REVIEW ARTICLE

Role of synaptotagmin in Ca^{2+} -triggered exocytosis

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The Ca^{2+} -binding synaptic-vesicle protein synaptotagmin I has attracted considerable interest as a potential Ca^{2+} sensor that regulates exocytosis from neurons and neuroendocrine cells. Recent studies have shed new light on the structure, biochemical/biophysical properties and function of synaptotagmin, and the emerging view is that it plays an important role in both exocytosis and endocytosis. At least a dozen additional isoforms exist, some of which are expressed outside of the nervous system, suggesting that synaptotagmins might regulate membrane traffic

in a variety of cell types. Here we provide an overview of the members of this gene family, with particular emphasis on the question of whether and how synaptotagmin I functions during the final stages of membrane fusion: does it regulate the Ca^{2+} -triggered opening and dilation of fusion pores?

Key words: calcium, C2-domain, exocytosis, membrane fusion, synapse.

Ca^{2+} TRIGGERS RAPID EXOCYTOSIS

The pioneering studies of Katz [1] showed that Ca^{2+} ions trigger the release of neurotransmitters in defined packets or quanta. This ‘quantal mechanism’ of Ca^{2+} -triggered exocytosis has been refined over the last 50 years (summarized in Figure 1), and it is now well established that transmitters are sequestered into synaptic vesicles (SVs), which are the smallest known membrane-bound organelles (≈ 30 – 50 nm in diameter) [2]. SVs are targeted to the pre-synaptic plasma membrane, where they dock close to the plasma membrane at specialized regions called active zones [3]. After docking, they then undergo a series of priming reactions that make them competent to fuse [4]. Fusion is triggered by Ca^{2+} influx via voltage-activated Ca^{2+} channels. The Ca^{2+} transient is rapid, and local $[\text{Ca}^{2+}]$ experienced by the fusion machinery is strongly dependent on the distance between SVs and channels [5]. Changes in the proximity of channels and SVs, during development for example, might modulate the efficiency of exocytosis.

Ca^{2+} triggers release by driving the abrupt opening of a ‘fusion pore’, which is the first aqueous connection between the lumen of the vesicle and the synaptic cleft (Figure 1). The pore is lined by either membrane proteins, lipids, or a combination of each, and in some cases has a conductance similar to that of large ion channels [6]. Once opened, fusion pores either close (‘kiss and run’ [7]), or dilate such that the vesicle membrane collapses into the plasma membrane, from which it is recycled via endocytosis [8] (Figure 1). Both modes of release are believed to occur, but their relative proportion during normal synaptic transmission remains an open issue.

Neuronal exocytosis is among the fastest processes in cell biology. The lag time between rises in pre-synaptic $[\text{Ca}^{2+}]$ and post-synaptic responses can be as short as 60–200 μs in some synapses [9,10]. Capacitance measurements revealed overall rates of secretion as fast as 5200 vesicles in 10 ms in certain synapses that process sensory information [11]. In contrast, other kinds of

synapses are limited to overall release rates of ≈ 15 – 30 vesicles/s, in part owing to the low probability of release and the limited size of the total vesicle pool [12]. An interesting finding is that even in these synapses, when a fusion occurs it occurs quickly, suggesting that there is considerable heterogeneity among docked vesicles (≈ 10 vesicles/release site in typical hippocampal synapses [13]). This might be due to the reversibility of priming reactions such that, at any instant in time, few vesicles are in a fusion-competent state. Slight adjustments in this equilibrium might have profound effects on synaptic efficacy. The speed of release dictates that only a handful of molecular rearrangements mediate the final stages of exocytosis, and the Ca^{2+} sensor must respond to Ca^{2+} with very rapid kinetics.

What is the Ca^{2+} -sensitivity of the Ca^{2+} sensor for synaptic exocytosis? For technical reasons, this value has been determined for only two synapses, yet the results from these studies have already revealed striking differences. In goldfish (*Carassius auratus*) retinal bipolar neurons, the threshold for detectable exocytosis is $\geq 20 \mu\text{M}$ Ca^{2+} , and half-maximal rates of secretion occur at $194 \mu\text{M}$ Ca^{2+} [14], while in the rat calyx of Held synapse the threshold for exocytosis is $\leq 1 \mu\text{M}$ Ca^{2+} [15,16]. One possible explanation for this difference is the differential expression of Ca^{2+} sensors with low and high affinities for Ca^{2+} respectively. More is known concerning the Ca^{2+} requirements for the fusion of large dense core vesicles (LDCVs), which appear to have low Ca^{2+} thresholds for release and in many cases exhibit half-maximal rates of release in the range 1 – $20 \mu\text{M}$ Ca^{2+} [17–20]. In most cases of Ca^{2+} -triggered exocytosis there is a steep exponential relationship between $[\text{Ca}^{2+}]$ and release, suggesting that occupancy of multiple Ca^{2+} binding sites drives fusion (see, e.g. [14,21]).

What is the molecular identity of the sensors that trigger exocytosis, and how do they operate? Over the last decade there has been a virtual explosion in the identification of proteins that play critical roles in the release process. The leading candidates to serve as the Ca^{2+} sensors that regulate fusion are members of

Abbreviations used: (c)PLA₂, (cytosolic) phospholipase A₂; FRET, fluorescence resonance energy transfer; LDCVs, large dense core vesicles; PKC, protein kinase C; PLC, phospholipase C- δ 1; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; SNAP, soluble N-ethylmaleimide-sensitive fusion protein attachment protein; SNARE, SNAP receptor; SV, synaptic vesicle; TMD, transmembrane domain.

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Figure 1 The SV cycle

Details are provided in the text.

the synaptotagmin gene family. The best characterized, and perhaps most abundant isoform is synaptotagmin I (referred to as synaptotagmin, unless otherwise indicated). Here, we will focus on studies aimed at determining whether and how synaptotagmin operates as the long-sought-after Ca^{2+} sensor.

DISCOVERY AND STRUCTURE OF SYNAPTOTAGMIN I

In 1981, Matthew et al. [22] reported a 65 kDa antigen (p65) present on the surface of SVs and LDCVs. The sequence of this marker protein was determined by Perin et al. [23], who later renamed it synaptotagmin [24]. Synaptotagmin attracted immediate attention because its cytoplasmic domain is almost completely composed of tandem C2-domains (Figures 2 and 3B); the N-terminal C2-domain is C2A; the C-terminal C2-domain is C2B. C2-domains were initially named after the conserved or constant sequence 2 among protein kinase C (PKC) isoforms. C2 homologous segments were found in PKC isoforms that interacted with Ca^{2+} and membranes, and were not initially found in Ca^{2+} -independent isoforms (but also see [25]), suggesting that they might form Ca^{2+} /lipid-binding domains [26]. Presently there are 123 C2-like sequences in the human genome [27]. The C2A region of synaptotagmin was the first example of a C2-like sequence that could autonomously fold into a Ca^{2+} and phospholipid-binding module, and is hence referred to as a C2-‘domain’ [28,29]. C2A was also the first C2-domain whose structure was determined [30]. Since then, a number of additional C2-domains have been characterized and found to conform to similar overall structures [31]. They are compact, rigid eight-stranded β -sandwiches; flexible loops protrude from the ends of these domains, including two flexible loops that co-ordinate three Ca^{2+} ions in C2A [32] and two Ca^{2+} ions in C2B [33] (shown in Figure 2A). In the absence of synaptotagmin binding partners, Ca^{2+} binds to these sites with low affinity (the K_d values for C2A are $\approx 60 \mu\text{M}$, $400 \mu\text{M}$, $> 1 \text{ mM}$ Ca^{2+} ; the K_d values for C2B are $\approx 400 \mu\text{M}$, $\approx 600 \mu\text{M}$ Ca^{2+}).

What is the function of the C2-domains in synaptotagmin? In some cases, the role played by C2-domains is clear. In cytosolic phospholipase A_2 (cPLA $_2$), a C2-domain mediates Ca^{2+} -triggered translocation to lipid bilayers, where a separate catalytic domain is pressed against the membrane such that it can

encounter and efficiently turn over membrane-delimited substrate (i.e. phospholipids) [31]. In this case, the C2-domain functions to enhance the overall rate of catalysis. Because synaptotagmin is anchored to the vesicle membrane through its lone N-terminal transmembrane domain (TMD), binding of its C2-domain to the plasma membrane could help to juxtapose the vesicle and target membranes, potentially speeding-up the rate of membrane fusion in response to Ca^{2+} . We will return to this idea in greater detail below.

SYNAPTOTAGMIN FUNCTION

The function of synaptotagmin has been addressed by micro-injecting anti-synaptotagmin antibodies and regions of the protein into living cells, where they have been shown to block exocytosis [34–37]. Genetic approaches have also established that synaptotagmin plays a critical role in SV trafficking. Initial synaptotagmin gene disruption studies in fruitflies (*Drosophila*), nematode worms (*Caenorhabditis elegans*) and mice revealed that the evoked release of neurotransmitter was compromised [38–41]. A critical question has been to determine whether this phenotype results from a loss of vesicles (either biosynthesis or recycling), impaired docking (and/or tethering near Ca^{2+} channels), impaired priming and/or stability of primed vesicles, or impaired Ca^{2+} -‘sensing’. In the following sections we provide an overview of these results, followed by discussion of the biochemical properties of synaptotagmin that may couple Ca^{2+} -influx to SV fusion.

A ROLE IN VESICLE DOCKING?

Morphological analysis of synaptotagmin I-null *Drosophila* larvae demonstrated a slight overall reduction in the number of SVs and a marked reduction in the number of docked vesicles [42]. These findings provide the best support for the idea that one function of synaptotagmin may be to facilitate docking. These defects were also apparent in larvae with one null allele and one *AD3* mutant allele. The *AD3* allele harbours a single substitution [43] that disrupts the Ca^{2+} -sensing ability of the C2B-domain [44]. While evoked secretion is always compromised in *AD3*

Figure 2 Structure of synaptotagmin and the mechanism of its binding to membranes

(A) Schematic diagrams showing the Ca^{2+} -binding sites within C2A and C2B of synaptotagmin I {modified from [157] [Copyright (1998) American Chemical Society] and [33] [Copyright (2001) Elsevier Science] with permission}. The position of the fluorescent reporters analysed in (B) are indicated in red, as are the Ca^{2+} ions. (B) Solution structures of C2A and C2B (from the description files in [157] and [33]) were rendered in WebLab Viewer Lite [Molecular Simulations Inc. (now Accelrys), San Diego, CA, U.S.A.]; the flexible linker that connects them together [85] was added with a drawing program. Ca^{2+} ions are shown in red, and the position of fluorescent reporters (residues 234 and 367 from A) are indicated as red ball-and-stick images. In the bottom panel, the fluorescence increases that occur upon penetration of the reporters into lipid bilayers in response to Ca^{2+} are shown (11 nM liposomes composed of 25% PtdSer/75% PtdCho) and occur with very rapid kinetics {reprinted, with permission, from [64]; Copyright (2002) National Academy of Sciences, U.S.A.}.

mutants, fruitflies are viable as adults when *AD3* is paired with certain other mutant alleles. Defects in vesicle docking are not observed in the *AD3* flies that possess a complimentary mutant synaptotagmin allele [44]. This could be due to a docking activity provided by the complimentary allele. However, recent studies indicate that hypertonic sucrose [45] releases the same number of quanta from the neuromuscular junction of wild-type versus *AD3*/null embryos (J. T. Littleton, personal communication). These latter findings suggest that the physio-

logical deficits exhibited by *AD3*-harbouring flies are not due to defects in docking, but rather arise from the impaired Ca^{2+} -sensing ability of the C2B domain [44].

In some synapses, vesicles appear to dock in close proximity to pre-synaptic Ca^{2+} channels, where they experience very high local Ca^{2+} concentrations in tiny domains near the mouth of the channel [46]. Synaptotagmin interacts directly with a variety of Ca^{2+} channels, and this interaction has been proposed to play a role in tethering vesicles to release sites [47]. However, this view

Figure 3 Regions of synaptotagmin that interact with other molecules

(A) Synaptotagmin binding partners: botulinum neurotoxin B (BoNT/B) [158], AP-2 [75], stoned A/B [77,80,82], PtdIns₄-PtdIns₆ ('IP_{4,6}') [159], β-SNAP [160] (but see also [18]), neurexins [86], syntaxin [104,117], SNAP-25 [18,118,119], tubulin [161], Na⁺ channels [162], N- and P/Q-type Ca²⁺ channels [163,164], PtdSer ('PS') [28,29,70] (note ^b: C2B binds tightly to PS only when it is tethered next to C2A [64]), PtdInsP₂ or PtdInsP₃ ('PIP_{2/3}') [76] (note ^a: C2A binds only at high mol% PtdInsP₂ ('PIP₂') [165]), SV2 [166], Ca²⁺-dependent oligomerization [69,71,72,125,135], Ca²⁺-independent oligomerization [90,96,120,130], calmodulin [86], and the Rab3 effector RIM [71,167]. See the text for details and additional references. It should be noted that C2B has recently been shown to harbour a tightly bound contaminant [105]; interactions that involve C2B may differ in the absence of this factor. (B) Differences between the C2-domains of distinct synaptotagmin isoforms. These are illustrated with only three isoforms and one effector, namely PtdSer ('PS'); differences among all isoforms are too extensive to list here. The isolated C2A domain of synaptotagmin I binds tightly to liposomes containing PtdSer in response to Ca²⁺ [95], while the C2B only binds tightly to PtdSer when tethered next to C2A ([64]; indicated by '^b'). In synaptotagmin IV, neither C2-domain binds PS in response to Ca²⁺ [64], while in synaptotagmin VII, each isolated C2-domain binds tightly to PtdSer [112].

has recently become more complicated [48]. A number of synapses are sensitive to slow Ca²⁺ chelators, indicating that short-lived domains, nanodomains, of high local Ca²⁺ concentration are not needed to drive release, and that channels and Ca²⁺ sensors for secretion are at some distance from one another [49,50]. During an action potential in the calyx of Held, the Ca²⁺ sensor experiences local [Ca²⁺] of only $\approx 10\text{--}25\ \mu\text{M}$ and operates below saturation [15,16]. These data provide additional evidence that that the release machinery is relatively far away from Ca²⁺ channels. Whether this is a common theme among different neurons remains to be explored. Another recent finding was that the kinetics of LDCV release from chromaffin cells are diminished in synaptotagmin I knock-outs, even when Ca²⁺ levels are increased in a uniform manner using caged Ca²⁺ [51]. Thus the defects observed in the cells are not due to a loss of synaptotagmin-channel interactions.

The region of Ca²⁺ channels that binds directly to synaptotagmin and syntaxin (called the 'synprint' peptide [47]) reduces exocytosis when introduced into synapses [52]. This finding was interpreted as support for the idea that proteins involved in exocytosis must be tethered to Ca²⁺ channels to experience high local Ca²⁺ concentrations. Synprint would uncouple this interaction, and the same amount of Ca²⁺ influx would drive less release because the release sites are further away. However, synprint is a potent inhibitor of exocytosis even in cracked secretory cells in which Ca²⁺ channels have been completely by-passed [53]. Synprint can also inhibit endocytosis, leading to a loss of vesicles [54]. Inhibition of both exo- and endo-cytosis might be mediated by the binding of synprint to the C2B-domain of synaptotagmin [53,54].

A POST-DOCKING FUNCTION

The first strong evidence that synaptotagmin exerts a function between docking and fusion came from genetic studies of synaptotagmin I-null mice. Disruption of synaptotagmin I largely abolished the rapid component of synaptic transmission [41]. A key finding was in a subsequent study where it was shown that the total number of vesicles that are released by hypertonic

sucrose was not compromised in these mutant synapses [55]. The mechanism by which hypertonic sucrose drives SV exocytosis is unclear, but it is independent of Ca²⁺ and is thought to act on only docked and primed vesicles, via SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins [56]. Interestingly, sucrose fails to drive release from munc13-null synapses, even though normal numbers of vesicles, observed by electron microscopy, were docked at active zones [56,57]. It was suggested that munc13 is needed for vesicles to prime and become fusion-competent. However, similar results are obtained when syntaxin is disrupted; at present syntaxin is not considered to be a priming protein, but rather may form the core of the fusion machine [2,58]. In any case, detailed studies in mice, and in the *AD3* mutant in flies described above, have established that mutations in synaptotagmin impair the ability of docked vesicles to fuse rapidly in response to stimulation.

Genetic studies also revealed that synaptotagmin inhibits spontaneous fusion: mutations in *Drosophila* synaptotagmin result in increases in mini-frequency [43,59], and overexpression of synaptotagmins I and IV in flies suppresses mini-frequency [60]. Similar observations have been made in cultured *Xenopus* neurons [61]. Thus one function of synaptotagmin may be to 'clamp' spontaneous fusion (potentially via its Ca²⁺-independent mode of binding SNAREs, detailed below), while another function may be to drive evoked synchronous fusion in response to Ca²⁺ (potentially via its Ca²⁺-dependent mode of binding SNAREs, membranes, and other copies of synaptotagmin, detailed below).

FORMATION/STABILIZATION OF THE READILY RELEASABLE POOL?

Time-resolved capacitance measurements from chromaffin cells isolated from synaptotagmin I-deficient mice have been carried out [51]. In control cells, a rapid burst and a slower release component have been resolved. The mutant cells showed a 50% decrease in the extent of exocytosis, and all of this could be ascribed to the complete loss of the rapid component of secretion.

Much longer delays between rises in Ca^{2+} and exocytosis were also observed, as expected from an overall decrease in the kinetics of secretion. Interestingly, sustained release was not compromised, indicating that vesicle supply is not critically dependent on synaptotagmin I, again arguing against a key role in vesicle mobilization/docking.

Loss of the rapid component of secretion from synaptotagmin I-null chromaffin cells prompted two models: synaptotagmin I could function to trigger the fusion of the rapidly releasable pool, or synaptotagmin I could be required for the formation/stability of the readily releasable pool [51]. These findings illustrate the difficulty inherent in trying to use genetics to determine the exact step in which a protein acts in an overall process. However, it is important to note that the Ca^{2+} -binding sites of synaptotagmin are probably only occupied during the micromolar Ca^{2+} transients that trigger release. Thus, if these sites are important for the function of synaptotagmin, they are likely to operate during the Ca^{2+} -triggered fusion step rather than during a priming or maintenance step.

REGULATION OF FUSION-PORE DYNAMICS

Data suggesting that synaptotagmin functions during the final steps of exocytosis, i.e. the opening and dilation of fusion pores, came from time-resolved amperometry experiments of LDCV exocytosis in PC12 cells [62]. Increases in the copy number of synaptotagmin IV, an isoform that senses Ca^{2+} weakly [60,63,64], decreases the kinetics of secretion and destabilizes fusion pores. In contrast, increasing the copy number of synaptotagmin I did not affect the kinetics of secretion, but stabilized fusion pores. PC12 cells express synaptotagmins I and IX; no other isoforms are detected, except for trace levels of synaptotagmin IV [65–67]. In these cells, synaptotagmins I and IX appear to function in a redundant manner [65,68]. Thus the effects of synaptotagmin IV on secretion is likely due to displacement of I and XI, while the effects of I on secretion might be due to displacement of IX. Because synaptotagmins oligomerize [24,53,69–73] and are likely to function *in vivo* as multimeric complexes [59], it is tempting to speculate that overexpressed isoforms hetero-oligomerize with the endogenous synaptotagmins to yield fusion complexes with altered properties.

The key finding here is that synaptotagmins can determine, at least in part, the time to opening and the dilation kinetics of fusion pores. The simplest interpretation of these data is that synaptotagmin interacts with, and directly modulates, the fusion apparatus. Future studies will make it possible to relate precise biochemical functions of synaptotagmin with its affect on fusion-pore dynamics.

ENDOCYTOSIS

In contrast with mouse and fly studies described above, morphology studies of *C. elegans* lacking synaptotagmin I (or a deletion of the C2B domain) revealed the marked loss ($\approx 80\%$) in the total number of SVs [74]. Control experiments suggested that vesicle biogenesis and transport were not affected, and it was concluded that the defect was due to failure to efficiently recycle vesicles after exocytosis. Indeed, the C2B-domain of synaptotagmin has been reported to bind, with high affinity, to a number of molecules that function in endocytosis, including the clathrin adaptor protein complex AP-2 [75], PtdInsP_2 [76], and proteins encoded by the *stoned* locus [77] (Figure 3A). A similar decrease in the total number of SVs was observed in fly photoreceptor synapses following extensive stimulation of exocytosis in mutants lacking the C2B-domain [44]. Reist et al. [42] reported a less marked decrease in the total number of vesicles in fly embryos

lacking synaptotagmin, but discovered a significant increase in the number of large, irregularly shaped vesicles. Together, these data strongly suggest that synaptotagmin functions in endocytosis.

Additional details regarding the role of the C2B-domain in endocytosis are beginning to emerge. The interaction between synaptotagmin and AP-2 was potentiated by tyrosine-based endocytosis motifs, suggesting that synaptotagmin could function as a 'master regulator' during endocytosis [54,78]. Acute disruption of C2B–AP-2 interactions with the synprint peptide inhibits endocytosis, and C2B immobilized to coated vesicles facilitates coat formation *in vitro* [54]. Genetic and biochemical evidence also indicate that protein products of the *stoned* locus interact with C2B to regulate endocytosis [79–83], perhaps by facilitating the uncoating of coated vesicles [77]. A recent study using chimaeric proteins containing short regions of synaptotagmin suggested that C2B does not mediate endocytosis, but rather regulates a C-terminal internalization signal [84]. However, structural studies of synaptotagmins indicate that what was proposed to be a C-terminal 'tail' is in fact part of the folded structure of the C2B domain [33,85]. Thus it is unclear whether studies in which this sequence is taken out of context reflect physiologically relevant interactions (the same caveat holds for studies reporting that the C-terminal tail binds neuroligins and calmodulin [86] and mediates vesicle docking [87]; however, there is evidence that native synaptotagmin can indeed bind calmodulin [88,89]). Transfection studies indicate the synaptotagmin constructs lacking the C2B-domain can inhibit the formation of clathrin-coated pits in fibroblasts [90]. Inhibition was correlated with the ability of the transfected protein to hetero-oligomerize via its N-terminal domain. One interpretation of these data is that copies of synaptotagmin that lack a C2B-domain form multimers that cannot efficiently recruit AP-2, resulting in dominant negative effects on endocytosis. C2B may also help to recruit PtdInsP_2 from the plasma membrane into sites of endocytosis, where it could facilitate the binding of AP-2 [91].

HOW DOES SYNAPTOTAGMIN OPERATE DURING EXOCYTOSIS?

The studies described above have shown that synaptotagmin plays distinct roles in vesicle trafficking. Among these, synaptotagmin clearly fulfills a post-docking function, and at least one of these functions concerns the opening and dilation kinetics of fusion pores. In the following sections we will outline studies on putative effector interactions that might mediate the function of synaptotagmin during the final steps of release (summarized in Figure 3A).

INTERACTIONS WITH MEMBRANES

The best characterized synaptotagmin interaction is with anionic phospholipids, and in particular, phosphatidylserine (PtdSer). Some of the first insights into the potential mechanism by which synaptotagmin interacts with membranes came from structural studies of phospholipase C- $\delta 1$ (PLC) [92,93]. Molecular modeling predicted that, in order for the catalytic domain of the enzyme to gain access to membrane-delimited substrate, the Ca^{2+} -binding 'jaws' of its sole C2-domain would point towards the surface of the bilayer. Additional structural studies revealed that metal-binding stabilized the open conformation of the Ca^{2+} -binding 'jaws' or loops of the PLC C2-domain. Opening involved the exposure of charged residues proposed to interact with lipid head groups, resulting in a model in which Ca^{2+} binding changes the electrostatic potential at the tip of the domain to alter its interaction with effectors [93]. The first test of this model was carried out by placing single fluorescent reporters on dif-

ferent surfaces of the C2A-domain of synaptotagmin. Using membrane-embedded fluorescence quenchers, it was found that reporters placed in one of the Ca^{2+} binding loops (loop 3) directly penetrated into bilayers [94]. These observations were extended to the second Ca^{2+} -binding loop (loop 1) [95] with both loops penetrating to the extent of about one-sixth into the hydrophobic phase of the bilayer [96] (Figure 2B). This mode of binding has been confirmed in studies of other C2-domains, including cPLA₂ and PKC [97–101]. It is notable that, despite the findings that side chains on the distal tips of loops 1 and 3 of C2A penetrate bilayers, C2A–membrane interactions are highly sensitive to ionic strength, indicating that binding is largely mediated by electrostatic interactions [94], which may explain the rapid response time of this C2-domain [95]. In contrast, the cPLA₂ C2-domain–membrane interaction appears to have a much stronger hydrophobic component for complex formation [98,99] and responds to changes in $[\text{Ca}^{2+}]$ with much slower kinetics [95,102,103].

Some interesting surprises regarding the mechanism by which the intact cytoplasmic domain of synaptotagmin binds membranes have recently surfaced. For example, the isolated C2B domain of synaptotagmin exhibits very low levels of Ca^{2+} -dependent binding to liposomes composed of PtdSer and phosphatidylcholine (PtdCho) [18,33,76]; when studied in isolation, its Ca^{2+} -binding loops do not penetrate lipid bilayers composed of PtdSer/PtdCho [64]. However, when C2B is tethered next to the C2A domain by a flexible linker, it binds very tightly to liposomes containing PtdSer, and Ca^{2+} binding loop 3 of C2B penetrates into bilayers to the same extent as the loops in C2A [64] (Figure 2B). This activity does not require Ca^{2+} -binding activity in the adjacent C2A domain. An important outcome of these studies is that robust Ca^{2+} -triggered lipid binding activity is somewhat ‘redundant’ between the tandem C2-domains of synaptotagmin; disruption of membrane binding requires Ca^{2+} -ligand mutations in both C2-domains [18,64]. These properties were not apparent in early studies of synaptotagmin [96,104] because the initial synaptotagmin cDNA [23] encoded a defective C2B domain (in which a conserved glycine residue at position 374 was substituted with a glutamate residue [53,105]).

The inability of isolated C2B to penetrate PtdSer/PtdCho bilayers is rather surprising, because isolated C2B binds with high affinity to liposomes containing PtdInsP₂ [76]. Subtle differences in the Ca^{2+} /membrane binding loops are likely to account for this difference. Synaptotagmin–PtdInsP₂ interactions are particularly intriguing, given the importance of PtdInsP₂ in both exo- and endo-cytosis [91,106]. We note that synaptotagmin reconstituted into proteoliposomes can penetrate the vesicle membrane (*cis*) or the target membrane (*trans*) equally well, suggesting that other factors may ‘steer’ the C2-domains to the appropriate membrane [96]. PtdInsP₂ has been recently shown to be preferentially localized to the plasma membrane [107,108]. C2B exhibits significant PtdInsP₂ binding activity, even in the absence of Ca^{2+} , and may steer the bilayer penetration activity of the C2-domains toward the plasma membrane. Such an interaction could mediate close apposition of the bilayers to facilitate fusion (discussed in detail below).

The Ca^{2+} requirements for synaptotagmin–membrane interactions depend on the assay conditions used to measure binding. For example, solution-based binding assays focused on the C2A-domain of synaptotagmin I yielded EC₅₀ values of 50–70 μM Ca^{2+} [95,102], whereas studies using synaptotagmin immobilized on beads yielded EC₅₀ values in the range of 5–20 μM Ca^{2+} [95,109]. Given that synaptotagmins are multimers anchored to membranes, the bead-immobilized protein may reflect conditions similar to synaptotagmin *in vivo*. Regardless, these data

suggest that this activity only occurs during the narrow window of time of the Ca^{2+} transient; to function, the C2-domains of synaptotagmin must have very fast response times. As alluded to above, kinetics studies revealed that the C2-domains of synaptotagmin are highly specialized for speed-of-response and readily meet the kinetic constraints of rapid exocytosis [64,95] (Figure 2B).

Another source of uncertainty as to the Ca^{2+} -sensitivity of synaptotagmin–membrane interactions concerns the finding that increases in the proportion of anionic phospholipids (usually PtdSer) decreases the apparent Ca^{2+} requirement for binding [70]. This is probably due to weak interactions between Ca^{2+} and the anionic lipid head groups that result in higher local $[\text{Ca}^{2+}]$ at the surface of the bilayer. Finally, a number of different bivalent cations can trigger release at different rates and extents (e.g. [110,111]). To date, the bivalent cation selectivity of synaptotagmin–membrane interactions remains an open issue. In some studies, 500 μM Sr^{2+} and Ba^{2+} triggered the same level of binding as 500 μM Ca^{2+} [28], but in another study, Sr^{2+} did not trigger membrane binding [112]. Future studies are needed to relate the bivalent-cation-specificity of release with synaptotagmin–effector interactions.

Do synaptotagmin–membrane interactions function during secretion? So far, the only functional evidence comes from a point mutation that increases the Ca^{2+} requirements for C2A–membrane interactions by twofold. Knock-in mice harbouring this mutation exhibit a two-fold reduction in exocytosis [113]. However, when synaptotagmins that completely lack all of the Ca^{2+} -ligands in C2A are expressed in synaptotagmin I-null cultured hippocampal neurons, robust fast synaptic transmission is rescued (J. Sullivan and C. Stevens, personal communication). This may be due to the redundancy of the Ca^{2+} ligands and lipid-binding activity between C2A and C2B described above [18,64]. While it seems likely that membrane interactions function in secretion, it has yet to be definitively demonstrated.

INTERACTIONS WITH SNARES

In the absence of synaptotagmin I, exocytotic membrane fusion persists, but does not occur rapidly or synchronously in response to stimulation. In contrast, SNARE proteins are obligatory for fusion, and the SNARE complex is thought to form the core of a conserved membrane-fusion machine [2]. In neurons, the SNARE complex is composed of the SV SNARE (v-SNARE), synaptobrevin and the target membrane SNAREs (t-SNAREs), syntaxin and SNAP-25 (SNAP is soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein) [114] (Figures 3A and 4).

There is a clearly established direct interaction between synaptotagmin and the t-SNAREs syntaxin and SNAP-25, providing a compelling biochemical connection between a Ca^{2+} -sensor and the fusion apparatus. The connection was originally established via immunoprecipitation studies of synaptotagmin from detergent extracts of rat brain. Syntaxin was found to be one of a limited number of proteins associated with synaptotagmin [115]. Syntaxin immunoprecipitations yielded the reciprocal finding: the only associated proteins were other components of the SNARE complex and synaptotagmin [116]. These findings establish the specificity of synaptotagmin–syntaxin interactions. Subsequent studies demonstrated that binding was promoted by Ca^{2+} and might serve as a coupling step in exocytosis [104,117]. Most proteins that bind to syntaxin also interact with the other neuronal t-SNARE SNAP-25, so it was not surprising that SNAP-25 was also shown to bind synaptotagmin [118] and to be regulated by Ca^{2+} in the same manner as syntaxin-binding [18,119]. Key findings were that mutations in SNAP-25 that

Figure 4 Model for the function of synaptotagmin during exocytosis

(1) Prior to the Ca^{2+} signal, synaptotagmin weakly interacts with PtdInsP_2 and t-SNAREs in the plasma membrane. (2) Ca^{2+} triggers the rapid penetration of the Ca^{2+} binding loops of synaptotagmin into the target membrane and increases the affinity of binding to t-SNAREs, resulting in (3) the formation of a hemi-fusion intermediate. Expansion of the hemi-fusion stalk/diaphragm while restricting lipid flow results in a fusion pore (4) that dilates (5) and either closes or expands to mediate complete fusion (6).

selectively reduce synaptotagmin binding result in diminished exocytosis from PC12 cells [66], and inhibitors of synaptotagmin binding to syntaxin or SNAP-25 rapidly block release from cracked PC12 cells [18], indicating that synaptotagmin must bind SNAREs in order for fusion to proceed.

The Ca^{2+} requirements for synaptotagmin–SNARE interactions range from 100–200 μM in some studies [44,104,119] to 200–400 μM in other studies ([117]; note, this latter study used the C2A domain of synaptotagmin, which binds SNAREs weakly [95]). However, cross-linking of proteins in permeabilized PC12 cells indicate that binding actually occurs at much lower Ca^{2+} -concentrations *in situ* ($\approx 10 \mu\text{M}$ [66]), perhaps owing the presence of intact lipid bilayers; anionic lipids appear to increase the affinity of synaptotagmin for Ca^{2+} [70]. Consistent with this idea, current evidence suggests that synaptotagmin binds the SNARE complex and membranes at the same time [95].

Because little is known concerning the Ca^{2+} -sensitivity of secretion at synapses, the level of Ca^{2+} that should drive *bona fide* coupling steps in exocytosis remains an open issue. Whether interactions should occur at 10 μM [15,16] or 200 μM Ca^{2+} [14] will depend on the synapse under study. Also, virtually nothing is known concerning the distribution and Ca^{2+} -sensitivity of the ≥ 13 isoforms of synaptotagmin. Current localization data are summarized in Table 1, where it can be seen that with the exception of isoform I, the localization of all other isoforms is controversial. Further studies are needed to establish whether there is a correlation between the Ca^{2+} -sensitivity of synaptotagmins with their presence in specific synapses.

Further insights into the putative function of synaptotagmin–SNARE interactions would be aided by a molecular understanding of how binding occurs. The first domain-mapping study showed that all high-affinity binding was localized to the C-terminal end of syntaxin, corresponding to the H3-domain, which is the region that assembles into SNARE complexes, and the TMD [104]. It is critical to note that deletion of the syntaxin TMD strongly reduces synaptotagmin-binding activity. This was initially a surprising observation, since this sequence is buried in the bilayer and cannot bind directly to the C2-domains of synaptotagmin. However, the presence or absence of the TMD affects the binding of all syntaxin-binding partners yet examined. Some partners bind more weakly (synaptotagmin, synapto-

brevin and α -SNAP), while other partners bind more strongly [SNAP-25, nsec-1 (Munc-18)], arguing that syntaxin is not simply misfolded due to the truncation [120]. Mutagenesis studies revealed that both the length and the sequence of the TMD are critical determinants for syntaxin protein–protein interactions. It is likely that the TMD affects the structure of the cytoplasmic domain and/or alters the disposition of the cytoplasmic domain with respect to the surface of a lipid bilayer or a micelle to affect its interactions with other molecules. Studies using the cytoplasmic domain of syntaxin must be interpreted cautiously, since these constructs exhibit only trace levels of binding to synaptotagmin (e.g. [121]).

Further confusion stems from NMR experiments in which an interaction between the isolated C2A-domain of synaptotagmin and the N-terminal region of syntaxin (called the Habc domain [122]) was characterized in detail [32,122,123]. However, the Habc domain is completely dispensable for binding [95,104,121,124]. Furthermore, both C2-domains of synaptotagmin are required for high-affinity binding to syntaxin [95,125] and SNAP-25 [119]. Because C2A has a propensity to adsorb to anionic surfaces, it is likely to have bound, non-specifically, to an anionic patch on the surface of Habc under the high-protein-concentration conditions used in the NMR studies. Interestingly, C2-domains possess a groove in their 'sides' that precisely matches the diameter of the SNARE complex four-helix bundle, prompting models in which synaptotagmin engages the SNARE complex via this groove [85,95]. This is an attractive model in that it would allow synaptotagmin to bind membranes and SNAREs at the same time.

There is now agreement that all synaptotagmin binding activity maps to H3 and TMD of syntaxin [95,104,121,124]. This is an important observation, because the H3 domain is 'where the action is' in terms of driving membrane fusion. In this light, it is tempting to speculate that synaptotagmin regulates the assembly of SNARE complexes. Support for this idea stems from the finding that mutations that disrupt the Ca^{2+} -sensing ability of synaptotagmin (e.g. the AD3 mutation, which corresponds to a Tyr³⁶⁴ → Asn substitution in *Drosophila* synaptotagmin I [43]) block the assembly of SNARE complexes *in vivo* [44]. A caveat of this interpretation are studies showing that disruption of other presynaptic proteins (e.g. SV2) also result in the loss of SNARE

Table 1 Reported localization of synaptotagmin isoforms

In most cases, localization was carried out by immunocytochemistry (ICC); the localization of synaptotagmins V and X–XIII is not known. Abbreviations: PM, plasma membrane; SLMV, synaptic-like microvesicle; ER, endoplasmic reticulum.

Synaptotagmin isoform	Organelle	Cells	Means of localization	Reference(s)
I	SV	Neurons		[22]
	LDCV	Hypothalamus, posterior pituitary		[168]
	LDCV	Chromaffin cells		[24]
II	SV	Neurons		[73,169]
	Amine-free lysosomes	Mast cells	Based on fractionation	[170]
III	SV	Neurons		[169]
	PM	Neurons	Based on fractionation	[171]
	Secretory granules	Pancreatic β cells	Based on fractionation, ICC and immuno-organelle isolation	[172–174]
	Granular structures	Pancreatic δ cells	Not seen in β cells	[175]
IV	SV	Neurons		[60,73]
	Immature LDCV	AT-20 cells		[176]
	SLMV	PC12 cells		[177]
	Golgi/neurites	PC12 cells		[178]
	Golgi	Pancreatic α cells		[175]
VI	ER/Golgi	COS-7 cells	Transfected	[179]
	Cytosol, PM, and internal membranes	COS-7 cells	Transfected, alternatively spliced form lacking a TMD	[179]
	PM	Brain	Based on fractionation	[171]
	Acrosome	Sperm head	Immunoelectron microscopy	[152]
VII	Lysosomes	NRK cells	Localized via ICC, density gradients and GFP-fusion proteins	[180]
	Secretory granules	Pancreatic β cells		[174]
	PM	Neurons	Immunoelectron microscopy	[137]
VIII	Acrosomal crescent	Sperm head		[153]
IX	LDCV	PC12 cells		[65]

complex assembly *in vivo* [126]. Indeed, a number of biochemical steps could occur between the binding of Ca^{2+} to synaptotagmin and the assembly of SNARE complexes. However, using purified cytoplasmic domains, synaptotagmin was shown to facilitate assembly of SNARE complexes, and Ca^{2+} -synaptotagmin drove the cross-linking of SNARE complexes into dimers, further suggesting that synaptotagmin may play a role in the assembly and clustering of SNARE complexes [44].

After fusion, α -SNAP probably displaces synaptotagmin to allow the *N*-ethylmaleimide-sensitive fusion protein to dissociate SNARE complexes [116] to prevent t-SNAREs from being carried into the endocytosed SV membrane [127]. Synaptotagmin can bind t-SNAREs at all stages of SNARE complex assembly: isolated t-SNAREs, t-SNARE heterodimers, as well as the fully assembled complex. Whether synaptotagmin preferentially binds to any of these intermediates has not been determined. An interesting possibility is that, in native membranes, synaptotagmin preferentially interacts with t-SNAREs and *trans*-SNARE complexes, then binds less tightly to *cis*-SNARE complexes that form as a consequence of fusion, to favour displacement by SNAPs prior to endocytosis.

Finally, while most studies indicate that synaptotagmin–SNARE interactions are promoted by Ca^{2+} , there are some exceptions [128,129]; the reasons for these differences remain to be explored.

SYNAPTOTAGMIN OLIGOMERIZATION

It is clearly established that at least some isoforms of synaptotagmin form homo- and hetero-oligomers [24,70], as predicted

by genetic studies in *Drosophila* [59]. At least three distinct modes of oligomerization have been reported, involving either the cytoplasmic, transmembrane or the luminal domain of the protein, depending on the isoform.

The initial biochemical characterization of synaptotagmin I showed that it was broadly distributed on density gradients, and was thought to be either a dimer or tetramer [24,70]. Cleavage of a hypersensitive proteolysis site between the membrane anchor and cytoplasmic domain [24,89] revealed that the N-terminus migrated as an oligomer [70]. This activity was demonstrated directly using purified recombinant proteins [96] and it was shown that the TMD was critical for oligomerization activity [120]. Mutagenesis of a set of cysteine residues within or near the TMD disrupted the co-immunoprecipitation of epitope-tagged versions of synaptotagmin I [130] or VII [90] generated via transfection into fibroblasts. A similar result was obtained using a fragment of synaptotagmin, corresponding to the luminal, TMD, linker and C2A domain, that was reconstituted into proteoliposomes. After reconstitution, the cysteine residues at the TMD/linker border cross-linked with one another, forming intermolecular disulphide bonds, probably as a consequence of a prior stable oligomerization within the plane of the bilayer [96]. This disulphide-bond formation does not occur with native synaptotagmin I, presumably because these residues are palmitoylated *in vivo* [131,132]. It should also be noted that, while deletion of the TMD inhibits N-terminal clustering activity [120], deletion of the linker that connects the TMD to C2A also disrupts oligomerization [130], indicating that both the TMD and linker region are necessary for Ca^{2+} -independent oligomerization, but neither domain is sufficient (Figure 3A).

Recent studies uncovered a novel mechanism of oligomerization. Some isoforms of synaptotagmin (III, V, VI and X) contain an intraluminal cysteine residue that mediates disulphide-bonded homodimerization as well as some combinations of heterodimers [133]. These dimers can be dissociated by boiling in SDS with reducing agents. Synaptotagmin I has been reported to form dimers that are resistant to SDS and reducing agents [130] in some studies, but these dimers are not apparent in other studies [72].

The mode of oligomerization that has gained the most attention is the Ca^{2+} -promoted self-association of the cytoplasmic domains of a number of synaptotagmin isoforms. Oligomerization of the cytoplasmic domain was first reported by Wendland and Scheller [134]. In their study, the isolated C2B domain was expressed in Aat-20 cells and found to co-immunoprecipitate with endogenous synaptotagmin. This finding was recapitulated using the immobilized recombinant C2B-domain to 'pull-down' native synaptotagmin from detergent extracts in response to Ca^{2+} [125,135]. However, recent studies established that recombinant C2B contains tightly bound bacterial contaminants that may influence the wide range of interactions summarized in Figure 3(A) [105]. The effects of the contaminant on all synaptotagmin-effector interactions are currently being evaluated. At present, the role of this contaminant in, for example, C2B-mediated oligomerization is unclear: in an NMR study, contaminant-free C2B failed to oligomerize [105], while in bead pull-down experiments, it retained the ability to bind native synaptotagmin in response to Ca^{2+} [71].

While there are concerns regarding studies of Ca^{2+} -triggered oligomerization of recombinant fragments of synaptotagmin, Ca^{2+} -triggered oligomerization of native synaptotagmin was elegantly demonstrated by Osborne et al. [73]. Ca^{2+} facilitated the co-immunoprecipitation of native synaptotagmin I and II from purified rat SVs (synaptotagmin IV was also found to co-localize and co-immunoprecipitate with synaptotagmin I, but to a much lesser degree; synaptotagmins I and IV are co-localized in flies [60]). This observation was recapitulated using recombinant cytoplasmic fragments of synaptotagmins I and II. The association of both native and recombinant proteins had the same $[\text{Ca}^{2+}]_{1/2}$, 5–6 μM , which is precisely the value reported in previous studies using immobilized synaptotagmin I to pull down native synaptotagmin I from detergent extracts of rat brain [125]. It should also be noted that a solution-based fluorescence resonance energy transfer (FRET) assay revealed a different Ca^{2+} -dependency, namely 140 μM Ca^{2+} [73], demonstrating the sensitivity of the Ca^{2+} requirements to the assay system being used (as shown in the analysis of synaptotagmin-membrane interactions in [95] discussed above).

A critical role for C2B in mediating oligomerization of synaptotagmin was independently suggested from analysis of the *AD3* mutant allele of synaptotagmin in *Drosophila*. This mutation lies in the C2B domain and it reduces the Ca^{2+} -triggered oligomerization of synaptotagmin that was produced in *Escherichia coli* [44] or in fibroblasts [136]. A key finding was that the *AD3* mutation results in a post-docking defect in exocytosis, indicating that the Ca^{2+} -sensing ability of C2B is critical for Ca^{2+} -triggered fusion [44]. Furthermore, reagents that bind to the C2B domain of native synaptotagmin, including the synprint peptide [47] and isolated recombinant C2B [71], disrupt oligomerization and inhibit exocytosis from cracked PC12 cells [53]. While these reagents may disrupt additional interactions to inhibit secretion, these findings are consistent with a model in which Ca^{2+} -triggered synaptotagmin oligomerization is a coupling step in exocytosis. We note recent studies suggesting that C2B from synaptotagmin I inhibits release from PC12 cells

less effectively than the C2A-domains from isoforms III and VII [112,137]. It was concluded that III and VII are plasma-membrane Ca^{2+} sensors in these cells. However, neither synaptotagmin III nor VII were detected in PC12 cells [66]. Interestingly, synaptotagmin VII appears to oligomerize via each of its C2-domains – C2A co-immunoprecipitates with other copies of C2A, and C2B co-immunoprecipitates with other copies of C2B [138]. These findings indicate that the biochemical properties of a given C2-domain cannot be predicted by whether it is C2A or C2B (note: the same principle may hold for synaptotagmin-membrane interactions, as illustrated in Figure 3B).

Virtually all synaptotagmins examined exhibit some form of homo- and hetero-oligomerization activity. The rules that govern possible combinations are not clear. In one study using recombinant cytoplasmic domains, all isoforms tested bound to one another [53], yet in another study using the co-transfection co-immunoprecipitation approach, some combinations of oligomers were not detected [72]. A caveat of this latter method is that there is little control over the expression levels of the synaptotagmin constructs. A negative result can occur as a consequence of low expression levels, or binding that occurs with a lower affinity. What is particularly compelling about the co-immunoprecipitation studies, however, was the finding that some oligomers could only be detected when tagged synaptotagmins were expressed in the same cell and did not form when independently transfected cells were solubilized with detergent and mixed [72]. It seems likely that, like other multimeric protein complexes (e.g. K^{+} channels), some synaptotagmin oligomers are probably stably associated during assembly in the endoplasmic reticulum.

It is not known whether Ca^{2+} -triggered oligomerization occurs within living cells. This might be resolved soon, with the advent of green-fluorescent-protein variants that are amenable to *in vivo* FRET experiments. Further studies are also needed to understand the mechanism of Ca^{2+} -triggered oligomerization and the structure/stoichiometry of the oligomeric complex. The precise function of oligomerization during exocytosis also remains an open question, but it is tempting to speculate that it functions to arrange the fusion apparatus into a ring-like fusion pore whose characteristics are determined by the $[\text{Ca}^{2+}]$ [62]. Indeed, recent studies indicate that the Ca^{2+} -sensing ability of the C2B domain of synaptotagmin I is critical for fast synaptic transmission. Mutation of some Ca^{2+} ligands in C2B disrupts the ability of synaptotagmin I to rescue rapid exocytosis from neurons cultured from synaptotagmin I-deficient mice (J. Sullivan and C. Stevens, personal communication).

MODEL FOR SYNAPTOTAGMIN FUNCTION DURING MEMBRANE FUSION

Membrane fusion is a poorly understood process, and a number of models have been proposed [2,6,139–141]. The ability of purified SNAREs to mediate the fusion of proteoliposomes *in vitro* suggests that simply pulling the bilayers together tightly enough is sufficient to catalyse exocytosis [142,143], as predicted by the 'proximity model' [139,144]. Apposition of the bilayers must be tight enough to overcome the hydration barrier, allowing contact between the membranes, resulting in a hemi-fusion intermediate in which the outer leaflets merge [140]. The hemi-fused region is thought to resemble either a stalk or diaphragm. Dilation of the hemi-fusion diaphragm, while restricting lipid flow into the diaphragm, would result in the opening of an aqueous fusion pore [140].

In the simplest model (detailed in Figure 4), PtdInsP_2 (or PtdInsP_3 ; [76]) steers the Ca^{2+} -triggered membrane-penetration

activity of the C2-domains of synaptotagmin toward the plasma membrane, helping to drive close apposition of the bilayers in response to Ca^{2+} . This may indirectly facilitate assembly of *trans*-SNARE complexes by simply bringing them together. Alternatively, synaptotagmin could drive SNARE assembly more directly, through its interactions with the regions of t-SNAREs that assemble into SNARE complexes [44]. It is also possible that synaptotagmin could affect SNARE function after assembly, for example by twisting the cytoplasmic domain of *trans*-complexes which would further pull lipid bilayers together [95].

It is sometimes overlooked that synaptotagmin binds SNAREs in the absence of Ca^{2+} , the affinity being just lower under these conditions [44,104,118,119]. Given the high local protein concentrations at release sites, it is likely that synaptotagmin interacts with SNAREs *in vivo* prior to the Ca^{2+} signal. It is possible that this interaction inhibits the assembly of SNARE complexes to reduce the frequency of spontaneous fusion events [43,59–61]. Ca^{2+} changes this mode of binding, as evidenced by the apparent increase in affinity of binding, releasing the inhibitory effect, and actively driving fusion.

Understanding the structure of membrane–synaptotagmin–SNARE complexes will help to determine the validity of these models. For example, synaptotagmin oligomers might form ring-like structures that cluster SNARE complexes into a ‘fusion pore’ composed of lipids and perhaps the membrane anchors of the SNARE proteins. Ca^{2+} -dependent conformational changes, mediated by copies of synaptotagmin within this structure, might regulate the dilation of fusion pores once they open [62], resulting in either complete fusion [8] or kiss-and-run exocytosis [7,145] (Figure 4). Support for this idea comes from recent studies, detailed above, showing that synaptotagmin can influence the stability of LDCV fusion pores [62]. Further studies are needed to determine whether synaptotagmins affect SV fusion pore dynamics. For example, the level of Ca^{2+} , acting through synaptotagmin, may influence the relative proportion of full fusion versus kiss-and-run exocytosis [146]. Future studies are also needed to determine whether PtdInsP_2 is also critical for SV exocytosis [147,148]. If this is the case, changes in the levels of PtdInsP_2 could strongly influence release probability.

CONCLUSIONS

Synaptotagmin now has an established post-docking role in secretion, and also appears to be a key component of the endocytosis machinery. Current evidence is consistent with the idea that synaptotagmin is a major Ca^{2+} sensor that triggers release. However, an unambiguous demonstration of this function is likely to require a reconstituted system composed of defined components [143,149].

What generalities can be drawn from studies of synaptotagmin I? In all, 13 isoforms of the protein have been described [150], and recent genomic analysis indicates that this family may actually consist of 19 isoforms, some of which exhibit alternative splicing [151]. Interestingly, synaptotagmin proteins have been detected in tissues outside of the nervous system. For example, synaptotagmin XIII has been found in sperm heads (localized to acrosomes) [152,153] and in the kidney [154]. Similarly, synaptotagmin VII has been detected not only in brain, but also in heart, lung and spleen [117] (but see also [137]). What do synaptotagmins do in these other cell types? Many, if not all, membrane-fusion events require Ca^{2+} (reviewed in [144]); even fibroblasts appear to exhibit Ca^{2+} -triggered exocytosis [155,156]. Thus synaptotagmins might be widespread regulatory proteins that confer the Ca^{2+} -sensitivity of fusion in a wide range of fusion events.

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