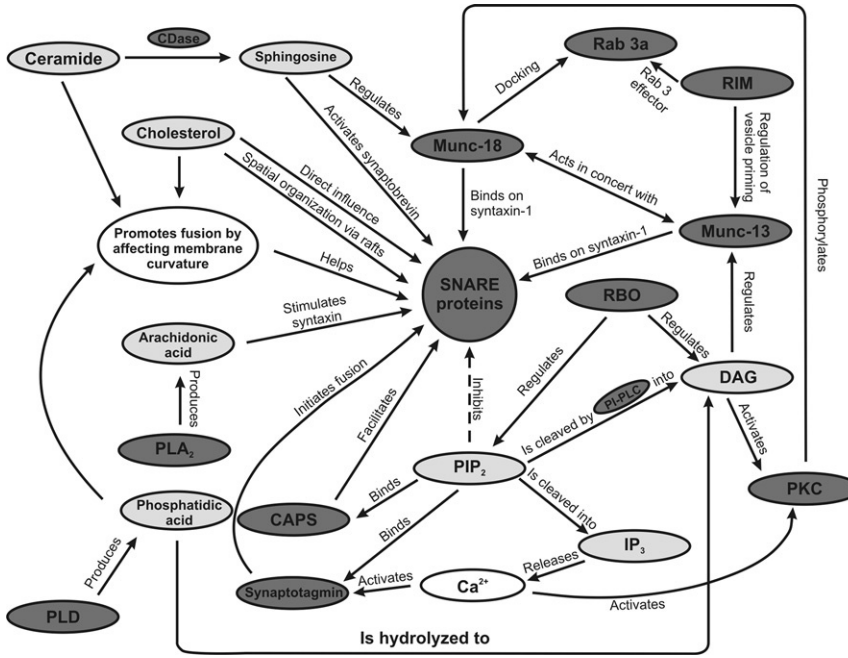

Figure 5 Interactions between lipids and proteins affecting exocytosis and fusion pore properties. Lipids and lipid derivatives are depicted in light gray, proteins are depicted in dark grey. Taken with permission from [27].

the repulsive electrostatic forces between phospholipid bilayers of the apposing membranes.

According to the "zipper hypothesis" [29], assembly is initiated at the N-terminal ends of the SNARE motifs and then progresses downhill an energy gradient toward the C-terminal membrane anchors, thus pulling the membranes together and initiating fusion. However, it is still contested as to how exactly SNARE zippering contributes to the membrane merger and in particular how these proteins relate to the fusion pore stability and the postfusion stages of exocytosis (Fig. 1).

For example, the trigger for secretions, an elevation of cytosolic [Ca^{2+}] is thought to be translated into dynamic fusion pore changes via the interaction of synaptotagmin-1, a Ca^{2+}-binding protein, with the SNARE complex [23]. Moreover, the SM family of proteins play a role in SNARE-mediated events, though their exact role(s) remains enigmatic. Neuronal isoform of mammalian Munc18, Munc18-1, was originally found to bind with high affinity to syntaxin-1, a SNARE member protein [30], which appears to have a role in determining fusion pore structure [31]. However, whether alterations in fusion pore dynamics involve specifically

changes in fusion pore conductance or/and fusion pore kinetics is unknown. The interpretation of fusion pore dynamics by SNARE mediated mechanisms is further complicated by the discovery that changing vesicle size affects its fusion pore [4,17]. In addition to proteins, negatively charged lipid molecules strongly affect the probability of exocytosis [22]. Thus, to understand how these complex mechanisms affect the dynamics of the fusion pore, one needs to consider a mechanism that can describe the stability of the fusion pore forming initially [17], and also predicts how interacting molecules (proteins and/or lipids) mediate changes in pore stability leading to fusion pore diameter enlargement. Which are the lipids that affect exocytosis and fusion pore properties? Do they modulate exocytosis indirectly (signaling) or directly?

6. Cytosolic Plasma Membrane PIP_2 Organizes Exocytotic Machinery

Phosphatidylinositol 4,5-biphosphate (PIP_2) is a member of membrane phospholipids of eukaryotic cells. Its inositol headgroup can be phosphorylated at single or multiple sites to give rise to a variety of phosphoinositides [32]. In addition to phosphorylation, hydrolysis of PIP_2 by PI-phospholipase C (PI-PLC) yields soluble inositol 1,4,5-tris-phosphate (IP_3) and membrane-resident diacylglycerol (DAG), both second messengers, playing multiple signaling roles [33]. PIP_2 is mainly located on the cytoplasmic leaflet of the plasma membrane and is known to be engaged in a series of regulated processes, including endocytic and exocytic membrane trafficking [34]. It is a necessary prerequisite for Ca^{2+}-dependent exocytosis [35]. Following activation of membrane fusion, plasma membrane PIP_2 becomes transiently depleted by phospholipase C activity downstream of Ca^{2+} signaling. Using PC12 cells, two enzymes (phosphatidylinositol transfer protein and phosphatidylinositol-4-phosphate-5-kinase) involved in metabolism of PIP_2, were identified to be required for vesicle priming [36]. Recently, it was shown that increase or decrease of plasmalemmal PIP_2 levels results in increase or decrease of primed vesicle pool [37] and that siRNA knockdown of previously mentioned enzymes results in impaired exocytosis of insulin [38]. PIP_2 self-organizes in lipid microdomains and colocalizes well with syntaxin clusters, which appears to be essential for Ca^{2+}-dependent exocytosis [39,40]. Interestingly, a substantial fraction of PIP_2 microdomains also colocalizes with calcium activated protein for secretion (CAPS) protein [40], which indicates that PIP_2 signals the recruitment of proteins required for exocytosis to sites necessary for vesicle docking and membrane merger.

Concentration of PIP_2 in lipid microdomains is estimated to be relatively high in comparison to the surrounding regions [41], which inhibits SNARE-dependent fusion in the absence of CAPS, most likely due to its inverted cone shape and positive curvature, which antagonizes highly negative curvature stalk models needed for transition to full fusion [41,42]. However, positively charged juxtamembrane regions of SNAREs may sequester PIP_2 from membrane regions that undergo high curvature transition during fusion, thus SNAREs themselves may help promote the curvature needed for completion of SNARE complex formation and transition to full fusion [43]. James *et al.* [41] suggest two mechanisms by which PIP_2 helps to regulate membrane fusion. First, inhibition is due to intrinsic positive curvature of PIP_2. Second, PIP_2 strongly facilitates rates of membrane fusion by PIP_2-binding proteins such as CAPS, synaptotagmin, or rabphilin which possess additional properties that promote SNARE function in fusion [41]. Thus, if one would regulate enzymes, that control PIP_2, then one could influence exocytosis. Indeed, the rolling blackout (*rbo*) gene encodes lipases, which are putative integral plasma membrane lipolytic enzymes that have an essential role in phospholipase C-dependent PIP_2/DAG signaling [44]. In rbo^{ts} (temperature-sensitive) *Drosophila* mutants, exocytosis appears to be blocked shortly before fusion step with accumulation of docked vesicles at presynaptic active zones, indicating a role for RBO protein downstream of vesicle docking [45], for example, through stabilization of the fusion pore in its narrow configuration which does not permit neurotransmitter release [17]. It is believed that RBO protein may be important for the local production of DAG and for the local regulation of PIP_2. Thus, if RBO protein is blocked, elevated concentrations of PIP_2 may negatively regulate Ca^{2+} channels thereby blocking Ca^{2+} influx and arresting docked synaptic vesicle fusion [45].

7. Polyunsaturated Fatty Acids Modulate Exocytosis via SNARE Proteins

Polyunsaturated fatty acid (PUFA) and phospholipases, which release them, have been known to play an important role in exocytosis [46]. Especially two types of PUFAs, omega-6 (arachidonic) and omega-3 (docosahexaeonic) variants, are essential in membrane fusion [47]. Besides ion channel modulation and cytoskeleton functions, PUFAs act on proteins that are pivotal in vesicle fusion—syntaxins. PUFAs are also major building blocks of cellular membranes [48] and possess favorable biophysical properties such as flexibility and solubility, which promote membrane fluidity [46]. It is known that a mutation of PUFA-related enzymes as well as PUFA deficient diet causes mental retardation [49] and deficient brain function

[50]. However, until recently not much has been known about mechanism of PUFAs actions. It was proposed [47] that omega-3 and omega-6 fatty acids act on syntaxin by changing its conformation thus allowing formation of Munc18-syntaxin-SNAP25 tripartite complex and promoting completion of SNARE complex formation [47,51].

8. Lipid Rafts, Ceramide, Sphingosine, and Cholesterol

Ceramide, a membrane sphingolipid, is unable to spontaneously transfer between lipid bilayers and has a tendency to self-aggregate into microdomains (lipid rafts) in association with other sphingolipids and cholesterol [52,53]. It was thought that ceramide acted as a secondary messenger. However, it is more likely that it acts by modifying interactions within lipid rafts, with consequences for raft coalescence, membrane curvature, and signaling emanating from these domains [54]. Additionally, with its small hydroxy headgroup and a cone shape, ceramide facilitates membrane fusion and fission by inducing negative membrane curvature [54].

Sphingosine is the backbone molecule of sphingolipids [55]. After being cleaved by ceramidase from ceramide, sphingosine is involved in many cellular functions, some of those being inhibition of voltage-operated calcium channels [56], modulation of excitability and/or transmitter release in the nervous system [57], control of apoptosis [58], and more. Recently, it was also discovered that sphingosine facilitates SNARE complex assembly by acting on synaptobrevin-2 [59] (Fig. 5) and that it regulates syntaxin-1 interaction with Munc18-1 [60], thus making it one of the most important lipids in regulation of exocytosis.

Cholesterol is a major lipid component of cellular membranes and is known to aggregate with saturated phospholipids and sphingolipids in lipid microdomains or rafts [61], where it plays a key organizing role in enhancing the tendency of saturated phospholipids and sphingolipids to segregate from unsaturated phospholipids [62]. Rafts themselves are considered to serve as sites for specific protein–lipid interactions [63] and several proteins necessary for the exocytotic process have been shown to associate with these cholesterol-rich domains, including SNAREs [64]. Thus, it is very likely that lipid rafts or specialized membrane sites serve as sites at or near which membrane fusion occurs [65,66].

There are several mechanisms by which cholesterol is capable of contributing to membrane fusion [67,68]. First, as a component of lipid rafts, cholesterol can organize essential proteins and lipids at the fusion site [67]. Removal of cholesterol from plasma/vesicle membrane breaks the structure of lipid microdomains causing dispersal of critical proteinaceous and lipidic

fusion machinery, thus interfering with efficient exocytosis. It was shown that removal of cholesterol by using methyl-β-cyclodextrin (MβCD) causes dose-dependent inhibition of the extent of triggered fusion and progressive shift to a higher [Ca^{2+}] [69]. Interestingly, the effect can be rescued by exogenous delivery of cholesterol or other molecules with similar or greater negative intrinsic curvature (α-tocoferol and dioleoylphosphatidyl-ethanolamine [DOPE]). However, only cholesterol can recover both the efficiency of fusion and the fundamental ability of vesicles to fuse [69,70]. Second important role of cholesterol in membrane fusion is its modulation of physical properties of the membrane, such as fluidity and/or curvature [71], the latter being extremely high in the fusion pore area [17]. Finally, as a functional ligand or cofactor, cholesterol can directly modulate the activity of proteins essential to the fusion process [67], for instance, synaptophysin/synaptobrevin interaction, which is necessary for efficient exocytosis, seems to critically depend on the cholesterol content at the fusion site [72]. At this site, cholesterol may participate in the relatively high density of anisotropic membrane components [17].

Cholesterol also appears to play an important role in fusion pore dynamics. It is believed that cholesterol regulates the persistence of the semi-stable fusion pore as shown electrophysiologically in single vesicle studies of pituitary peptidergic vesicles [17]. It is interesting to note that it is the cholesterol located in the cytosolic leaflet that may stabilize the fusion pore and not the one in the extracellular leaflet [73]. Wang *et al.* [73] indicate that all three previously depicted properties of cholesterol: viscosity of membrane, stiffness of lipid monolayer, negative intrinsic molecular curvature, and modulation of fusion proteins may contribute to this effect. These mechanisms may generate an anisotropically rich environment that is required for highly curved membrane structures [17]. Together, these properties make it energetically favorable to form and maintain the narrow waistline of the fusion pore. Thus, cholesterol removal on the cytoplasmic leaflet of the fusion pore lowers the overall probability for successful fusion [73].

9. Conclusions

We here discussed the fusion pore, a membranous intermediate that is formed upon the merger of vesicle and plasma membranes. Initially, it is an energetically stable, but release unproductive structure, if its diameter is in the subnanometer domain. The subsequent steps may lead to fusion pore dilation or even into full fusion stage, where the vesicle membrane collapses into the plasma membrane. The later stages are characterized by an enlarged pore diameter and are release productive. The stable and narrow fusion pore is likely established by accumulation of anisotropic membrane constituents

within the neck of the fusion pore. Which molecules exactly contribute to the anisotropic nature of the fusion pore is presently unknown; however, we here discussed some key proteins and lipids that play significant role. Future studies will focus in delineating the exact role of classes of molecules relevant for stabilizing the pore and also address the questions of narrow pore widening upon a physiological trigger.

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