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BREAKTHROUGHS AND VIEWS

Synaptotagmins: More Isoforms Than Functions?

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Constitutive trafficking of proteins and lipids requires the continous production and the subsequent fusion of transport vesicles with the target membrane. This property is shared by all cell types and allows the secretion of soluble molecules into the extracellular space. Neurons and endocrine cells possess, in addition to constitutive secretion, exocytic pathways characterised by their very precise regulation of fusion. The most regulated of all these processes is the release of neurotransmitters into the intersynaptic space. Neuroexocytosis is triggered by the transient increase of cytosolic calcium in response to an action potential. This calcium increase activates the lipid and protein machinery controlling the fusion of synaptic vesicles at the active site of the presynaptic membrane. Systematic characterisation of the protein components of small synaptic vesicles (SSV) led to the isolation of the first member of a family of proteins , named synaptotagmins. The members of this family are the best available candidates for the role of calcium sensors in neuroexocytosis and, more generally, in regulated secretion.

Synaptotagmins are a large family of membrane proteins with a wide distribution in nervous tissue and other organs. These proteins are characterised by a single transmembrane domain and by a large cytoplasmic portion that constitutes the majority of the protein mass. The cytosolic portion contains two copies of a domain known to bind calcium and acidic phospholipids, termed C2 (1-3). This structural motif, initially discovered in protein kinase C (PKC), was later identified in many proteins with different functions, such as phospholipases, lipid kinases, rab-specific GTPase activating proteins (rabGAP), the ubiquitin protein ligase NEDD-4 (4), the copines (5) and perforins (6). In synaptotagmins, the two C2 domains are responsible for different interactions, both calcium-dependent and calcium-independent.

STRUCTURAL ASPECTS

The first synaptotagmin family member (Syt I) was identified in the eighties with a strategy based on the use of a monoclonal antibody specific for an antigen of the synaptic membrane (7). The cloning of this protein revealed that Syt I is localised on a vesicular compartment and that its structure (schematised in Fig. 1) is conserved in vertebrates and invertebrates (8, 9). Syt has a small intraluminal N-terminal domain (domain 1) that is highly variable between isoforms and is glycosylated (10). It is connected to a single trans-membrane segment (domain 2) and to a cysteine-rich region that is palmitoylated in some isoforms (domain 3) (11, 12). The large cytoplasmic region contains the two C2 domains (1, 2, 8). The first of these, termed C2A (domain 5) is connected to the cysteine-rich region and the membrane-spanning domain via a hydrophylic highly-charged segment (domain 4) and to the second C2 domain or C2B (domain 7) via a short hinge region. Syt ends at the C-terminus with a highly conserved region known to interact with members of the neurexin family (13, 14).

The two C2 domains constitute, from a structural point of view, the *core* of Syt. Recently, the structure of C2A has been determined with both chrystallographic and NMR methods (15-17). The C2A domain is characterised by a very compact structure composed of two β-sheets. Each β-sheet can be sub-divided into 4 antiparallel β-strands, linked by very flexible loops. This β-sheet rich unit constitutes the *core* of the molecule and provides high structural stability. The high sequence homology between the C2 domains suggests that their structure is highly conserved in distinct proteins. In addition, the different biochemical properties

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Abbreviations: NSF, N-ethylmaleimide sensitive factor; PIs, phosphoinositides; PKC, protein kinase C; PS, phosphatidylserine; SNAP, soluble NSF attachment protein; SNARE, SNAP receptors; SSV, small synaptic vesicles; Syt, synaptotagmin.

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of C2 domains are determined by the flexible loops, which are characterised by a much lower sequence homology. In the C2A domain, these loops form a new calcium-binding structure, called C2 motif. The binding of two calcium ions is mediated by five aspartic acid residues conserved in all but one of the Syt sequences (14, 16, 17). The presence of these calcium-binding residues in a region of Syt characterised by local high flexibility is responsible for the absence of a large conformational change after calcium binding. The interaction with calcium results instead in a large change in the molecule's electrostatic potential, which provides the basis for the Syt binding to different ligands (proteins or acidic phospholipids).

SYT ISOFORMS AND DISTRIBUTION

In mammals, the Syt family is composed of at least twelve different genes (*syt* I-XII) each with a distinct tissue distribution (9). The presence of alternative splicing sites allows the formation of a higher number of isoform variants. All synaptotagmins resemble the general structure presented in Fig. 1 and show high homology in the cytoplasmic portion, particularly at the level of the C2 domains and the C-terminal segment. The differences between C2A and C2B are conserved in different isoforms, thus suggesting distinct functions for these two domains. The intravesicular (domain 1), the membrane-spanning (domain 2), and the highly-charged regions (domain 4), are instead characterised by a very low similarity and different dimensions. In one case (Syt X), the cysteine-rich region is absent and in other members (Syt II and VI) has a reduced size. Between the different isoforms, Syt I and II are the most strictly related with an identity of 75%, whilst all the others show a less pronounced homology (Table 1) (18).

Despite all synaptotagmins being enriched in the nervous system, their abundancy and distribution is strictly isoform-specific. Five isoforms (Syt I, II, III, V and X) are expressed exclusively in the nervous sytem and endocrine cells, whilst the others have an ubiquitous distribution. In particular, Syt I and Syt II show a complementary pattern of expression. Syt I is enriched in the cortex and the rostral part of the brain, whilst Syt II is predominantly (but not exclusively) expressed in the cerebellum and spinal cord (19). In contrast, Syt III is expressed all through the central nervous system and overlaps with both Syt I and Syt II distributions. Syt expression is also triggered by hormones: Sytr1/Srg-1 was recently cloned from a screening of genes activated by thyroid hormone (20). Its expression pattern is exclusively neuronal and it is particularly abundant in the cortex, amygdala, hippocampus and granular cell layer of cerebellum. Synaptotagmins expression can be regulated in pathological states, such as experimental epilepsy (21), a condition

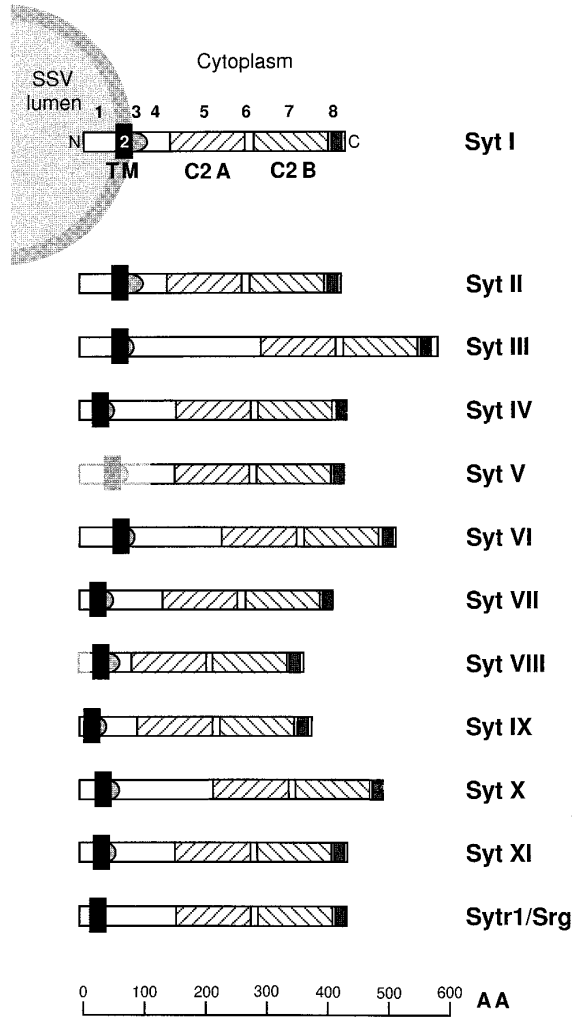

FIG. 1. Scheme of the structure of Syt isoforms. Syt can be divided in eight functional domains: (1) intralumenal; (2) membrane-spanning (TM); (3) cysteine-rich; (4) non-conserved highly charged portion; (5) C2A domain; (6) hinge region; (7) C2B domain; (8) conserved C-terminus. These domains are present in all isoforms, with the exception of the cysteine-rich portion that is absent from Sytr1/Srg1. Syt V and VIII are incomplete and their likely structure is reported in light grey. All isoforms are from rat (except Syt VIII, mouse) and the nomenclature adopted is from Südhof and Rizo, (1996) (Syt I-IX) and chronologically thereafter.

that promotes the synthesis of a newly identified Syt isoform (Syt X) (22). Although present also outside the CNS, some synaptotagmins show an enrichement in particular brain or cerebellar areas (i.e. Syt IV and VI). In addition, it is clear that some cerebral nuclei express a quite large repertoire of synaptotagmins (23, 24). The precise meaning of this functional overlapping is not known, but recent evidence suggests that association of distinct synaptotagmin isoforms may be important in determining the calcium threshold for the release of a SSV. Moreover, their distinct biochemical features may indicate a diverse role for different synaptotagmin

TABLE 1

Sequence Identity Matrix for the Different Syt Isoforms Based on a Multiple Alignment Constructed by the CLUSTAL_X Program (18)

	Similarity												
Identity	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	r1	B/K
SytI	—	81.4	34.5	39.2	41.8	41.6	45.6	44.3	64.9	40.2	42.2	35.5	38.0
SytII	74.7	—	34.4	39.2	41.3	40.7	46.7	45.3	62.6	39.5	39.6	36.8	38.7
SytIII	25.1	25.8	—	30.9	36.2	53.6	32.3	25.8	32.4	53.3	31.7	25.9	28.5
SytIV	28.3	28.4	21.8	—	36.1	37.0	45.2	31.0	39.4	37.2	66.0	36.3	33.7
SytV	31.7	31.6	30.8	26.8	—	44.4	39.2	35.5	44.8	45.2	35.8	31.2	30.3
SytVI	28.2	28.1	43.7	25.6	38.7	—	38.4	29.1	40.0	73.2	36.9	32.3	34.1
SytVII	31.4	34.6	23.8	33.0	30.6	26.6	—	31.7	42.9	39.0	48.6	34.2	36.6
SytVIII	32.3	32.6	17.5	20.4	24.7	18.8	21.0	—	45.3	27.5	29.9	29.6	31.0
SytIX	52.6	50.2	24.4	28.4	33.0	28.5	28.3	33.4	—	39.0	39.4	38.4	35.4
SytX	27.6	27.7	42.7	26.5	39.4	62.8	26.2	18.0	27.3	—	37.3	32.9	32.9
SytXI	27.8	27.3	21.4	53.7	26.1	25.0	35.1	19.0	27.8	25.7	—	35.6	34.7
Sytr1	20.4	21.6	15.7	21.8	20.0	21.2	20.3	19.2	24.0	20.8	21.8	—	30.4
SytB/K	26.5	26.5	20.8	23.3	21.6	23.3	25.9	20.4	24.8	21.9	23.0	20.2	—

The accession numbers for the different isoforms are: SytI, P21707; SytII, M64488; SytIII, D28512; SytIV, U14398, L38247; SytV, U20108, D62748; SytVI, U20105; SytVII, U20106; SytVIII, U20107, U20109; SytIX, U26402, P47861, X84884; SytX, U85513; SytXI, AF000423, Sytr1/Srg1, U71294; SytB/K, S68695, U30831.

isoforms in the general framework of neurotransmitter release (25).

Recently, other proteins with striking structural similarities to the members of the Syt family but not containing a membrane-spanning domain have been identified. Their structures are schematised in Fig. 2. Out of these, rabphilin3A is a soluble rab3-binding protein specific for the GTP-bound form and is suggested to modulate the cellular functions of the rab proteins. At the nerve terminal, rabphilin 3A is present in two pools, one cytoplasmic and the other associated with SSVs. In addition to the two C2 domains rabphilin 3A is characterised by an N-terminal extension containing a zinc finger domain. Rabphilin 3A also interacts with cytoskeletal proteins and could be important for actin remodelling. Another protein highly similar to synaptotagmins, but not containing a membrane-spanning domain is DOC2 (double C2) (26). DOC2 is present in two isoforms with different localisations. DOC2A is neuron-specific and associated with SSVs, whilst DOC2B is ubiquitous. Two different proteins were shown to interact with DOC2: Munc18, a syntaxin-binding protein that may regulate formation of core SNARE complexes during SSV docking and Munc13, another protein con-

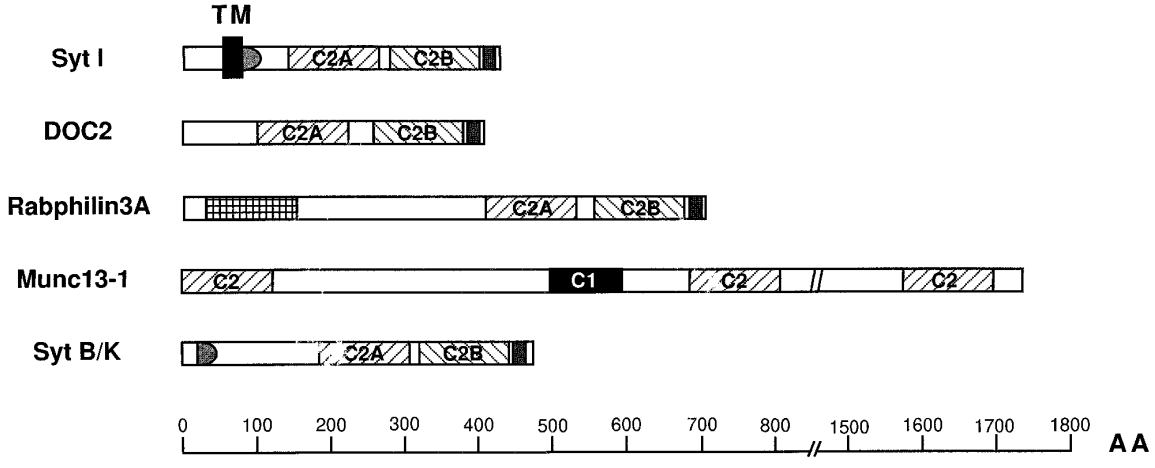

FIG. 2. Comparison of the structures of proteins containing double C2 domains. All proteins presented in this scheme (with the exception of Syt I) lack a membrane-spanning domain. Syt B/K contains a cysteine-rich region (light grey) that, analogous to Syt I, could be palmitoylated, thus mediating its interaction with lipid bilayers. Rabphilin 3A binds rab 3 via a N-terminal domain (cross-hatched area), whilst Munc13-1 contains, in addition to the C2 domains, also a single C1 domain (filled area). The conserved C-terminus domain (domain 8) of Syt is also indicated (dark grey area).

taining a double C2 motif and presented in Fig. 2. Munc13 is the mammalian homologue of the *unc*13 gene product in *C. elegans*, mutation of which is characterised by abnormal neuronal connections and modified synaptic transmission (27). In addition to a double C2 domain, Munc13 also contains a C1 domain, responsible for the phorbol ester-dependent high-affinity interaction with phospholipids. Three isoforms, characterised by a divergent N-terminus, but conserved C-terminus containing the C1 and C2 domains are presently known (28). Of these, Munc13-1 is characterised by the presence of an extra C2 domain at the very N-terminus (28). All members of this new protein family are expressed in brain and are associated with the presynaptic membrane. Munc13 interacts with both DOC2 (in a process stimulated by phorbol esters through the C1 domain) and syntaxin in a still poorly defined equilibrium that may regulate SSV docking (29). The last protein presented in Fig. 2 is synaptotagmin B/K, a molecule expressed only in brain and kidney and containing an extended N-terminal cysteine-rich region (30). In analogy with Syt I, this region is likely to be fatty acid-modified *in vivo*, thus providing the means for membrane anchoring.

In contrast with other synaptic proteins involved in neurotransmitter release, like VAMP or syntaxin, synaptotagmin appears not to be conserved phylogenetically. In fact, no synaptotagmin homologues have been identified in organisms lower than *C. elegans* (Pseudocelomates) on the evolutionary scale. In the yeast genome there are three large hypothetical proteins containing multiple C2 domains and one predicted N-terminal transmembrane domain. Although no functional data are presently available, this finding could suggest that proteins with a synaptotagmin-like topology may act in biological systems lacking calcium-dependent secretory pathways.

PHYSIOLOGICAL ASPECTS

The role of synaptotagmins at the CNS synapse has been investigated using a variety of model systems, ranging from classical biochemistry to molecular genetics and electrophysiology. Neurotransmitter release is the key-event in the life cycle of the SSV, representing the fastest and best regulated vesicular fusion event (31). The life cycle of a synaptic vesicle can start at the level of an endocytic recycling compartment, from which it buds (32). Alternatively, the SSV could be recycled directly from the plasma membrane without a sorting event (33). In both cases, SSV are loaded with neurotransmitters and subsequently recruited to specialised areas of the plasma membrane, termed active zones (31). This specific binding is a multi-step process mediated by several protein-protein and lipid-protein interactions and is thought to progress from an early event, termed tethering, to a more proper vesicle docking. At this point, the SSV is in close proximity with the calcium channels in the synaptic plasma membrane. Docking is followed by an ATP-dependent priming step that makes the SSV competent for the fusion. This last process is strictly dependent on a rise in the cytosolic calcium concentration and entails either complete bilayer membrane fusion or the opening of a fusion pore. The exocytic phase is very fast, lasting less than 0.5 ms. Studies on the dependency of neurotransmitter release on calcium concentration indicate that the threshold to initiate SSV fusion in a synapse of the central nervous system is in the range of 20 μM Ca^{2+}, although the maximal rate of release is reached only at 200 μM Ca^{2+}. More than a single calcium ion is required for a single SSV fusion event to occur, thus suggesting the presence of multiple calcium binding sites (from three to six) cooperating in this process. Experimental evidence indicates a central role for synaptotagmins in vesicle fusion. Genetic analysis *in C. elegans, Drosophila* and mouse demonstrates that Syt I ablation or mutation results in a common phenotype characterised by a strong decrease in the calcium dependency of neurotransmitter release (34-38). This dependency is not entirely lost, suggesting that Syt is not the only calcium sensor in regulated exocytosis. Mice lacking Syt I present a lethal phenotype in the early days after birth, despite being morphologically indistinguishable from their wild-type litter mates (36). This result demonstrates that synaptotagmin function(s) is essential for the correct performance of the nervous system, but not for its development. In addition, this finding indicates that, from birth, different synaptotagmins isoforms have non-redundant functions. In isolated neurons from mice embryos lacking Syt I, the rapid phase of neurotransmitter release is almost completely abolished, while the asynchronous release is untouched. Moreover, agents capable of stimulating neurotransmitter release with a mechanism independent from calcium are still active, thus indicating that, even in the absence of Syt I, SSVs are able to fuse with the presynaptic membrane (38). Impairments of synaptotagmin functions in neurons via the microinjection of peptides and antibodies are in agreement with the results presented above (39-41). Taken together, these findings suggest that Syt I is likely to be the main calcium-sensor of the fast-phase of neurotransmitter release in the CNS, probably by clamping vesicle fusion in a calcium-dependent manner.

MOLECULAR INTERACTIONS OF SYNAPTOTAGMIN

Several molecules have been proposed as functional ligands of Syt in processes both dependent and independent from calcium and involving distinct Syt domains. At the molecular level, the ligands of Syt could be divided in two large categories: proteins and lipids. The most studied Syt interaction is with artificial liposomes con-

taining phosphatidylserine (PS) (42-44). This interaction is promoted by calcium ions with an EC_{50} of 3-6 μM and requires a high concentration of PS in the liposomes (>25%). The specificity of the interaction is demonstrated by the fact that other negative phospholipids (such as phosphatidic acid and phosphatidylinositol) show a negligible or much less pronounced interaction. The C2A domain is responsible for PS binding. In fact, using a recombinant protein containing only the C2A domain, it is possible to have PS binding identical to that observed with the entire cytoplasmic domain. Recently, synaptotagmins have been shown to interact with another class of negative phospholipids, the phosphoinositides (PIs). Only the highly phosphorylated members of this lipid family bind Syt I with high affinity. In particular, the two bisphosphorylated isomers, PIns(3,4)P_2 and PIns(4,5)P_2, and the trisphosphorylated PIns(3,4,5)P_3, promote the interaction of Syt I with liposomes in a very specific and efficient manner (45). Syt I interacts with PIs independently of their incorporation in lipid bilayers, as demonstrated by the binding of PIns(4,5)P_2 in detergent micelles and by the interaction with inositol polyphosphates, compounds mimicking the soluble headgroups of PIs (45). These compounds, of which the most representative member is inositol hexaphosphate, compete efficiently with PIs, thus suggesting that these molecules share the same binding site. At variance with PS, the putative binding site of PIs and inositol polyphosphates is not located on the C2A domain, but on the C2B. The specificity of the interaction with different PIs is strictly dependent on the calcium concentration. At submicromolar levels of calcium, Syt binds PIns(3,4,5)P_3, but not PIns(4,5)P_2. An increase of calcium to a level higher than micromolar reverses the specificity of the binding. In this condition, the interaction of Syt I with PIns(3,4,5)P_3 becomes negligible, whilst the binding with PIns(4,5)P_2 increase until reaching the maximum at calcium concentrations between 20 μM and 100 μM Ca^{2+} (45).

Several proteins have been indicated as functional partners of Syt at the synapse, both in processes related to the exocytic phase and to the retrieval of SSV after fusion with the plasma membrane. The calcium-independent high-affinity interaction of the C2B domain of Syt I with the clathrin adaptor AP-2 seems to play an essential role in this last process (46). Another equilibrium independent from calcium is with β-SNAP, the neuronal isoform of a family of cytosolic proteins termed soluble NSF attachment protein (SNAP) (47). These protein adaptors mediate the interaction of N-ethylmaleimide sensitive factor (NSF) with membrane-bound receptors. NSF is an ATP-ase involved in the process of vesicular transport and fusion. The interaction of β-SNAP with Syt is likely to be important, because it catalyses the formation of a large protein particle (47) including NSF, the ubiquitous SNAP isoform α-SNAP and the synaptic protein receptors of SNAPs and NSF, termed SNAREs (SNAP receptors) (48). The SNARE receptors specific for neurotransmitter release are three integral membrane proteins, localised on two different cellular compartments. VAMP/synaptobrevin is a protein specifically localised on SSV (v-SNARE), while syntaxin and SNAP-25 are present mainly on the presynaptic plasma membrane (t-SNAREs) (48). These proteins form a high affinity complex characterised by high thermodynamic stability and are essential for neurotransmitter release to occur (48-50). In addition to β-SNAP, another two components of this protein complex interact directly with Syt. In fact, Syt binds syntaxin with a calcium-dependent mechanism (EC_{50}= 200 μM) via the C2A domain (17, 24, 51-53) and SNAP-25 via the C2B domain (54-56). This last interaction is only weakly dependent on calcium (56). The capability of Syt to bind directly both with β-SNAP and the two t-SNAREs suffices to re-define Syt as a specialised v-SNARE for calcium-dependent trafficking events (47). The ability of Syt to bind both PS and syntaxin is remarkably different for the various isoforms. These binding features are the basis for the classification of synaptotagmins into four distinct groups (9).

Another level of complexity in the study of the synaptotagmin dynamics is introduced by its ability to self-associate by forming both homo and hetero-dimers in a process mediated by calcium (42, 57-59). The self association is mediated by the C2B domain, but requires for its full extent, the integrity of the entire molecule. The formation of hetero-dimers was recently demonstrated for Syt I and II (59). Although these two isoforms are characterised by only a limited overlapping distribution, it is possible to isolate SSVs from brain cortex containing both isoforms. Calcium triggers effectively the association between Syt I and II with an EC_{50} of 3 μM, reaching the maximal binding at 100 μM (59). These results raise the possibility that the formation of homo- and hetero-dimers of synaptotagmins may have a role in the calcium-dependent phase of exocytosis. In addition, the self-association of synaptotagmins with different calcium-binding features could create a variety of calcium-sensors characterised by distinct calcium sensitivity. This combinatorial hypothesis predicts that the probability of a single SSV exocytic event is determined by both the repertoire of synaptotagmins present on the SSV surface and the gating properties of the calcium channels at the synapse.

In this regard, an increasing number of evidence links synaptotagmin with the voltage-gated calcium channels (51, 60-68) in a process likely to be important for the physiology of the nerve terminal. In *Xenopus* oocytes, Syt I expression alters the steady-state voltage inactivation of the N-type calcium channel, and fully restores the syntaxin-modified current amplitude and inactivation kinetics in a calcium dependent manner (67). Syt binds the N-type calcium channel directly via a cytosolic region comprising amino acids 710-1090 (68). Immunoprecipitation experiments suggest that

Syt also associates with P- and Q-type calcium channels which, together with the N-type, trigger rapid neurotransmitter release at many mammalian synapses (64, 65). The complex of Syt and calcium channels recruits also the SNARE proteins syntaxin, SNAP-25 and VAMP/synaptobrevin (69-71). At equilibrium, a large percentage of the total amount of calcium channels present at the synaptic terminal are engaged in this association. These results suggest that these proteins may constitute an isolated exocytotic complex in which the calcium channel tightly interacts with a synaptic vesicle docking site. The central role of the SNAREs-Syt complex in the regulation of nerve terminal functions is also supported by its association with the muscarinic ACh autoreceptor (mACaR), a presynaptic protein responsible for the feedback inhibition of transmitter release (72). The interaction between mAChRs and both syntaxin and SNAP-25 is modulated by depolarization, with a mechanism compatible for controlling the rapid, synchronous neurotransmitter release at nerve terminals.

In addition to the role of the cytoplasmic domain of synaptotagmin in calcium-regulated exocytosis, recent studies demonstrate that its intralumenal domain could be used as acceptor for exogenous ligands during during the exocytosis and recycling fo SSVs. During neurotransmitter release, the lumen of SSV becomes accessible to the external milieu, as demonstrated by the labelling of SSV with antibodies directed against the N-terminus of Syt I added to the extracellular medium (73). Botulinum neurotoxin type B (BoNT/B) exploits this property in its strategy to block the neuromuscular junction. BoNT/B binds the N-terminus of Syt II and could in this way enter the synapse in the lumen of SSV (74, 75). During the recycling of SSV, in a process mediated by the acidification of the vesicle lumen, the active fragment of BoNT/B could then translocate to the cytoplasm where it blocks neurotransmitter release. Support for this model is provided by experimental evidence indicating that tetanus toxin, another member of the clostridial neurotoxins family, enters hippocampal neurons in vesicles indistinguishable from SSV (76).

SYNAPTOTAGMINS AND NEUROTRANSMITTER RELEASE

At the present time, no unitary view exists on the molecular details of the processes of tethering, docking, priming and fusion of a SSV with the presynaptic membrane and the role(s) of synaptotagmins in this mechanism. Two points deserve to be discussed here. The first concerns the central role of synaptotagmins not only as calcium sensors, but also as the centre of a network of lipid-protein and protein-protein interactions with structural implications in the process of neuroexocytosis. The interaction between synaptotagmins on the membrane of SSV and the two presynaptic t-SNAREs, syntaxin and SNAP-25, constitutes another aspect of the tethering and docking processes. This complex, alone or including VAMP/synaptobrevin, is important for the interaction with another structural element of the presynaptic membrane, the calcium channel (69-71). As well as modulating the gating properties of the channel (67), the interaction between these proteins is functional in localising the SSV close to the site of calcium entry. The intracellular concentration of calcium decreases steeply with the distance from the mouth of the calcium channel. For this reason, the SSV must be docked in its immediate vicinity to be exposed to the calcium concentrations required for exocytosis (31). The complex between synaptotagmins, t-SNAREs and calcium channels could provide both the requirements for correct spatial localisation and all the known elements necessary for exocytosis, namely the calcium sensor, the proteins required for docking and fusion (SNAREs) and the calcium entry site. Furthermore, the Syt-mediated recruitment of β-SNAP, α-SNAP and NSF provides the means for the correct localisation of the soluble proteins needed for the activation of the SNAREs (47).

The existence of such a large number of interactions between elements of the SSV and proteins localised on the pre-synaptic membrane may explain why many different treatments destroying the integrity of SNAREs cause an increase rather than a decrease of docked SSVs (50, 77, 78). In fact, both v-and t-SNAREs are needed for the final fusion process of the paired membranes. A disruption of any of them will block the exocytic process at this stage. However, due to the presence of this network of interactions, the apparent tethering and docking will appear normal, but will be completely non-functional.

The second point concerns the possible role played by the lipid components in neuroexocytosis. Experimental evidence suggests that disruption of the PI pool results in a complete block of exocytosis at the priming step. The calcium-dependent interaction between Syt I and PIs offers a possible link between these lipids and the machinery of neuroexocytosis at the nerve terminal. At $Ca^{2+}>10$ μM, Syt interacts with the membrane at the level of both C2 domains, binding $PIns(4,5)P_2$ via the C2B and PS via the C2A region. The lipid interactions of Syt anchor the entire molecule to the membrane, thus creating an intimate interaction between the protein machinery for exocytosis and the membrane bilayer. In addition, Syt dimerises with formation of homo- and hetero- complexes. At this sub-optimal calcium concentration, the lipid and protein particle formed by Syts, v- and t-SNAREs could constitute a pre-active metastable fusion pore. In this model, the intrinsic ability of the SNARE complex to trigger membrane fusion (79) would be counteracted by Syt, serving as a calcium-sensitive clamp. The opening of a calcium

channel would then increase the calcium concentration thus allowing two further protein-protein interactions: the completion of Syt homo- and hetero- dimerisation and Syt-syntaxin binding. These new interactions may re-arrange the lipid and protein fusion pore into a transient open state functional for the release of the SSV contents. The intracellular reduction of calcium concentration to resting levels could then mediate the closure of the fusion pore (80, 81). In this case, the release of neurotransmitters occurs without a complete fusion between SSV and presynaptic membranes. This fact limits the intermixing between components of the two compartments. As a result, the SSV recycling does not require a complex protein sorting step, thus fulfilling the requirement of the "kiss and run" hypothesis for neurotransmitter release (33, 82). Alternatively, the fusion pore could disassemble during the open state, allowing full compartmental fusion. This event is likely to be linked to the clathrin-mediated endocytosis of SSV that occurs via a recycling endosomal-like compartment (32).

The discussion on the physiological role of synaptotagmins cannot be concluded without underlying the needs for future studies on the roles of the different isoforms. Their presence in cellular systems lacking calcium-regulated exocytosis argues in favour of the existence of isoform-specific modulators of Syt other than calcium and suggests a more general function of synaptotagmins in intracellular trafficking events.

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