Journal of Neurochemistry, 2001, **77**, 972–985

REVIEW

Functional and physical coupling of voltage-sensitive calcium channels with exocytotic proteins: ramifications for the secretion mechanism

Daphne Atlas

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

Abstract

The secretion of neurotransmitters is a rapid Ca^{2+}-regulated process that brings about vesicle fusion with the plasma membrane. This rapid process (< 100 μs) involves multiple proteins located at the plasma and vesicular membranes. Because of their homology to proteins participating in constitutive secretion and protein trafficking, they have been characterized extensively. The sequential events that lead these proteins to vesicle docking and fusion are still unclear. We will review recent studies that demonstrate the operative role played by voltage-sensitive Ca^{2+} channels and discuss the relevance for the process of evoked transmitter release. The regulation of Ca^{2+} influx by syntaxin, synaptosome-associated protein of 25 kDa (SNAP-25) and synaptotagmin, and the reciprocity of these proteins in controlling the kinetic properties of the channel will be discussed. Calcium channel and synaptic proteins expressed in *Xenopus* oocytes demonstrate a strong functional interaction, which could be pertinent to the mechanism of secretion. First, the voltage-sensitive Ca^{2+} channels are negatively modulated by syntaxin: this inhibition is reversed by synaptotagmin. Second, the modulation of N-type Ca^{2+} channel activation kinetics strongly suggests that the vesicle could be docked at the plasma membrane through direct interaction with synaptotagmin. Finally, these interactions provide evidence for the assembly of the voltage-sensitive Ca^{2+} channel with syntaxin 1A, SNAP-25 and synaptotagmin into an excitosome complex: a putative fusion complex with a potential role in the final stages of secretion. Studies suggest that cross-talk between the synaptic proteins and the channel in a tightly organized complex may enable a rapid secretory response to an incoming signal such as membrane depolarization.

Keywords: calcium channels, exocytosis, secretion, synaptotagmin, syntaxin 1A, vesicle fusion.

J. Neurochem. (2001) **77**, 972–985.

In recent years, multiple specialized proteins that mediate this tightly controlled process have been identified, although their precise role in the process is still unclear (see reviews: Burgoyne *et al*. 1993; Bennett 1997; Goda 1997; Hanson *et al*. 1997; Stanley 1997; Bajjalieh 1999; Fossier *et al*. 1999). Three membrane proteins, syntaxin, synaptosome-associated protein of 25 kDa (SNAP-25) and synaptobrevin (VAMP), also known as soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) were shown to be key players in the fusion process. These proteins assemble with equal molar stoichiometry into a tight stable ternary complex. Three models have been postulated for the role of the SNARE proteins in secretion: the ternary complex together with α-SNAP and NSF form a 20S complex. This complex dissociates upon ATP hydrolysis and leads to membrane fusion via a mechanism that is still not fully understood (Söllner *et al*. 1993; Pevsner *et al*. 1994; Hanson *et al*. 1997; Lin and Scheller 1997). This model was modified and it now seems that after SNARE proteins form a complex and fusion takes place, dissociation of the complex through recruitment of α-SNAP and NSF, is needed as means of priming the SNAREs for another round of fusion (Hanson *et al*. 1997; Fasshauer *et al*. 1998). A more recent model suggests that Ca^{2+}, through binding to a low-affinity site, initiates pairing of SNARE proteins on apposing membranes, and the generated high-affinity trans-SNARE complex leads to fusion of bilayers (Chen *et al*. 1999). This complex is stabilized by the interactions of the

Received October 15, 2000; revised manuscript received November 12, 2000; accepted December 8, 2000.

Address correspondence and reprint requests to Dr D. Atlas, Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: datlas@vms.huji.ac.il

Abbreviations used: LDCVs, large dense core vesicles; NSF, *N*-ethylmaleimide-sensitive factor; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor; SSVs, small clear synaptic vesicles; VAMP, synaptobrevin.

 © 2001 International Society for Neurochemistry, *Journal of Neurochemistry*, **77**, 972–985

transmembrane domains of the SNAREs (Poirier *et al*. 1998a, b; Margittai *et al*. 1999). The third model shows the essential role of a voltage-sensitive Ca^{2+} channel assembled with syntaxin, SNAP-25 and synaptotagmin in generating the excitosome complex. Upon membrane depolarization, by an as yet uncharacterized mechanism, the excitosome complex transmits the signal via the channel to the synaptic proteins, inducing membrane fusion and secretion (Wiser *et al*. 1996, 1997, 1999; Tobi *et al*. 1998).

This review has considered recent data that demonstrates the physical and functional interaction of voltage-sensitive Ca^{2+} channels, $Ca_v1.2$ (Lc-type), $Ca_v2.1$ (P/Q-type), and $Ca_v2.2$ (N-type) with synaptic proteins. It focuses on functional assignments of the synaptic proteins as calcium channel modulators and discusses evidence for the generation of the excitosome complex. It begins with a rationale for the importance of a two–way interaction between Ca^{2+} channels and proteins of the secretory apparatus. It will summarize current studies on physical and functional interactions of N-, Lc- and P/Q-type Ca^{2+} channels with SNARE proteins, and will describe synaptotagmin modification of the SNARE interaction with the channel. It will also discuss possible regulation of the release process by synaptic proteins, not only through promoting lipid fusion but also by controlling Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. Finally, the formation and putative role of the excitosome complex in bringing the vesicle and plasma membrane together in preparation for fusion will be discussed.

Co-localization of the Ca^{2+} channel and exocytotic machinery: a rationale for channel interaction with the secretory apparatus

The rapid kinetics of synaptic transmission requires an influx of calcium ions, which is supplied by voltage-sensitive Ca^{2+} channels during depolarization. Exocytosis is triggered by membrane depolarization followed by Ca^{2+} entry in which three or four cations act cooperatively at high concentrations (Llinas *et al*. 1992; Dodge and Rahamimoff 1967) to rapidly (0.2–0.5 ms) induce transmitter release. The local Ca^{2+} concentration at Ca^{2+} entry sites within 100 nm or less of the Ca^{2+} binding sites (Adler *et al*. 1991) suggests a morphological association of the channels with the exocytotic machinery and a relatively low affinity of Ca^{2+} within the release site (Llinas *et al*. 1992). These constraints suggest that the 10 nm particles seen in the freeze fracture images of the active zone (Heuser and Reese 1973) could be Ca^{2+} channels (Llinas *et al*. 1992), although so far there is no direct evidence that these particles are indeed calcium channels. Extensive studies of the rabbit retina showed a total of 100–200, 9–11-nm particles assembled at < 60 nm from the vesicle docking sites. The particles believed to be Ca^{2+} channels are strategically located close to the site that is most likely to ensure efficient transmission (Mennerick and Matthews 1996; Sakaba *et al*. 1997). Cellular co-localization of Ca^{2+} channels and release sites were found in a number of cells using combined electrophysiological and imaging techniques (Stanley 1993; Schroeder *et al*. 1994; Bokvist *et al*. 1995; Robinson *et al*. 1995; Elhamdani *et al*. 1998; Macleod *et al*. 1999). Carbon fiber microelectrodes placed at the cell surface, identified exocytosis release sites alternating with silent sites at the cell surface of bovine chromaffin cells (Finnegan *et al*. 1996; Schroeder *et al*. 1994). Voltage-clamp and capacitance measurements combined with quinacrine fluorescence imaging of single pancreatic β-cells, showed clusters of L-type Ca^{2+} channels in the β-cell region that contained the secretory granules (Bokvist *et al*. 1995).

A combination of a transient high-intensity illumination of pulsed laser light used to excite the fluorescent Ca^{2+} indicator, together with carbon fiber amperometry, showed a co-localization of $[Ca^{2+}]$ with catecholamine secretion (Robinson *et al*. 1995, 1996). Other studies in bovine chromaffin cells, using FM1-43 and dopamine-β-hydroxylase antibodies, showed a co-localization of the secretory apparatus with clusters of voltage sensitive Ca^{2+} channels, providing further support for the theory that evoked secretion occurs in unique cellular regions (‘hot spots’) (Cuchillo-Ibanez *et al*. 1999).

The question arises whether, in addition to high $[Ca^{2+}]$ at the release site, the close proximity provides a physical link between the Ca^{2+} channel and the exocytotic machinery, establishing regulation of Ca^{2+} entry and modulation of the release process.

Reconstitution of synaptic protein interactions and membrane fusion

The difficulty involved in reconstituting evoked secretion *in vitro* constitutes a major obstacle in understanding the molecular mechanism of transmitter release (Edwardson 1998; Avery *et al*. 1999; reviews). Unlike the reconstitution of signaling pathways such as G-protein coupled-receptors, in which the receptor, transducer and effector reside on the same membrane, the release process involves fusion of two independent membranes, transiently in contact with each other. This obstacle was overcome when an efficient fusion of phospholipid vesicles by the α helical core of the SNARE complex was recently demonstrated (Weber *et al*. 1999; Nickel *et al*. 1999; Parlati *et al*. 1999). However, other reconstitution studies question the role of the SNARE complex in the sequence of events leading to membrane fusion for the following reasons. First, yeast vacuole fusion demonstrated that while the SNARE complex is necessary at an early stage, it can later be dissociated without affecting the fusion process (Ungermann *et al*. 1998). Secondly, the

© 2001 International Society for Neurochemistry,

Table 1 Diagram of the channel interaction with synaptic proteins is intended to serve as (a) an organizational framework for knowledge so far gleaned on protein–protein interaction and (b) as a testable hypothesis

	N-channel				Lc-channel				P/Q-channel
	Peak (nA)	τ act (ms)	τ inact (sec)	E50 (mV)	Peak (nA)	τ act (ms)	τ inact (sec)	E50 (mV)	E50 (mV)
channel	-1024±130[a] -117±15[c]	59±2[e]	1.5±0.19[a] 1.2±0.1[b] 3.3±0.3[c]	-12.8±7[a] -19±1[b] 5.4±1.1[c] -83.9±3.3[f]	-1600±240[a] -3200±300[d]	4.0±0.8[d]	2.04±0.24[a] 2.00±0.06[h]	-25±4.4[a] -10±2.8[d]	-17.2±1.2[g] -63.0±1.5[f]
+ syntaxin 1A (Sx)	-308±60[a] -54±14[c]	79±3[e]	2.9±0.27[a] 3.2±0.3[b] 5.7±0.7[c]	-12.6±6[a] -104.0±2.8[f] 9.0±1.7[c]	-900±80[a] -950±50[d]	6.2±1.47[d]	2.48±0.13[a] 2.48±0.07[h]	-35±2.0[a] -24±1.0[h]	-18.9±3.0[g] -83.2±3.1[f]
+ SNAP-25 (Sp)	-725±146[a] -160±35[c]	64±1[e]	1.5±0.18[a]	-0.0±0.7[a] 9.8±2.4[c]	-1262±153[a]		1.33±0.07[a] 1.32±0.04[h]	-21±1.5[a] -25±2.7[h]	-26.5±0.9[g]
+ Sx/Sp	-804±104[a] -152±32[c]	66±2[e]	2.96±0.27[a] 5.3±0.5[c]	-5.3±3.0[c]	-947±155[a]		1.33±0.13[a] 1.38±0.04[h]	-32±2.2[a] -38±2.8[h]	-22.8±2.2[g]
+ synaptotagmin (St)	-113±21[c]	37±4[e]	3.8±0.3[c]	-9±2[b] 8.2±0.9[c]	-3240±200[d]	4.0±0.55[d]	1.86±0.98[h]	-23±2.9[h]	-18.8±2.5[g]
+ Sx/St	-169±25[c]	52±2[e]	2.5±0.3[b] 3.2±0.4[c]	3.4±4.5[c]	-2700±400[d]	4.17±0.8[d]	1.43±0.06[h]	-15±1.5[h]	
+ Sx/Sp/St (Excitosome Complex)	-268±35[c]	42±3[e]	3.1±0.5[c]	-3.5±2.0[c]			2.84±0.15[h]	-21±1.4[d]	-19.2±0.9[g]

[a]Wiser *et al*. 1996, [b]1997; [c]Tobi *et al*. 1998; [d]Wiser *et al*. 1999; [e]Fig. 1; [f]Zhong *et al*. 1999; [g]Bezprozvanny *et al*. 1995; [h]Fig. 2.

fusion of cortical vesicles in sea urchin eggs showed disruption of the SNARE coiled-coil complex by Ca^{2+}, implying that the SNARE complex primes vesicles does respond to Ca^{2+} although it is not essential at the time of fusion (Coorssen *et al*. 1998; Tahara *et al*. 1998). Furthermore, SNAREs interactions are not selective and could alter membrane fusion specificity (Bock and Scheller 1999; Yang *et al*. 1999a). Thirdly, the reconstitution of SNAREs binding to the C2A domain, the first C2 domain of synaptotagmin (Rizo and Südhof 1998), in vesicles containing anionic lipids supports yet another model. In this model the core of the SNAREs that are localized to specific subcellular compartments are not sufficient in themselves to cause membrane fusion, only synaptotagmin could trigger secretion through interaction with both membranes and SNARE complexes (Davis *et al*. 1999). These studies contradict data derived from intracellular fusion between liposome with minimal synaptic SNAREs (Parlati *et al*. 1999; Weber *et al*. 1999), and indicate difficulties regarding the reconstitution of membrane fusion. Finally, synaptic proteins and voltage-sensitive Ca^{2+} channels reconstituted in *Xenopus* oocytes demonstrated the assembly of syntaxin, SNAP-25, synaptotagmin and the Ca^{2+} channel into a functionally distinct complex named excitosome (Tobi *et al*. 1998; Wiser *et al*. 1999; Fig. 2e below). Previously, a neuroscretosome complex was reported based on immunoprecipitation studies (O'Connor *et al*. 1993) and was modeled as a secretion unit acting at the active zone (Bennett *et al*. 1997, 2000). This complex, unlike the excitosome, does not contain SNAP-25 but contains neurexin, which makes it structurally different from the excitosome. In recent years, neurexin has been identified as the α-latrotoxin receptor, which similar to CIRL acts at the plasma membrane independently of the calcium channel (Sugita *et al*. 1999). The composition of the excitosome, however, was determined functionally and the kinetic properties of the channel within the excitosome were measured, displaying distinct time constants of activation, inactivation and a unique steady-state inactivation profile (Table 1; Tobi *et al*. 1998:; Wiser *et al*. 1999). Although conceptually, a complex that acts as a secretory unit at the active zone is similar, the excitosome is structurally and functionally different from the neurosecretosome. The mechanism by which the excitosome operates is unclear but a two-way interaction between the Ca^{2+} source and the exocytotic proteins is apparent, and this could be critical to the temporal and spatial onset of the release process.

Xenopus oocytes as a reconstitution assay to study protein–protein interactions

The *Xenopus* oocyte expression system has proved particularly useful in studying membrane receptors, membrane transporters, ionic- and ligand-gated channels. Using this system it has been unequivocally established that translated proteins are targeted to their final cellular destination and maintain specificity and activity levels similar to that of native channels in intact mammalian cells. Heterogously expressed ion channels provide higher current densities than channels endogenous to oocytes, so that the background remains negligible. Changes in the well-characterized electric behavior of Ca^{2+} channels analyzed in voltage-clamped oocytes, when co-expressed with synaptic proteins, indicate a functional interaction. These facts, combined with the ease of injection into large cells, make frog oocytes the preferred choice for studying functional protein–protein interactions.

In fact, the oocytes act as 'living test tubes' for demonstrating proximity and predicting physiological activity and potential interactions of exogenously expressed proteins.

Functional interaction of the N- and Lc-type voltage-sensitive Ca^{2+} channels and the SNARE proteins syntaxin 1A and SNAP-25, reconstituted in *Xenopus* oocytes

Ca^{2+} channels are multisubunit (α1,β, $\alpha 2\delta$ and γ) membrane proteins, classified according to biophysical, pharmacological and structural characteristics (see the followowing reviews on voltage-sensitive Ca^{2+} channels: Varadi *et al*. 1995; Catterall *et al*. 1998; Walker and De Waard 1998; Meir *et al*. 1999; Randall and Benham 1999). The $Ca_v2.2$ (N-type), $Ca_v2.1$ (P/Q-type), and $Ca_v2.3$ (R-type) channels support depolarization-induced release in neuronal cells. $Ca_v1.2$ (Lc cardiac and neuronal rbC-I and rbC-II; Snutch *et al*. 1991), $Ca_v1.3$ (LD-type) support evoked release in neuroendocrine cells (e.g. epinephrine release in chromaffin cells, glutamate release in retinal bipolar cells and insulin release in pancreatic β-cells). $Ca_v1.1$ (Ls-type) channels do not support secretion. The involvement of $Ca_v3.1$ (T-type) in evoked release in the absence of a selective channel blocker, is not determined.

Over the past decade, studies aimed at characterizing the Ca^{2+} channel interaction with synaptic proteins such as syntaxin 1A and SNAP-25 have begun to increase. Syntaxin 1A plays a major role in membrane fusion and transmitter release, as determined in genetic studies using loss-of-function mutations in the *Drosophila* syntaxin 1A gene (Broadie *et al*. 1995; Schulze *et al*. 1995; Wu *et al*. 1998, 1999a) and lesions in alleles that map to sites at syntaxin in *C. elegans* (Saifee *et al*. 1998). Additional evidence was obtained from biochemical studies. Botulinum C1 specifically cleaved syntaxin 1A and inhibited exocytosis (Schiavo *et al.* 1995, 1997; Blasi *et al*. 1993; Hayashi *et al*. 1994; Marsal *et al*. 1997; O'Connor *et al*. 1997). Anti-syntaxin antibodies injected into PC12 cells impaired release (Bennett *et al*. 1993), and PC12 cells expressing reduced syntaxin 1A levels showed an increase in evoked release (Watanabe *et al*. 1999).

The association of syntaxin 1A with N-type voltage-sensitive Ca^{2+} channels was shown through co-immunoprecipitation (Saisu *et al*. 1991; Bennett *et al*. 1992; Morita *et al*. 1992). Recently, the co-localization of the fluorescent-tagged-α1D subunit and syntaxin 1A in pancreatic β-cells was reported (Yang *et al*. 1999b)

Alterations in Ca^{2+} channel activity induced by SNARE proteins were monitored using standard two-electrode voltage-clamp measurements in oocytes co-expressing various syntaxin isoforms: syntaxin 1A, 1B and syntaxin 2, together with either N- or Lc-type Ca^{2+} channels. Syntaxin 1A and 1B, although not syntaxin 2, dramatically inhibit inward N- and Lc-currents (Wiser *et al*. 1996). Syntaxin 1A decreases α1C subunit ($\alpha_1$1.2)-mediated current amplitude, in the absence of auxiliary subunits (Wiser *et al*. 1996). This result indicates the direct interaction of syntaxin 1A with the $\alpha_1$1.2, possibly at the II-III cytosolic domain. In addition syntaxin 2, like syntaxin 1A, lowered the rate of activation of Lc- and N-type channels to a similar extent (Fig. 1), whereas syntaxin 1A also reduced the rate of inactivation and modified the steady state of inactivation (Table 1; Wiser *et al*. 1996). Furthermore, syntaxin 1A shifted the steady-state inactivation curve of the N-type channel when these proteins were co-expressed in *Xenopus* oocytes, supporting an interaction with the channel that affects yet another intrinsic kinetic property of the channel (Bezprozvanny *et al*. 1995; Wiser *et al*. 1996).

Modulation of voltage-sensitive calcium channels by syntaxin 1A is characterized by the following features: (i) it requires the transmembrane domain; (ii) not only does it not diminish, but it in fact increases the number of voltage-sensitive Ca^{2+} channels in the plasma membrane; (iii) Munc-18 does not reverse its inhibitory effect (Wiser *et al*. 1999) and (iv) in addition to current amplitude it also modifies intrinsic kinetic properties of voltage-sensitive Ca^{2+} channels.

Changes in kinetic properties, such as rate of activation, rate of inactivation and steady-state inactivation, may be accounted for by interaction of the channel with syntaxin, rather than by a reduction in the number of channels.

Syntaxin 1A also modifies CFTR (Naren *et al*. 1997), amyloride-sensitive Na^+ channels (ENaC) (Qi *et al*. 1999; Saxena *et al*. 1999) glycine transporters (Geerlings *et al*. 2000) and GAT1 transporter (Deken *et al*. 2000). Similar to its interaction with voltage-sensitive Ca^{2+} channels,

syntaxin 1A is also involved in targeting these membranous proteins to the cell membrane as part of a natural process, which is crucial to their activity. In these cases, its activity is reversed by Munc-18 (n-Sec1), a cytosolic protein that specifically interacts with syntaxin 1A (Pevsner *et al*. 1994; Dresbach *et al*. 1998; Wu *et al*. 1998; Haynes *et al*. 1999). Recently, syntaxin 1A was shown to be a positive modulator of GABA transporter (GAT1). It increased GAT1 expression in the oocytes plasma membrane but acted as a negative regulator of GABA transport, reminiscent of its effects on calcium channels (Deken *et al*. 2000).

SNAP-25 modifies the kinetic properties of N- and Lc-type channels differently to syntaxin 1A: it increases the rate of inactivation of Lc-type channels without affecting current amplitude, and slightly decreases the N-channel amplitude ($< 30\%$) without affecting either its rate of activation or inactivation (Table 1; Fig. 1). However, the SNAP-25/syntaxin 1A binary complex (Chapman *et al*. 1994; Fasshauer *et al*. 1997; Vogel *et al*. 2000), generated by co-expressing the two SNAREs, abolishes the syntaxin 1A inhibitory effect on N-type current amplitude but does not affect the rate of inactivation (Table 1).

The Lc-channel on the other hand, was modified differently by the binary complex. Combined with syntaxin/SNAP-25, Lc-currents were inhibited while the rate of inactivation remained the same; suggesting that the addition of SNAP-25 reversed the inhibitory action of syntaxin 1A on the rate of inactivation, though not on current amplitude (Table 1). These results show first, that N-channels (present in neuronal cells) and Lc-channels (expressed mostly in neuroendocrine cells) are regulated by syntaxin 1A, SNAP-25 and the binary syntaxin/SNAP-25 complex. Secondly, the two channels are modulated differently by SNAREs proteins. Although the physiological function of these interactions in the release process has yet to be established, they predict juxtaposition of N- and Lc-type, Ca^{2+} channels with the release proteins. Clusters of N- or P/Q-type channels at preferential sites, namely at active zones, is well established in neuronal cells, whereas clusters of Lc-type channels at unique sites (hot spots) in neuroendocrine cells was inferred from various studies (Bokvist *et al*. 1995; Robinson *et al*. 1995; Elhamdani *et al*. 1998). The ultrafast exocytosis in retinal bipolar cells (0.5–15 ms), which uses L-channel for Ca^{2+} influx, suggests spatial and temporal requirements similar to those of neuronal cells (Mennerick and Matthews 1996; Zenisek *et al*. 2000). Likewise, Lc-type Ca^{2+} channels co-localize with release sites (13 nm) in adrenal chromaffin cells and display exocytosis at a rate (mean delay time ~3 ms) similar to the rate observed in neuronal cells (Elhamdani *et al*. 1998). Lc-channel coupling to SNAREs can be predicted from functional interactions carried out in *Xenopus* oocytes revealing Lc-channels, like N-type channels, sufficiently close to tSNAREs to allow protein–protein interaction.

Physical interaction of voltage-sensitive Ca^{2+} channels and tSNAREs proteins

The close proximity of Ca^{2+} channels with synaptic proteins was demonstrated biochemically, by co-immunoprecipitation of N- and P/Q-type channels with syntaxin 1A and synaptotagmin (Saisu *et al*. 1991; Bennett *et al*. 1992; Inou *et al*. 1992; Morita *et al*. 1992; Yoshida *et al*. 1992; Leveque *et al*. 1994; Martin-Moutot *et al*. 1996; Charvin *et al*. 1997).

A more detailed analysis of synaptic protein interaction sites at the Ca^{2+} channel was obtained using recombinant fusion-proteins. These *in vitro* assays demonstrated a specific binding of the cytosolic domain that links repeats II and III (synprint; N-loop) of the N-type channel $\alpha 1$ subunits ($\alpha_1 2.2$; aa 718–963; $N_{718-963}$) and P/Q(BI)-type ($\alpha_1 2.1a$ aa722–1036; $P/Q_{722-1036}$) channels to syntaxin 1A and SNAP-25 (Sheng *et al*. 1994, 1996). The synprint domain of $\alpha_1 2.2$ that binds to syntaxin 1A and SNAP-25 is a bell-shaped [Ca^{2+}] sensitivity curve. It does not bind when phosphorylated with PKC or CaM KII (Yokoyama *et al*. 1997). A larger sized N-loop ($N_{710-1090}$) of $\alpha_1 2.2$ binds syntaxin 1A and SNAP-25 (Wiser *et al*. 1997; Tobi *et al*. 1998). P/Q-type channels contain two distinct $\alpha_1 2.1a$ species isoforms in the rabbit brain (BI; Mori *et al*. 1991) and rat brain (rbA; Starr *et al*. 1991). Alhough a higher homology is found in the II–III domains of the two isoforms, than in the II–III domains of $\alpha_1 1.2$ and $\alpha_1 2.2$, the II–III domain of BI isoform binds both syntaxin 1A and SNAP-25, whereas the rbA form binds only SNAP-25 (Rettig *et al*. 1996). The II–III domain of the Lc-channel $\alpha 1$ subunit ($\alpha_1 1.2$; aa753–893; $Lc_{753-893}$) also binds the SNARE proteins, SNAP-25 and syntaxin 1A, displaying a ~five-fold lower affinity as compared with $N_{710-1090}$ (Wiser *et al*. 1999). In contrast, the II–III domain of the α_1 subunit of $Ca_v 1.2$ (Ls skeletal channel; $Ls_{670-800}$) does not bind synaptic proteins (Sheng *et al*. 1994). Hence, the II–III cytosolic domain of calcium channels that support evoked secretion serves as a specific interaction site with synaptic proteins.

Another key player in the fusion process is synaptobrevin/VAMPII, a v-SNARE which forms a ternary sodium dodecyl sulfate-resistant complex with syntaxin 1A and SNAP-25. Binding studies showed that VAMPII does not bind to the N-loop$_{718-963}$ (Sheng *et al*. 1996). We demonstrated that VAMPII expressed together with syntaxin 1A in *Xenopus* oocytes potentiated the inhibitory effect of syntaxin 1A (Nahon, Wiser and Atlas unpublished observations).

The *in vitro* binding studies together with specific functional N- and Lc–channel interaction with synaptic proteins that were reconstituted in *Xenopus* oocytes established a cross-talk between the Ca^{2+} channel and the SNAREs. Indeed, injection of the II–III cytosolic domain of the N-channel ($N_{718-983}$) and Lc-channel ($Lc_{753-893}$) into

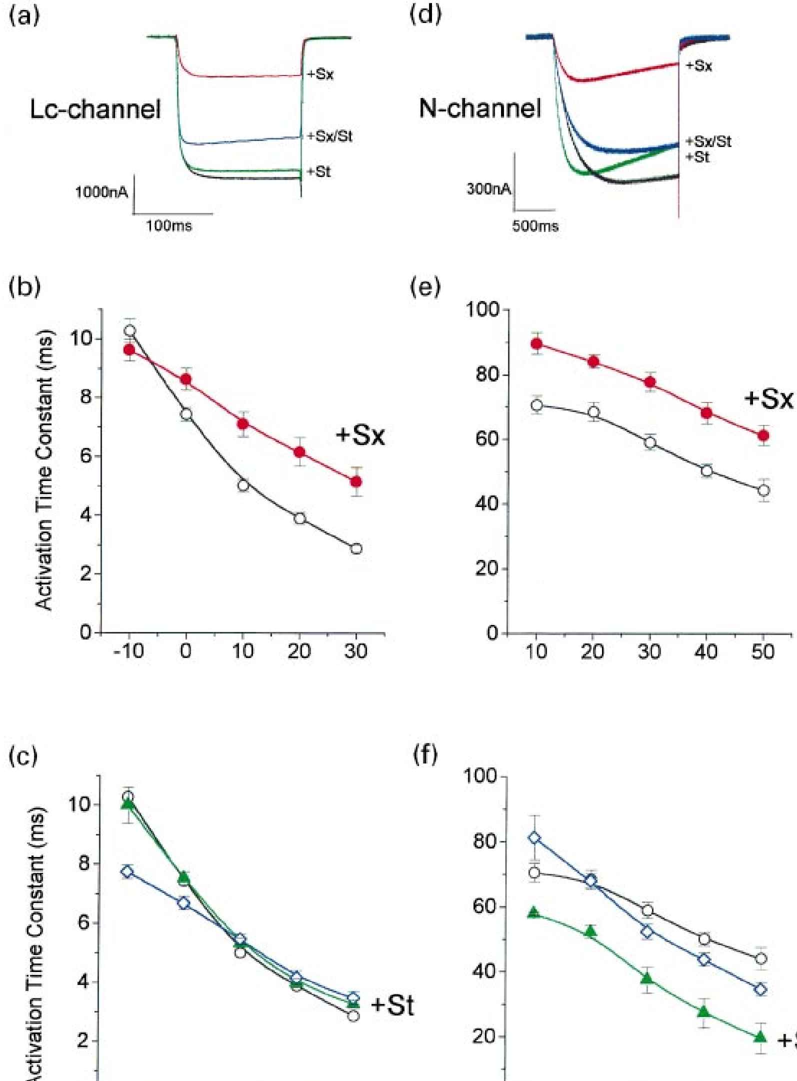

Fig. 1 The activation kinetics of N- and Lc-type Ca^{2+} channels are modified interchangeably by syntaxin and synaptotagmin. The rate of activation of α1C/α2δ/β2A and α1B/α2δ/β2A alone or in combination with syntaxin 1A (Sx, ○), synaptotagmin (St, △), and Sx combined with St (◇), were monitored in *Xenopus* oocytes ($n = 6$–15). Lc channel currents were evoked from a holding potential of ~80 mV in response to a 100-ms pulse (a, b and c) and N-type channels in response to a 1200-ms pulse for (d, e and f) to various test potentials. (a) Normalized traces of Lc- and (d) N-type channels. Time constant of activation (τact) of Lc-channel (b and c) and of N-type channel as a function of membrane potential (e and f) (see also Table 1).

secreting cells (Mochida *et al*. 1996; Rettig *et al*. 1997; Wiser *et al*. 1999) interfered with Ca^{2+}-dependent exocytosis. Synaptic transmission in $N_{718-963}$ injected SCG neurons (Mochida *et al*. 1996) and in embryonic spinal neurons of early blastomere of *Xenopus,* was reduced by approximately 25–40% at physiological [Ca^{2+}] (Rettig *et al*. 1997). Based on model-derived figures, Rettig *et al*. proposed that in peptide-injected cells, the fraction of uncoupled vesicles from the channels is larger than the measured reduction in release. Rettig *et al*. suggested that vesicles uncoupled from the channel are able to secret their content, provided Ca^{2+} influx is increased. Thus, competition between the N-peptide and the binding domain of the N-type channel causes a reduction in transmission efficiency caused by a shift to higher [Ca^{2+}] values. Likewise, the $\alpha_1$1.2-derived peptide $Lc_{753-893}$, effectively abolished depolarization-evoked secretion, which was measured as a decrease in membrane capacitance in single pancreatic β-cells (Wiser *et al*. 1999). $Lc_{753-893}$ affected neither Ca^{2+} entry nor release mediated by train of pulses. $Lc_{753-893}$, therefore, disrupts the fast release of insulin in β-cells by uncoupling the Lc-channel from release-competent vesicles (Wiser *et al*. 1999). Competion between $Lc_{753-893}$ and the endogenous channel most likely interferes with binding to syntaxin 1A or SNAP-25, synaptotagmin, or some combination of these. Evoked release by caged Ca^{2+}, in which vesicle coupling with the channel is bypassed, was not affected by the presence of $Lc_{753-893}$. It perhaps suggests different characteristics for release mediated by caged Ca^{2+} as opposed to depolarization-induced opening of Ca^{2+} channels (Oberhauser *et al*. 1995). Taken together, the results indicate that inhibition of release by $Lc_{753-893}$ is confined to a restricted population of vesicles, which are possibly the release-ready vesicles that

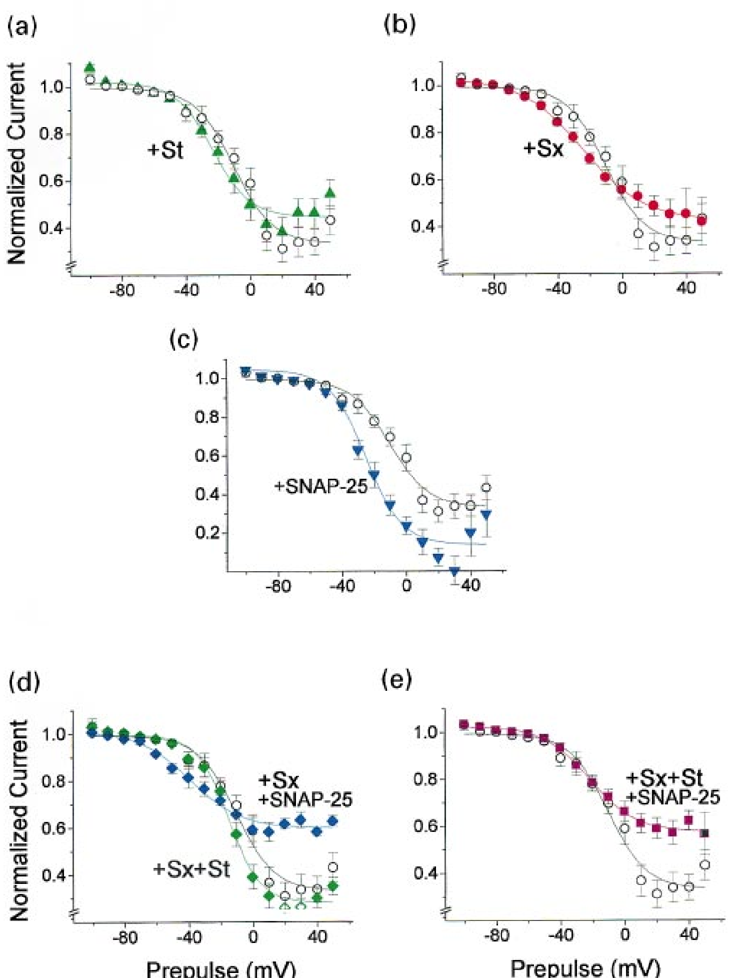

Fig. 2 Functional interaction of syntaxin, SNAP-25, synaptotagmin and combinations with Lc-type Ca^{2+} channel. Inactivation curves of steady-state normalized inward currents, generated by the Lc-channel (α1C/α2δ/β2A, ○) (a) in the presence of synaptotagmin (St, △), (b) syntaxin 1A (Sx, ○), (c) SNAP-25 (▼), and (d and e) combinations as indicated. The peak-normalized currents were fitted to Boltzman inactivation curves represented by the smooth curves. The data points correspond to the mean ± SEM of currents. The curve profiles show that the channel is affected by the combination of the three synaptic proteins differently than by the individual, or the binary combinations, and support the existence of the excitosome complex.

associate with the channel in a putative primed ready-to-fuse complex (e.g. excitosome; Wiser *et al*. 1999; see below). These results confirm previous studies categorizing vesicles in the cell according to distance from the voltage-sensitive Ca^{2+} channels (Moser and Neher 1997; Voets *et al*. 1999).

Physical and functional interaction of synaptotagmin with voltage-sensitive Ca^{2+} channels

How do Ca^{2+} channels, SNAREs and synaptotagmin cooperate in the sequential events leading to vesicle fusion?

Synaptotagmins constitute a family of Ca^{2+}-binding proteins that are not present in the yeast genome. Synaptotagmin is composed of an N-terminal domain anchored at the vesicular membrane and two homologous C2 domains, C2A and C2B, the 3D structure of which was recently reported (Rizo and Südhof 1998; Sutton *et al*. 1998, 1999). Its importance in cellular exocytosis has been established genetically and biochemically over the past few years (Geppert *et al*. 1994; Littleton *et al*. 1994; Thomas and Elferink 1998). The unique Ca^{2+} binding site within the C2A domain and the Ca^{2+}-dependent binding to phospholipid, strongly support a major role for synaptotagmin in exocytosis (Davletov and Südhof 1994).

In vitro, synaptotagmin interacts with several proteins that are involved in exocytosis and endocytosis. Recombinant synaptotagmin binds syntaxin 1A at the C2A domain; this binding is strongly dependent on Ca^{2+} (K_D~200 μM; Chapman *et al*. 1995; Li *et al*. 1995; Kee and Scheller 1996), whereas synaptotagmin binding to SNAP-25 is Ca^{2+} independent and involves the C2B domain (Schiavo *et al*. 1997).

Synaptotagmin, like syntaxin 1A and SNAP-25, binds to the II–III cytosolic domain of N-, P/Q- and Lc-type Ca^{2+} channels (Charvin *et al*. 1997; Sheng *et al*. 1997; Wiser *et al*. 1997, 1999; Tobi *et al*. 1998). The binding either of the full-length synaptotagmin C2A or C2B domains to

$N_{710-1090}$ is Ca^{2+}-independent (Wiser *et al*. 1997; Tobi *et al*. 1998). A shorter version, $N_{718-963}$, binds to C2B, but not to the C2A domain (Sheng *et al*. 1997). Inositol polyphosphate (IP_6) decreases the $N_{710-1090}$ binding to full-length synaptotagmin and to the C2B domain ($IC_{50} = 0.6$ and 3 μM, respectively; Fukuda *et al*. 1995), while hardly affecting C2A binding at all ($IC_{50} > 30$ μM; Tobi *et al*. 1998). Distinct affinities of various α1 subunits to synaptotagmin, as well as differences in Ca^{2+} dependency and sensitivity to inositol polyphosphates, could be relevant to Ca^{2+}-regulated processes within the cell (e.g. evoked secretion).

Gel overlay experiments revealed binding of synaptotagmin I to a single region (aa780–969) within the II–III loop of the P/Q-type channel (Charvin *et al*. 1997). Interestingly, the II–III domain of $\alpha_1$2.1a, the α1 subunit of the rat brain isoform of P/Q-type channel, binds synaptotagmin in a Ca^{2+}-dependent manner, with maximum affinity at 10–20 μM Ca^{2+} (Kim and Catterall 1997). Meanwhile, the same domain of the rabbit-brain splice variant, interacts specifically with synaptotagmin and C2B in a Ca^{2+}-independent manner (Rettig *et al*. 1996).

The Lc-type channel binds synaptotagmin and either of its C2 domains, as shown with the recombinant II–III loop, $Lc_{753-893}$ (Wiser *et al*. 1999). In competition experiments $Lc_{753-893}$ displayed a ∼ 10-fold lower affinity for synaptotagmin as compared with $N_{710-1090}$, the intracellular loop of the N-type channel (Wiser *et al*. 1999). $Lc_{753-893}$ interaction with synaptotagmin was also confirmed in 'pull-down' experiments using GST-$Lc_{753-893}$ (Wiser *et al*. 1999). A physical coupling between synaptotagmin, N-, P/Q- and Lc-type channels provide biochemical evidence to support the intimacy of Ca^{2+} channel and synaptic vesicles, as predicted from the unique organization of active zones and hot spots in neuronal and neuroendocrine cells, respectively.

Additional support for such conclusions was found in functional assay. Co-expression of synaptotagmin with N- and Lc-type channels in *Xenopus* oocytes, showed no significant change in current amplitude or rate of inactivation, with minor alterations in the steady-state of inactivation of both N- and Lc-channels (Table 1; Fig. 2). However, the rate of activation of the N-channel, though not the Lc-channel, was increased considerably by synaptotagmin, as demonstrated by the decrease in the time constant of activation (from $\tau act = 59 \pm 2$ to 37 ± 4 ms; Table 1; Fig. 1).

If applied to secretory cells, could this interaction imply tethering (or docking) of a synaptic vesicle to the channel?

The docking of vesicles to the plasma membranes through the N-type Ca^{2+} channel induces acceleration of the activation kinetics. This change occurs during vesicle priming and represents a new state of the channel that distinguishes vesicle-coupled from vesicle uncoupled channels (Fig. 3). When the channel is coupled to a vesicle, it expresses maximal current amplitude and faster activation kinetics, compared with a syntaxin-coupled channel. A docking role for synaptotagmin was demonstrated by ultrastructural analysis of synaptotagmin mutants. These synaptotagmin mutants revealed a significant reduction in the number of vesicles adjacent to the plasma membrane in the active zone (Reist *et al*. 1998).

Could synaptotagmin modify the channel indirectly, via syntaxin?

The N- and Lc-Ca^{2+} current amplitudes are reduced by syntaxin 1A, whereas the addition of synaptotagmin causes restoration of current amplitude and the rate of activation (Tobi *et al*. 1998; Wiser *et al*. 1999). Similarly, the inhibited rate of activation of the two-type channels returned to normal rates by synaptotagmin (Wiser *et al*. 1999; Fig. 2). Reversal of syntaxin 1A inhibition by synaptotagmin was highly dependent on the degree of inhibition. Syntaxin 1A and synaptotagmin appear to coordinate a delicate balance of inward current amplitudes and activation kinetics in N- and Lc-type channels (Wiser *et al*. 1999; Fig. 1). Synaptotagmin and syntaxin 1A could compete with each other to interact with the channel, alternatively a synaptotagmin/syntaxin 1A complex could affect the channel differently. The changes in steady-state inactivation strongly argues for a putative syntaxin/synaptotagmin complex interaction with the channel (Wiser *et al*. 1999; Fig. 2e). Similarly, the negative shift in the steady-state inactivation kinetics of P/Q-type channel by SNAP-25 in HEK293 cells was reversed by co-expressing syntaxin 1A and synaptotagmin (Zhong *et al*. 1999). Recently, a Ca^{2+}-dependent assembly of synaptotagmin onto the base of the ternary SNARE complex within the H3/transmembrane domain of syntaxin, was reported (Davies *et al*. 1999). Together, these results support formation of syntaxin/synaptotagmin complex. The relevance of these interactions to secretion is illustrated when the delicate balance between synaptotagmin and syntaxin (Tobi *et al*. 1998; Wiser *et al*. 1999) or SNAP-25 and syntaxin (Zhong *et al*. 1999) is tilted. For example, overexpression of syntaxin 1A reduces transmitter release (Wu *et al*. 1998), whereas lowering syntaxin level in PC12 cells has been shown to increase transmitter release (Watanabe *et al*. 1999). These results support an inhibitory action of syntaxin on exocytosis via the calcium channel.

A detailed analysis of the steady-state inactivation profiles of N- and L-type Ca^{2+} channels is consistent with a model in which the channel simultaneously interacts with syntaxin, SNAP-25 and synaptotagmin to generate the excitosome complex (Tobi *et al*. 1998; Wiser *et al*. 1999). Within the

© 2001 International Society for Neurochemistry,

excitosome complex, a vesicle is tethered to the channel directly or indirectly, via syntaxin 1A (Fig. 3). The channel then expresses maximal current amplitude and maximal rate of activation; a state that would help promote an efficient ready-to-fuse state (Figs 2 and 3). Possibly, in neuronal or neuroendocrine cells, a vesicle associated with syntaxin, SNAP-25 and the channel would be primed to fuse upon the arrival of a signal, unlike a channel associated with either one of these proteins. These changes maintain an integral role for the channel in the exocytotic process, where synaptotagmin interacts with syntaxin 1A and SNAP-25 generation: a metastable state in the form of the excitosome complex. At this metastable-state Ca^{2+} entry implement a rapidly triggered exocytosis.

There is no direct evidence that different channel populations associated either with syntaxin 1A and/or SNAP-25 and/or synaptotagmin really exist in nerve terminals. However, the abundance of syntaxin 1A (1% membrane protein) and syntaxin/SNAP-25 binary complex (Vogel *et al*. 2000) compared with the low density of Ca^{2+} channels at the plasmalemma would indirectly support the probability that the channel is assembled into complexes with synaptic proteins. Furthermore, at the active zone, where a large number of vesicles are packed close to clusters of calcium channels, a physical association of synaptotagmin with the channel is almost inevitable. Can we predict the exact sequence of events that are thought to occur between docking and fusion, and in what order do the various synaptic proteins assemble with the calcium channel? Based on the kinetic properties of the calcium channel obtained in the reconstitution experiments, it is tempting to draw up a tentative order of events; such an order is schematically demonstrated in Fig. 3. Outside the active zone (or hot spots), the channel is inhibited because it is associated with syntaxin. At the active zone, the channel could be combined either with syntaxin/SNAP-25 or with syntaxin 1A and p65. Within these two complexes, the channel is in a partially open conformation ('primed'), but assumes a conformation that could lead to fusion if associated with all three proteins together. When the delicate balance between synaptotagmin and syntaxin (Tobi *et al*. 1998; Wiser *et al*. 1999) or SNAP-25 and syntaxin (Zhong *et al*. 1999) is tilted, transmitter release is significantly affected. For example, overexpression of syntaxin 1A reduces transmitter release (Wu *et al*. 1998), whereas lowering syntaxin level in PC12 cells has been shown to increase transmitter release (Watanabe *et al*. 1999). These results support an inhibitory action of syntaxin on exocytosis via the calcium channel.

Can these results be extended to explain differences between secretion in neuronal and neuroendocrine cells?
A decline in the effective $[Ca^{2+}]$ at the release site is expected when the distance between the Ca^{2+} source and the exocytotic machinery is increased. Does a slower release imply that the Lc-channel, as opposed to the N-channel, is far removed from the exocytotic machinery? Are the channels clustered differently at the release site? Do the channels interact differently with synaptic proteins?

In neuroendocrine cells, secretion takes place from at least two distinct types of secretory vesicles, large dense core vesicles (LDCVs) and small clear synaptic vesicles (SSVs). In both chromaffin and PC12 cells, acetylcholine packages into SSV, whereas monoamines, such as dopamine and epinephrine, are preferentially stored in LDCVs.

Presynaptic terminals contain, in addition to synaptic vesicles, dense-core secretory granules for the secretion of hormones and peptides. The LDCVs of neuroendocrine cells release with a high Ca^{2+} affinity (~190 μM for bipolar cells; 10–20 μM for adrenal chromaffin and pituitary cells) similar to the LDCVs of dorsal root ganglion cells (3–10 μM; Huang and Neher 1996). In neuronal cells, a brief increase of $[Ca^{2+}]_i$ up to 10 μM was shown to be sufficient to elicit transmitter release from synaptic vesicle in crayfish release bouton (Ravin *et al*. 1999), brainstem synapse (Bollmann *et al*. 2000) and in synaptic terminals of calyx of Held (Schneggenburger and Neher 2000). Hence, the Ca^{2+} concentration for half-maximal activation of exocytosis in nerve terminals and endocrine cells appears to be similar. The differences that lie in the timing of synaptic release vs LDCVs release could be related either to multiple calcium sensors (Burgoyne and Morgan 1998) and/or changes in the interaction of Ca^{2+} channels with the calcium sensor, as predicted by Ca^{2+} channel modulation by synaptotagmin (see above and Wiser *et al*. 1997, 1999; Tobi *et al*. 1998) or differences in the organization of the channels *vis-á-vis* the synaptic proteins. Furthermore, reconstitution experiments of calcium channels and synaptic proteins in *Xenopus* oocytes showed that modulations of the kinetic properties of N- and L-channels by syntaxin/SNAP-25 binary complexes are not the same. Inhibition of the N-channel by syntaxin 1A is partly reversed by syntaxin/SNAP-25 binary complex (Wiser *et al*. 1996). In contrast, Lc-current remained inhibited by syntaxin/SNAP complex (Wiser *et al*. 1997, 1999; Tobi *et al*. 1998). Hence, N-channel, although not the Lc-channel, appears to harbor an intermediate step that facilitates removal of the syntaxin 1A inhibitory action. This difference could partially explain faster exocytosis in neuronal cells and the need for a stronger stimuli through Lc-channel to elicit secretion in neuroendocrine cells (facilitation channels: Artalejo *et al*. 1994; Elhamdani *et al*. 1998; see also Borst and Sakmann 1999; Wu *et al*. 1999b). Overall, despite differences in protein–protein interaction of SNAP-25, syntaxin 1A and synaptotagmin with the Lc- and N-type channels, the mechanism of release in neuronal and neuroendocrine cells are likely to be fundamentally very similar.

Conclusions

The hypothetical sequence of events leading to exocytosis presented above is largely based on functional interactions between synaptic proteins and voltage-sensitive Ca^{2+} channels. Syntaxin, SNAP-25 and synaptotagmin alone, or in various combinations, variously modify the kinetic properties of N- and Lc-Ca^{2+} channels. Outside the active zone, the probability of having a channel associated with syntaxin 1A only is much greater than a channel associated with both syntaxin 1A and synaptotagmin, thus keeping Ca^{2+} channels silent. At the active zone, the channel is associated with the SNAREs and the vesicle (via synaptotagmin). The channel-docked vesicles are likely to undergo fusion as the channel is in a conformation that yields larger currents and a faster activation rate, and more particularly brings syntaxin 1A and synaptotagmin together to facilitate release. In response to a depolarization signal, followed by Ca^{2+} influx, a rapid conformational change in the excitosome complex would trigger fusion of vesicles tethered to the channel ('release-ready'). The excitosome could provide perfect timing for a Ca^{2+}-dependent vesicle fusion as it accommodates both the calcium channel and the docked vesicle, whereas the rate of release of vesicles that are not coupled to the channel within the excitosome complex is predicted to be slower.

There appears to be a twofold regulation of transmitter release by syntaxin, SNAP-25 and synaptotagmin: first, these proteins act directly on channel kinetics during transient signals and second, they act as a rate-limiting step during sustained depolarization as soon as new excitosome complexes are generated. The two approaches of protein expression in oocytes and *in vitro* binding of recombinant proteins proved to be useful in studying the synaptic protein modulation of Ca^{2+} channel activity. The observed changes are consistent with modulation of secretion in secreting cells. This has led to a better understanding of the molecular aspects of Ca^{2+}-mediated synaptic transmission.

Fig. 3 Assembly of calcium channel with synaptic proteins: implication to vesicle fusion. The calcium channel is postulated to assemble with synaptic proteins to generate three putative complexes. (i) The 'rest' state represents a channel/syntaxin 1A complex. Current amplitude is inhibited and the rate of activation is slow (Fig. 1). (ii) The 'primed'-state represents a channel/synatxin/SNAP-25 or channel/syntaxin/synaptotagmin (p65) complexes. The channel kinetics within these complexes varies. It displays either faster kinetics and slow inactivation or faster kinetics and faster activation than in 'rest' state (see Table 1; Fig. 1). (iii) The 'ready' state represents the excitosome complex, channel/syntaxin/SNAP-25/synaptotagmin. Within the excitosome complex, the channel conducts currents with maximal amplitude and faster rate of activation in comparison with the 'rest' state.

Acknowledgements

I thank Dr Ofer Wiser, Dr Michael Trus and Sharon Simmer for constructive comments and suggestions and This work was supported in part by a grant from The H. L. Lauterbach Fund and the J. J. Berreby Fund.

References

Adler E. M. Augustine G. J., Duffy S. N. and Charlton M. P. (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *J. Neurosci.* **11**, 1496–1507.

Artalejo C. R., Adams M. E. and Fox A. P. (1994) Three types of Ca2+ channel trigger secretion with different efficacies in chromaffin cells. *Nature* **367**, 72–76.

Avery J., Jahn R. and Edwardson J. M. (1999) Reconstitution of regulated exocytosis in cell-free systems: a critical appraisal. *Annu. Rev. Physiol.* **61**, 777–807.

Bajjalieh S. M. (1999) Synaptic vesicle docking and fusion. *Curr. Opin. Neurobiol.* **9**, 321–328.

Bennett M. K. (1997) Ca^{2+} and the regulation of neurotransmitter secretion. *Curr. Opin. Neurobiol.* **7**, 316–322.

Bennett M. K., Calakos N. and Scheller R. H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**, 255–260.

Bennett M. K., Garcia-Arras J. E., Elferink L. A., Peterson K., Fleming A. M., Hazuka C. D. and Scheller R. H. (1993) The syntaxin family of vesicular transport receptors. *Cell* **74**, 863–873.

Bennett M. R., Gibson W. G. and Robinson J. (1997) Probabilistic secretion of quanta and the synaptosecretosome hypothesis: evoked release at active zones of varicosities, boutons, and endplates. *Biophys. J.* **73**, 1815–1829.

Bennett M. R., Farnell L. and Gibson W. G. (2000) The probability of quantal secretion near a single calcium channel of an active zone. *Biophys. J.* **78**, 2201–2221.

Bezprozvanny I., Scheller R. H. and Tsien R. W. (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* **378**, 623–626.

Blasi J., Chapman E. R., Yamasaki S., Binz T., Niemann H. and Jahn R. (1993) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.* **12**, 4821–4828.

Bock J. B. and Scheller R. H. (1999) SNARE proteins mediate lipid bilayer fusion. *Proc. Natl Acad. Sci. USA* **96**, 12227–12229.

Bokvist K., Eliasson L., Ammala C., Renström E. and Rorsman P. (1995) Co-localization of L-type Ca^{2+} channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic B-cells. *EMBO J.* **14**, 50–57.

Bollmann J. H., Sakmann B. and Borst J. G. (2000) Calcium sensitivity of glutamate release in a calyx-typeterminal. *Science* **289**, 953–957.

Borst J. G. and Sakmann B. (1999) Effect of changes in action potential shape on calcium currents andtransmitter release in a calyx-type synapse of the rat auditory brainstem. *Philos Trans R Soc. Lond. B. Biol. Sci.* **354**, 347–355.

Broadie K., Prokop A., Bellen J. H., O'Kane C. J., Schulze K. L. and Sweeney S. T. (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila*. *Neuron* **15**, 663–673.

Burgoyne R. D. and Morgan A. (1998) Calcium sensors in regulated exocytosis. *Cell Calcium.* **24**, 367–376.

Burgoyne R. D., Morgan A., Robinson I., Pender N. and Cheek T. R. (1993) Exocytosis in adrenal chromaffin cells. *J. Anat.* **183**, 309–314.

Catterall W. A. (1998) Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. *Cell Calcium.* **24**, 307–323.

Chapman E. R., An S., Barton A. S. and Jahn R. (1994) SNAP-25, a t-SNARE which bind to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.* **269**, 27427–27432.

Chapman E. R., Hanson P. I., An S. and Jahn R. (1995) Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. *J. Biol. Chem.* **270**, 23667–23671.

Charvin N., Leveque C., Walker D., Berton F., Raymond C., Kataoka M., Shoji-Kasai Y., Takahashi M., De Waard M. and Seagar M. J. (1997) Direct interaction of the calcium sensor protein synaptotagmin I with a cytoplasmic domain of the alpha1A subunit of the P/Q-type calcium channel. *EMBO J.* **16**, 4591–4596.

Chen Y. A., Scales S. J., Patel S. M., Doung Y. C. and Scheller R. H. (1999) SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. *Cell* **97**, 165–174.

Coorssen J. R., Blank P. S., Tahara M. and Zimmerberg J. (1998) Biochemical and functional studies of cortical vesicle fusion: the SNARE complex and Ca^{2+} sensitivity. *J. Cell Biol.* **143**, 1845–1857.

Cuchillo-Ibanez I., Michelena P., Albillos A. and Garcia A. G. (1999) A preferential pole for exocytosis in cultured chromaffin cells revealed by confocal microscopy. *FEBS Lett.* **459**, 22–26.

Davis A. F., Bai J., Fasshauer D., Wolowick M. J., Lewis J. L. and Chapman E. R. (1999) Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes. *Neuron* **24**, 363–376.

Davletov B. A. and Südhof T. C. (1994) Ca^{2+}-dependent conformational change in synaptotagmin I. *J. Biol. Chem.* **269**, 28547–28550.

Deken S. L., Beckman M. L., Boos L. and Quick M. W. (2000) Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat. Neurosci.* **3**, 998–1003.

Dodge F. A. and Rahamimoff R. (1967) Co-operative action calcium ions in transmitter release at the neuromuscular junction. *J. Physiol. (Lond.)* **193**, 419–432.

Dresbach T., Burns M. E., O'Connor V., DeBello W. M., Betz H. and Augustine G. J. (1998) A neuronal Sec1 homolog regulates neurotransmitter release at the squid giant synapse. *J. Neurosci* **18**, 2923–2932.

Edwardson J. M. (1998) A cell-free system for Ca^{2+} regulated exocytosis. *Meth Enzymol.* **16**, 209–214.

Elhamdani A., Zhou Z. and Artalejo C. R. (1998) Timing of dense-core vesicle exocytosis depends on the facilitation L-type Ca channel in adrenal chromaffin cells. *J. Neurosci.* **18**, 6230–6240.

Fasshauer D., Bruns D., Shen B., Jahn R. and Brünger A. T. (1997) A structural change occurs upon binding of syntaxin to SNAP-25. *J. Biol. Chem.* **272**, 4582–4590.

Fasshauer D., Eliason W. K., Brünger A. T. and Jahn R. (1998) Identification of minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. *Biochem.* **37**, 10354–10362.

Finnegan J. M., Pihel K., Cahill P. S., Huang L., Zerby S. E., Ewing A. G., Kennedy R. T. and Wightman R. M. (1996) Vesicular quantal size measured by amperometry at chromaffin, mast, pheochromocytoma, and pancreatic beta-cells. *J. Neurochem.* **66**, 1914–1923.

Fossier P., Tauc L. and Baux G. (1999) Calcium transients and neurotransmitter release at an identified synapse. *Trends Neurosci.* **22**, 161–166.

Fukuda M., Moreira J. E., Lewis F. M., Sugimori M., Niinobe M., Mikoshiba K. and Llinas R. (1995) Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal. *Proc. Natl Acad. Sci. USA* **92**, 10708–10712.

Geerlings A., Lopez-Corcuera B. and Aragon C. (2000) Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. *FEBS Lett.* **470**, 51–54.

Geppert M., Goda Y., Hammer R. E., Li C., Rosahl T. W., Stevens C. F. and Südhof T. C. (1994) Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. *Cell* **79**, 717–727.

Goda Y. (1997) SNAREs and regulated vesicle exocytosis. *Proc. Natl Acad. Sci. USA* **94**, 769–772.

Hanson P. I., HouseJ. E. and Jahn R. (1997) Neurotransmitter release-four years of SNAREs complexes. *Curr. Opin. Neurobiol.* **7**, 310–315.

Hayashi T., McMahon H., Yamasaki S., Binz T., Hata Y., Südhof T. S. and Niemann H. (1994) Synaptic membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* **13**, 5051–5061.

Haynes L. P., Morgan A. and Burgoyne R. D. (1999) nSec-1 (munc-18) interacts with both primed and unprimed syntaxin 1A and associates in a dimeric complex on adrenal chromaffin granules. *Biochem. J.* **342**, 707–714.

Heuser J. E. and Reese T. S. (1973) Evidence for recycling of synaptic vesicle membranes during transmitter release at the frog neuromuscular junction after transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **57**, 315–344.

Huang L., Y. and Neher E. (1996) Ca (2+)-dependent exocytosis in the somata of dorsal root ganglion neurons. *Neuron* **1**, 35–145.

Inoue A., Obata K. and Akagawa K. (1992) Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. *J. Biol. Chem.* **267**, 10613–10619.

Kee Y. and Scheller R. H. (1996) Localization of synaptotagmin-binding domains on syntaxin. *J. Neurosci* **16**, 1975–1981.

Kim D. K. and Catterall W. A. (1997) Ca^{2+}-dependent and –independent interactions of the isoforms of the alpha1A subunit of brain Ca2+ channels with presynaptic SNARE proteins. *Proc. Natl Acad. Sci. USA* **94**, 14782–14786.

Leveque C., el Far O., Martin-Moutot N., Sato K., Kato R., Takahashi M. and Seagar M. J. (1994) Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. *J. Biol. Chem.* **269**, 6306–6312.

Li C., Ullrich J. Z., Anderson R. G. W., Brose N. and Südhof T. C. (1995) Ca^{2+} dependent and independent activities of neuronal and non neuronal synaptotagmines. *Nature* **375**, 594–599.

Lin R. C. and Scheller R. H. (1997) Structural organization of the synaptic exocytosis core complex. *Neuron* **19**, 1087–1094.

Littleton J. T., Stern M., Perin M. and Bellen H. J. (1994) Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. *Proc. Natl Acad. Sci. USA* **91**, 10888–10892.

Llinas R., Sugimori M. and Silver R. B. (1992) Microdomains of high calcium concentration in a presynaptic terminal. *Science* **256**, 677–679.

Macleod G. T., Gan J. and Bennett M. R. (1999) Vesicle-associated proteins and quantal release at single active zones of amphibian (Bufo marinus) motor-nerve terminals. *J. Neurophysiol.* **82**, 1133–1146.

Margittai M., Otto H. and Jahn R. (1999) A stable interaction between syntaxin 1a and synaptobrevin 2 mediated by their transmembrane domains. *FEBS Lett* **446**, 40–44.

Marsal J., Ruiz-Montasell B., Blasi J., Moreira J. E., Contreras D., Sugimori M. and Llinas R. (1997) Block of transmitter release by botulinum C1 action on syntaxin at the squid giant synapse. *Proc. Natl Acad. Sci. USA* **94**, 14871–14876.

Martin-Moutot N., Charvin N., Leveque C., Sato K., Nishiki T., Kozaki S., Takahashi M. and Seagar M. J. (1996) Interaction of SNARE complexes with P/Q-type calcium channels in rat cerebellar synaptosomes. *J. Biol. Chem.* **271**, 6567–6570.

Meir A., Ginsburg S., Butkevich A., Kachalsky S. G., Kaiserman I., Ahdut R., Demirgoren S. and Rahamimoff R. (1999) Ion channels in presynaptic nerve terminals and control of transmitter release. *Physiol. Rev.* **79**, 1019–1088.

Mennerick S. and Matthews G. (1996) Ultrafast exocytosis elicited by calcium current in synaptic terminalsof retinal bipolar neurons. *Neuron* **17**, 1241–1249.

Mochida S., Sheng Zu. H., Baker C., Kobayashi H. and Catterall W. A. (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of the N-type Ca^{2+} channels. *Neuron* **17**, 781–788.

Mori Y., Friedrich T., Kim M. S., Mikami A., Nakai J., Ruth P., Bosse E., Hofmann F., Flockerzi V., Furuichi T., .Mikoshiba K., Imoto K., Tanabe T. and Numa S. (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* **350**, 398–402.

Morita T., Mori H., Sakimura K., Mishina M., Sekine Y., Tsugita A., Odani S., Horikawa H. P. M., Saisu H. and Abe T. (1992) Synaptocalin I, a protein associated with brain ω-conotoxin-sensitive calcium channels. *Biomed. Res.* **13**, 357–362.

Moser T. and Neher E. (1997) Rapid exocytosis in single chromaffin cells recorded from mouse adrenal slices. *J. Neurosci.* **17**, 2314–2323.

Naren A. P., Nelson D. J., Xie W., Jovov B., Pevsner J., Bennett M. K., Benos D. J., Quick M. W. and Kirk K. L. (1997) Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature* **390**, 302–305.

Nickel W., Weber T., McNew J. A., Parlati F., Söllner T. H. and Rothman J. E. (1999) Content mixing and membrane integrity during membrane fusion driven by pairing of isolated v-SNAREs and t-SNAREs. *Proc. Natl Acad. Sci. USA* **96**, 12571–12576.

Oberhauser A. F., Robinson I. M. and Fernandez J. M. (1995) Do caged-Ca2+ compounds mimic the physiological stimulus for secretion? *J. Physiol. (Paris)* **89**, 71–75.

O'Connor V. M., Shamotienko O., Grishin E. and Betz H. (1993) On the structure of the 'synaptosecretosome'. Evidence for aneurexin/synaptotagmin/syntaxin/Ca2+ channel complex. *FEBS Lett.* **326**, 255–260.

O'Connor V., Heuss C., De Bello W. M., Dresbach T., Charlton M. P., Hunt J. H., Pellegrini L. L., Hodel A., Burger M. M. and Betz H. Augustine G. J. and Schafer T. (1997) Disruption of syntaxin–mediated protein interactions blocks neurotransmitter secretion. *Proc. Natl Acad. Sci. USA* **94**, 12186–12191.

Parlati F., Weber T., McNew J. A., Westermann B., Söllner T. H. and Rothman J. E. (1999) Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain. *Proc. Natl Acad. Sci. USA* **96**, 12565–12570.

Pevsner J., Hsu S., Braun J. E. A., Calakos N., Ting E., Bennett M. K. and Scheller R. H. (1994) Specificity and regulation of a synaptic vesicle docking complex. *Neuron* **13**, 353–361.

Poirier M. A., Hao J. C., Malkus P. N., Chan C., Moore M. F., King D. S. and Bennett M. K. (1998a) Protease resistance of syntaxin SNAP-25 VAMP complexes. Implication for assembly and structure. *J. Biol. Chem* **273**, 11370–11377.

Poirier M. A., Xiao W., Macosko J. C., Chan C., Shin Y. K. and Bennett M. K. (1998b) The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Biol.* **5**, 765–769.

Qi J., Peters K. W., Liu C., Wang J. M., Edinger R. S., Johnson J. P., Watkins S. C. and Frizzell R. A. (1999) Regulation of the amiloride-sensitive epithelial sodium channel by syntaxin 1A. *J. Biol. Chem.* **274**, 30345–30348.

Randall A. and Benham C. D. (1999) Recent advances in the molecular understanding of voltage-gatedCa^{2+} channels. *Mol. Cell Neurosci.* **4**, 255–272.

Ravin R., Parnas H., Spira M. E., Volfovsky N. and Parnas I. (1999) Simultaneous measurement of evoked release and $[Ca^{2+}]_i$ in a crayfishrelease bouton reveals high affinity of release to Ca^{2+}. *J. Neurophysiol.* **81**, 634–642.

Reist N. E., Buchanan J., Li J., DiAntonio A., Buxton E. M. and Schwarz. T. L. (1998) Morphologically docked synaptic vesicles are reduced in synaptotagmin mutants of *Drosophila*. *J. Neurosci.* **18**, 7662–7673.

Rettig J., Sheng Z. H., Kim D. K., Hodson C. D., Snutch T. P. and Catterall W. A. (1996) Isoform–specific interaction of the alpha1A subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. *Proc. Natl Acad. Sci. USA* **93**, 7363–7368.

Rettig J., Heinemann C., Asheri U., Sheng Zu H., Yokoyama C. T., Catterall W. A. and Neher E. (1997) Alteration of Ca^{2+} dependence of neurotransmitter release by disruption of Ca^{2+} channel/syntaxin interaction. *J. Neurosci* **17**, 6647–6656.

Rizo J. and Südhof T. C. (1998) C2-domains, structure and function of a universal Ca^{2+}-binding domain. *J. Biol. Chem.* **273**, 15879–15882.

Robinson I. M., Finnegan J. M., Monck J. R., Wightman R. M. and Fernandez J. M. (1995) Colocalization of calcium entry and exocytotic release sites in adrenal chromaffin cells. *Proc. Natl Acad. Sci. USA* **92**, 2474–2478.

Robinson I. M., Yamada M., Carrion-Vazquez M., Lennon V. A. and Fernandez J. M. (1996) Specialized release zones in chromaffin cells examined with pulsed-laser imaging. *Cell Calcium* **20**, 181–201.

Saifee O., Wei L. and Nonet M. (1998) The *Caenorhabditis elegans unc*-64 locus encodes a syntaxin that interacts genetically with synaptobrevin. *Mol Biol. Cell* **9**, 1235–1252.

Saisu H., Ibaraki K., Yamaguchi T., Sekine Y. and Abe T. (1991) Monoclonal antibodies immunoprecipitating ω-conotoxin-sensitive calcium channel molecules recognize two novel proteins localized in the nervous system. *Biochem. Biophys. Res. Commun.* **181**, 346–350.

Sakaba T., Tachibana M., Matsui K. and Minami N. (1997) Two components of transmitter release in retinal bipolar cells: exocytosis and mobilization of synaptic vesicles. *Neurosci Res.* **27**, 357–370.

Saxena S., Quick M. W., Tousson A., Oh Y. and Warnock D. G. (1999) Interaction of syntaxins with the amiloride-sensitive epithelial sodium channel. *J. Biol. Chem.* **274**, 20812–20817.

Schiavo G., Shone C. C., Bennett M. K., Scheller R. H. and Montecucco C. (1995) Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem* **270**, 10566–10570.

Schiavo G., Stenbeck G., Rothman J. E. and Söllner T. H. (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc. Natl Acad. Sci. USA* **94**, 997–1001.

Schneggenburger R. and Neher E. (2000) Intracellular calcium dependence of transmitter release rates at a fast central synapse. *Nature* **406**, 889–893.

Schroeder T. J., Jankowski J. A., Senyshyn J., Holz R. W. and Wightman R. M. (1994) Zones of exocytotic release on bovine adrenal medullary cells in culture. *J. Biol. Chem.* **269**, 17215–17220.

Schulze K. L., Broadie K., Perin M. S. and Bellen H. J. (1995) Genetic and electrophysiologic studies of *Drosophila* syntaxin 1A demonstrates its role in nonneuronal secretion and neurotransmission. *Cell* **80**, 311–320.

Sheng Zu H., Rettig J., Takahashi M. and Catterall W. A. (1994) Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303–1313.

Sheng Zu H., Rettig J., Cook T. and Catterall W. A. (1996) Calcium–dependent interaction of N-type calcium channels with the synaptic core complex. *Nature* **379**, 451–454.

Sheng Zu H., Yokoyama C. T. and Catterall W. A. (1997) Interaction of the synprint site of N-type Ca^{2+} channels with the C2B domain of synaptotagmin I. *Proc. Natl Acad. Sci. USA* **94**, 5405–5410.

Snutch T. P., Tomlinson W. J., Leonard J. P. and Gilbert M. M. (1991) Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. *Neuron* **7**, 45–57.

Söllner T., Bennett M. K., Whiteheart S. W., Scheller R. H. and Rothman J. E. (1993) A protein assemblyñdisassembly pathway in vitro that may correspond to sequential steps of synaptic vesicles docking, activation and fusion. *Cell* **75**, 409–418.

Stanley E. F. (1993) Single calcium channels and acetylcholine release at a presynaptic nerve terminal. *Neuron.* **11**, 1007–1011.

Stanley E. F. (1997) The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci.* **20**, 404–409.

Starr T. V., Prystay W. and Snutch T. P. (1991) Primary structure of a calcium channel that is highly expressed inthe rat cerebellum. *Proc. Natl Acad. Sci. USA* **88**, 5621–5625.

Sugita S., Ichtchenko K., Khvotchev M. and Sudhof T.C. (1998) alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein not required for triggering exocytosis. *J. Biol. Chem.* **273**, 32715–32724.

Sutton R. B., Fasshauer D., Jahn R. and Brünger A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395**, 347–353.

Sutton R. B., Ernst J. A. and Brünger A. T. (1999) Crystal structure of the cytosolicC2A-C2B domains of synaptotagmin III: implications for Ca^{2+}-independent SNARE complex interaction. *J. Cell. Biol.* **147**, 589–598.

Tahara M., Coorssen J. R., Timmers K., Blank P. S., Whalley T., Scheller R. H. and Zimmerberg J. (1998) Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. *J. Biol. Chem.* **273**, 33667–33673.

Thomas D. M. and Elferink L. A. (1998) Functional analysis of the C2A domain of synaptotagmin 1: implications for calcium-regulated secretion. *J. Neurosci.* **18**, 3511–3520.

Tobi D., Wiser O., Trus M. and Atlas D. (1998) N-type voltage-sensitive calcium channel interacts with syntaxin, synaptotagmin and SNAP-25 in a multiprotein complex. *Receptors Channels* **6**, 89–98.

Ungermann C., Sato K. and Wickner W. (1998) Defining the functions of trans-SNARE pairs. *Nature* **396**, 543–548.

Varadi G., Mori Y., Mikala G. and Schwartz A. (1995) Moleculare determinants of Ca2+ channel function and drug action. *Trends Pharmacol. Sci.* **16**, 43–49.

Voets T., Neher E. and Moser T. (1999) Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. *Neuron.* **23**, 607–615.

Vogel K., Cabaniol J. P. and Roche P. A. (2000) Targeting of SNAP-25 to membranes is mediated by its association with the target SNARE syntaxin. *J. Biol. Chem.* **275**, 2965.

Walker D. and De Waard M. (1998) Subunit interaction sites in voltage-dependent Ca2+ channels: role in channel function. *Trends Neurosci.* **21**, 148–154.

Watanabe T., Fujiwara T., Komazaki S., Yamaguchi K., Tajima O. and Akagawa K. (1999) HPC-1/syntaxin 1A suppresses exocytosis of PC12 cells. *J. Biochem. (Tokyo)* **125**, 685–689.

Weber T., Zemelman B. V., McNew J. A., Westermann B., .Gmachl M., Parlati F., Söllner T. H. and Rothman J. E. (1999) SNAREins minimal machinery for membrane fusion. *Cell* **92**, 759–772.

Wiser O., Bennett M. K. and Atlas D. (1996) Functional interaction of syntaxin and SNAP-25 with voltage sensitive L- and N-type Ca^{2+} channels. *EMBO J.* **15**, 4100–4110.

Wiser O., Tobi D., Trus M. and Atlas D. (1997) Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. *FEBS Lett* **404**, 203–207.

Wiser O., Trus M., Hernandez A., Renström E., Barg S., Rorsman P. and Atlas D. (1999) The voltage sensitive L-type Ca^{2+} channel is functionally coupled to the exocytotic machinery. *Proc. Natl Acad. Sci. USA* **96**, 248–253.

Wu M. N., Littleton J. T., Bhat M. A., Prokop A. and Bellen H. J. (1998) ROP, the Drosophila Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. *EMBO J.* **17**, 127–139.

Wu M. N., Fergestad T., Lloyd T. E., He Y., Broadie K. and Bellen H. J. (1999a) Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo. *Neuron* **23**, 593–605.

Wu L. G., Westenbroek R. E., Borst J. G. G., Catterall W. A. and Sakmann B. (1999b) Calcium channel types with distinct

presynaptic localization couple differentially to transmitter release in single calyx-type synapses. *J. Neurosci.* **19**, 726–736.

Yang B., Gonzalez L., Prekeris R., Steegmaier M., Advani R. J. and Scheller R. H. (1999a) SNARE interactions are not selective. Implications for membrane fusion specificity. *J. Biol. Chem.* **274**, 5649–5653.

Yang S. N., Larsson O., Branstrom R., Bertorello A. M., Leibiger B., Leibiger I. B., Moede T., Kohler M., Meister B. and Berggren P. O. (1999b) Syntaxin 1 interacts with the L (D) subtype of voltage-gated Ca (2+) channels in pancreatic beta cells. *Proc. Natl Acad. Sci. USA.* **96**, 10164–10169.

Yokoyama C. T., Sheng Z. H. and Catterall W. A. (1997) Phosphorylation of the synaptic protein interaction site on N-type calcium channels inhibits interactions with SNARE proteins. *J. Neurosci.* **17**, 6929–6938.

Yoshida A., Oho C., Omori A., Kuwahara R., Ito T. and Takahashi M. (1992) HPC-1 is associated with synaptotagmin and omega-conotoxin receptor. *J. Biol. Chem.* **267**, 24295–24928.

Zenisek D., Steyer J. A. and Almers W. (2000) Transport, capture and exocytosis of single synaptic vesicles at active zones. *Nature* **406**, 849–854.

Zhong H., Yokoyama C. T., Scheuer T. and Catterall W. A. (1999) Reciprocal regulation of P/Q-type Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. *Nature Neurosci* **2**, 939–941.