How does calcium trigger neurotransmitter release?

George J Augustine

Recent work has established that different geometric arrangements of calcium channels are found at different presynaptic terminals, leading to a wide spectrum of calcium signals for triggering neurotransmitter release. These calcium signals are apparently transduced by synaptotagmins – calcium-binding proteins found in synaptic vesicles. New biochemical results indicate that all synaptotagmins undergo calcium-dependent interactions with membrane lipids and a number of other presynaptic proteins, but which of these interactions is responsible for calcium-triggered transmitter release remains unclear.

Addresses
Department of Neurobiology, Box 3209, Duke University Medical Center, Durham, NC 27710, USA; e-mail: georgea@neuro.duke.edu

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Abbreviations
SNAP-25 25 kDa synaptosomal associated protein
SNARE soluble NSF-attachment protein receptor

Introduction

Synaptic transmission results from the calcium-regulated release of quantal packets of neurotransmitter, with release arising from the exocytotic discharge of neurotransmitter after the fusion of synaptic vesicles with the presynaptic plasma membrane [1]. Given these facts, one might imagine that it would be straightforward to purify synaptic vesicles, identify their molecular constituents, and thereby understand the molecular basis of neurotransmitter release.

Unfortunately, the problem has turned out to be more complex than appreciated initially. Nearly all of the proteins of synaptic vesicles have been identified and sequenced. Many of their binding partners within the presynaptic terminal — either on the vesicles, the plasma membrane, or the presynaptic cytoplasm — are now known. Despite this largess of information, a satisfying picture of the molecular basis of exocytosis has so far eluded us.

Part of the problem is that neurotransmitter release involves many different trafficking reactions and each of these requires a substantial number of protein–protein interactions. In this review, I would like to try to reduce the problem of calcium-regulated neurotransmitter release to its simplest possible terms by concentrating on the final exocytotic reactions that are triggered by calcium ions (Ca^{2+}). My focus will be on recent progress that addresses two related questions: first, how much Ca^{2+} is needed to trigger transmitter release? Second, to which protein(s) does Ca^{2+} bind to trigger release?

A brief introduction to diffusion of Ca^{2+}

To appreciate how much Ca^{2+} is required to trigger transmitter release, it is important to understand the diffusion of Ca^{2+} once it enters the presynaptic terminal. Mathematical models indicate that diffusion yields an immediate accumulation of calcium ions within an area covering tens of nanometers around the mouth of open Ca^{2+} channels [2,3]. This calcium signal can be damped by the buffering action of Ca^{2+}-binding proteins [4]. Such unitary Ca^{2+} influx events will result in qualitatively different types of intracellular signals, depending on the spatial arrangement of Ca^{2+} channels relative to each other and to the relevant Ca^{2+}-binding proteins (Figure 1; and [5,6]).

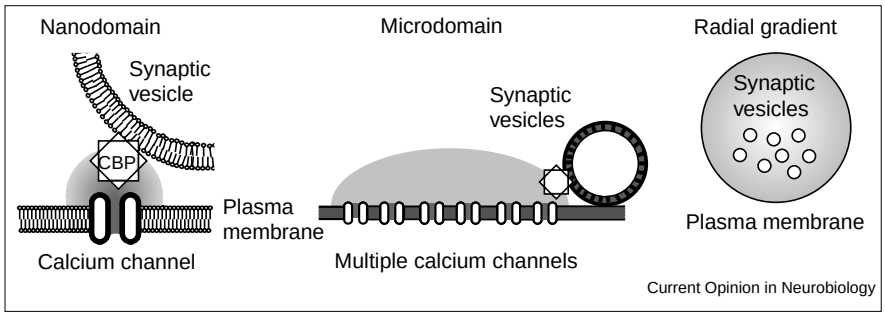
If the Ca^{2+}-binding proteins are within a few nanometers of open Ca^{2+} channels, the signaling arrangement is called a 'calcium domain' [2] or, in reference to the spatial dimensions involved, a 'nanodomain' [5]. In a single nanodomain, local Ca^{2+} concentrations vary steeply with distance but can be as high as hundreds of micromolar [3]. When many Ca^{2+} channels are clustered together in a membrane area of roughly 1 μm^2, a Ca^{2+}-binding protein positioned within the cluster will sense a 'microdomain' of Ca^{2+} [5,7]. Free Ca^{2+} concentration within microdomains can range from tens of micromolar to hundreds of micromolar, depending on the amount of summation of individual nanodomains arising from multiple Ca^{2+} channels [7,8].

A third type of Ca^{2+} signal arises when Ca^{2+} channels are 1 μm or further from target Ca^{2+}-binding proteins. In this situation, 'radial gradients' of concentration occur as Ca^{2+} diffuses from the plasma membrane into the interior of the cell [9,10]. Because of the resulting dilution, Ca^{2+} levels at the target proteins will be much lower than in the previous two cases and may be in the range of a few micromolar or less

Highly localized Ca^{2+} signaling in presynaptic terminals

Until recently, there was relatively good agreement that the release of fast-acting neurotransmitters (such as glutamate and acetylcholine) relies on nanodomain signaling [11,12]. This consensus was constructed from many lines of evidence. First, injection of Ca^{2+} buffers with different rates of Ca^{2+} binding produces differential effects on transmitter release. Specifically, BAPTA, a rapidly binding chelator, efficiently blocks release, whereas EGTA, a slower chelator, does not [13]. Second, high presynaptic Ca^{2+} concentrations, of the order of 100–200 μM, are necessary to produce rapid rates of secretion [14,15]. Third, titration of presynaptic Ca^{2+} signals, by injecting Ca^{2+} buffers with varying affinities, yields similar estimates of Ca^{2+} levels during transmitter release [13]. Fourth, measurements of local Ca^{2+} concentration with a low-affinity indicator protein suggest very high Ca^{2+} concentrations during

Figure 1



Types of presynaptic Ca^{2+} signals. 'Nanodomains' arise from local diffusion from single open Ca^{2+} channels, 'microdomains' from multiple open Ca^{2+} channels, and 'radial gradients' from long-distance movements of Ca^{2+} away from the channels. CBP indicates a Ca^{2+}-binding protein that translates Ca^{2+} entry into vesicle fusion. Modified with permission from [6].

transmitter release [7]. Fifth, the use of Ca^{2+}-activated potassium channels as monitors suggests that single action potentials raise presynaptic Ca^{2+} concentration to about 150 μM [16••]. Sixth, modifying the number of open Ca^{2+} channels causes changes in transmitter release that are consistent with nanodomain Ca^{2+} signaling [11,17]. Last, measurements of the opening of individual Ca^{2+} channels suggest that only one channel needs to open to release transmitter [12].

From these findings, the evidence seemed strong that nanodomains trigger the rapid release of fast-acting transmitters. Equally compelling data indicate that microdomain-type Ca^{2+} signals mediate the secretion of transmitters at terminals that may release transmitter tonically, such as sensory synapses [8,18]. Furthermore, radial shell-type Ca^{2+} signals seem to operate at peptide-secreting terminals [19,20]. Thus, the picture seemed clear: very local, nanodomain Ca^{2+} signals are used when optimizing for speed, microdomains are used at slightly slower synapses, and radial shells mediate the delayed secretion of peptides.

Microdomain Ca^{2+} signaling at the Calyx of Held

Recent studies have turned this simplification on its head, however, by providing strong evidence that microdomain Ca^{2+} signaling triggers the very rapid secretion of transmitter at the Calyx of Held synapse in mammals. This specialized 'giant' synapse serves as a key relay in the auditory system. One early indication that things may be different from the picture described above came from the observation that EGTA is almost as potent as BAPTA in blocking transmitter release at this synapse [21]. This suggests that there is a substantial distance between the open Ca^{2+} channels and the relevant Ca^{2+}-binding protein, as would be expected if influx from several channels was required for transmitter release at this synapse.

This notion has been reinforced by very recent explorations of the intracellular Ca^{2+} requirements for transmitter release at the Calyx synapse (Figure 2). Two groups have used Ca^{2+} uncaging experiments to determine how much Ca^{2+} is required in the Calyx terminal to cause transmitter release [22••,23••]. Both Bollmann *et al.* [22••] and Schneggenburger and Neher [23••] found significant amounts of transmitter release at Ca^{2+} levels as low as a few micromolar, and saw signs of saturation at Ca^{2+} concentrations greater than 20–30 μM (Figure 2a). This relationship is markedly different from the Ca^{2+} requirements for transmitter release from other synapses; for example, approximately 20-fold higher Ca^{2+} concentrations are required at the goldfish bipolar cell terminal (Figure 2a).

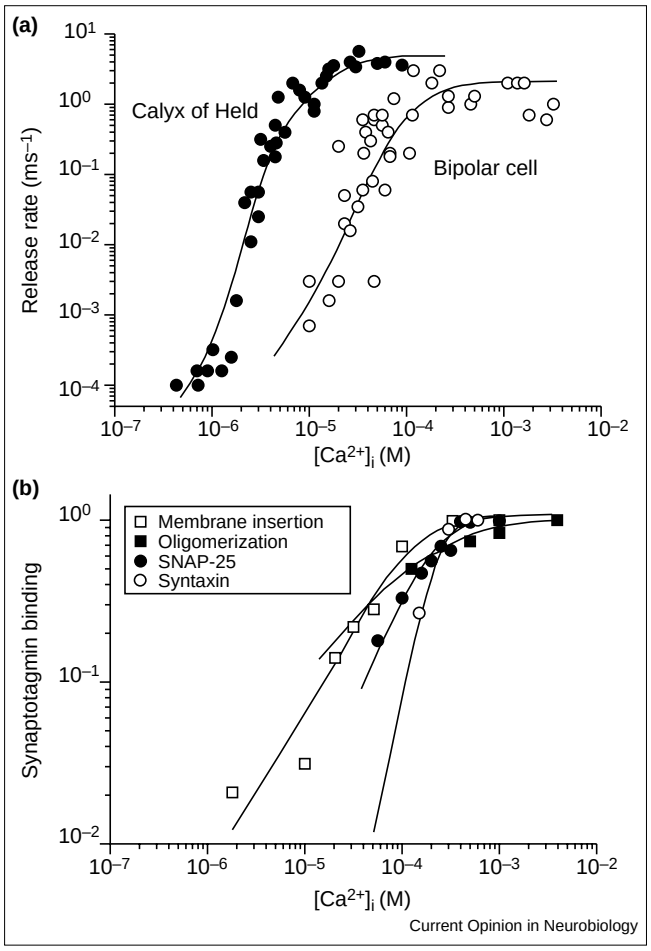
Although these results indicate that a low concentration of Ca^{2+} is needed to trigger transmitter release, they do not necessarily mean that small Ca^{2+} signals are produced during transmitter release. It is possible that large rises in Ca^{2+} concentration still occur, but saturate the Ca^{2+}-dependent release apparatus. Other experiments in these papers indicate that this is not the case [22••,23••]. This point was established by comparing the rates of neurotransmitter release to those produced by elevating presynaptic Ca^{2+} to various concentrations. This null-point method provided estimates of 5–25 μM for the levels of Ca^{2+} reached during a presynaptic action potential.

Again, this contrasts with comparable experiments done in other systems; for example, uncaging 80 μM Ca^{2+} at the squid giant synapse causes a lower rate of transmitter release than is produced by an action potential [15].Thus, presynaptic Ca^{2+} signals differ even among fast-releasing synapses. At the Calyx presynaptic terminal, presynaptic Ca^{2+} concentration reaches only relatively low levels during an action potential. But the relatively high Ca^{2+} affinity of exocytosis allows this small signal to release large amounts of transmitter.

These results are compatible with a microdomain style of Ca^{2+} signaling, with several channels contributing to the triggering of transmitter release. Evidence from some other fast-acting synapses also indicates that transmitter release occurs within microdomains containing many Ca^{2+} channels. For example, by proposing that several types of Ca^{2+} channels are present in these clusters, it is possible to account for the synergistic actions of Ca^{2+} channel toxins in blocking release [24,25].

A microdomain model also is the simplest way to explain the supralinear increase in transmitter release that results

Figure 2



Ca^{2+}-dependent presynaptic reactions. Ca^{2+} requirements (a) for neurotransmitter release and (b) the binding of synaptotagmin to various presynaptic constituents. Note logarithmic scales on all axes. Measurements of the rates of transmitter release from the Calyx of Held synapse are from [22••], and measurements of release rates at bipolar cell terminals are from [14]. Data from the Ca^{2+}-dependent binding of synaptotagmin *in vitro* comes from a number of sources: membrane binding is from [38••]; binding to other synaptotagmin molecules is from [41•]; binding to SNAP-25 is from [58••]; and binding to syntaxin is from [37].

from broadening the presynaptic action potential at central synapses [26,27]. Finally, the concept of Ca^{2+} microdomains makes it possible to understand how low micromolar amounts of Ca^{2+} can trigger transmitter release at crayfish neuromuscular synapses [28] and PC12 cells [29••].

In summary, even for the case of fast release of neurotransmitters it is now clear that not all presynaptic terminals require Ca^{2+} concentrations in excess of 100 μM. It appears that terminals vary markedly in their calcium requirements, with rapid exocytosis triggered by physiological Ca^{2+} levels as low as 5–10 μM or as great as 100–200 μM. These variations arise, in part, from differences in the structural organization of Ca^{2+} channels at presynaptic active zones and, in part, from differences in the Ca^{2+} sensitivity of exocytosis. The latter indicates that presynaptic terminals must use different Ca^{2+}-binding proteins that vary in their affinity for Ca^{2+}.

Synaptotagmin as a presynaptic Ca^{2+} trigger

Although there is no consensus on the identity of the Ca^{2+}-binding protein responsible for transmitter release, for some years the most popular candidate has remained synaptotagmin, an integral protein of synaptic vesicles. Twelve different synaptotagmin isoforms have been identified in mammalian genomes, and the properties of this interesting protein family have been summarized in a number of excellent reviews [30,31,32].

The molecular structures of cytoplasmic domains of some synaptotagmins have been solved and the structure of one of these, synaptotagmin III, is shown in Figure 3. Of particular note is that synaptotagmins have two repeating structures, called C2 domains. The precise locations of the Ca^{2+}-binding sites of synaptotagmin are still being identified, but it is clear that the C2A and C2B domains of synaptotagmin are each capable of binding more than one Ca^{2+} ion [33,34•]. Ca^{2+} binding to acidic residues on the C2 domains of synaptotagmin promotes a number of reactions. Because synaptotagmin is a remarkably 'sticky' protein that tends to bind indiscriminantly, the biological significance of these *in vitro* observations is not yet clear [35•]. Here I will list a few of the most interesting binding reactions and the evidence hinting at their possible roles in Ca^{2+}-triggered neurotransmitter release.

Membrane insertion

Ca^{2+} binding to the C2A domain of synaptotagmin causes the protein to bind to membranes that contain acidic phospholipids [36]. Although it was thought initially that this lipid binding requires only small amounts of Ca^{2+} — in the range of 5–10 μM [36,37] — the most recent and accurate determinations indicate that half-maximal binding to membranes occurs at Ca^{2+} concentrations of about 20–70 μM (Figure 2b; [38••]). Chapman and co-workers [38••,39] have shown that Ca^{2+} acts by binding to two loops that represent the Ca^{2+}-coordination sites of C2A, and thus induces the insertion of these regions of the molecule into the membrane. The Ca^{2+}-dependent insertion of synaptotagmin into membranes is remarkably rapid, occurring within milliseconds [38••]. This speed arises because Ca^{2+} acts by interacting directly with negative charges on synaptotagmin, rather than by inducing a slow conformational change in the protein [33].

The Ca^{2+}-dependent ability of synaptotagmin to bind to membranes fits nicely with observations made on synapses that have high Ca^{2+} requirements for rapid transmitter release (such as the bipolar terminals shown in Figure 2a). These parallels argue strongly that insertion of synaptotagmin participates in the membrane reorganization that underlies exocytotic fusion. Another observation consistent with this idea is that neurotransmitter release is

reduced by overexpressing synaptotagmin IV — a synaptotagmin isoform that does not exhibit Ca^{2+}-dependent lipid binding [40•]. Arguing against this idea is an observation by Kishimoto *et al.* [29••] that the selectivity of exocytosis for divalent cations does not match the divalent selectivity of insertion of synaptotagmin into membranes. Another is that mutating the acidic residues in the Ca^{2+}-binding loops of the C2A domain does not appear to affect transmitter release (I Robinson, T Schwarz, personal communication). Clearly, further tests of the *in vivo* importance of Ca^{2+}-dependent insertion of synaptotagmin into membranes will be necessary.

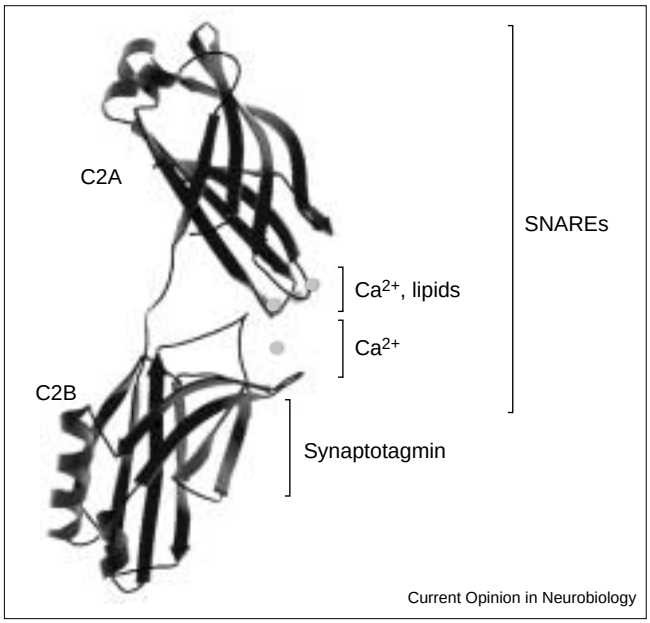
Oligomerization

Many synaptotagmins can bind to each other to form heterooligomers or homooligomers [39,41•,42,43•]. In some cases, this oligomerization is dependent on Ca^{2+}. For example, the binding of synaptotagmin I to synaptotagmin II is half-maximal at Ca^{2+} concentrations of roughly 100 μM (Figure 2b). This self-association occurs at the C2B domains and results from calcium binding to acidic motifs that are homologous to those responsible for Ca^{2+} binding to the C2A domain [39,44•]. Unlike the effects of Ca^{2+} on the C2A domain, the calcium-induced self-association involves a conformational change in C2B domains [44•]. Despite this requirement for a conformational change, synaptotagmin oligomerization still can occur within milliseconds [38••]. Binding of the C2B domains to each other seems to occur at a pocket distinguished by repeated lysine residues [39,44•].

These biochemical properties also parallel release at synapses with high Ca^{2+} requirements and suggest a physiological function for Ca^{2+}-induced oligomerization of synaptotagmin. Indeed, several studies point toward such a role in synaptic neurotransmitter release. The earliest indication came from the observation that injecting a peptide from the lysine-rich pocket of C2B inhibits neurotransmitter release by blocking a late step in vesicle fusion [45]. Likewise, a larger fragment of the C2B domain can serve as a dominant-negative inhibitor of a late, Ca^{2+}-dependent step required for exocytosis in PC12 cells — an effect that is abolished by mutating the lysine residues required for oligomerization [44•]. Perhaps the most compelling evidence of a role for synaptotagmin oligomerization in synaptic neurotransmitter release are observations in *Drosophila* that mutation of the residues required for oligomerization inhibits transmitter release [46] and prevents rescue of neurotransmitter release in synaptotagmin-null synapses [47•].

Together with the biochemical observations described above, these results make a strong case for the importance of Ca^{2+}-dependent oligomerization of synaptotagmin in the presynaptic actions of Ca^{2+}. It should be noted, however, that the same region of the C2B domain that is required for Ca^{2+}-dependent oligomerization is also involved in other reactions, such as the Ca^{2+}-independent binding of synaptotagmin to inositol polyphosphates [48]

Figure 3



Structure of the cytoplasmic region of synaptotagmin III, including the C2A and C2B domains. Brackets indicate the binding sites for various presynaptic constituents. Modified with permission from [34•].

and to the clathrin adapter protein AP-2 [39,49]. Thus, it is possible that some of the functional effects described above are due to perturbation of reactions other than oligomerization of synaptotagmin. Nonetheless, at the moment the Ca^{2+}-dependent oligomerization of synaptotagmin must be considered as a prime candidate for the action of Ca^{2+} during neurotransmitter release.

SNARE binding

SNARE (soluble NSF-attachment protein receptor) proteins have critical roles in neurotransmitter release and many other forms of membrane fusion [50]. Indeed, reconstitution experiments indicate that the formation of SNARE protein complexes that span two membranes is sufficient to catalyze membrane fusion [51]. Although SNARE protein complexes apparently possess a divalent-cation-binding site [52], none of the known SNARE-mediated reactions is influenced by Ca^{2+}. Thus, it is more likely that Ca^{2+} sensitivity is conferred to SNARE-based membrane fusion by synaptotagmin or some other Ca^{2+}-binding protein. With this in mind, much effort has gone into examining the Ca^{2+}-dependent interactions between synaptotagmin and SNARE proteins.

Available evidence suggests that Ca^{2+} binding to synaptotagmin can alter interactions with the SNARE proteins in several ways. A large number of experiments have documented that Ca^{2+} binding enhances the association between synaptotagmin and the plasma-membrane SNARE, syntaxin [37,53]. The amount of Ca^{2+} required for this interaction varies among different isoforms of

synaptotagmin, which would account nicely for the different Ca^{2+} requirements for exocytosis from different presynaptic terminals [37]. The Ca^{2+} dependence of binding of synaptotagmin I, the most abundant member of this family, closely parallels the other Ca^{2+}-regulated interactions shown in Figure 2b. This interaction occurs by binding of Ca^{2+} to the C2A domain, although the C2B domain is also required for maximal association with syntaxin [38••].

Although there have been few published tests of the functional importance of this interaction, Wu *et al.* [54•] have shown that neurotransmitter release is reduced by mutations that prevent the binding of synaptotagmin (and several other proteins) to syntaxin. Secretion from PC12 cells is inhibited by microinjecting fragments of the C2A domain of synaptotagmin; this inhibition might be caused by the disruption of interactions between synaptotagmin and SNARE proteins [42]. Alternatively, there are indications that the Ca^{2+}-dependent binding of synaptotagmin to syntaxin may not be important for transmitter release ([35•,55]; I Robinson, T Schwarz, personal communication). Indeed, there is even a report that Ca^{2+} causes synaptotagmin to dissociate from syntaxin in synaptosomes [56•]. Thus, the significance of the Ca^{2+}-regulated binding of synaptotagmin to syntaxin is presently unclear.

Schiaro *et al.* [57] and Gerona *et al.* [58••] have found that synaptotagmin also undergoes a Ca^{2+}-regulated association with SNAP-25 (25 kDa synaptosomal associated protein) — another plasma-membrane SNARE protein. The Ca^{2+} requirements for this interaction are similar to those of the other Ca^{2+}-regulated protein–protein interactions of SNAP-25 (Figure 2b) and the interaction involves the binding of SNAP-25 to the C2A domain of synaptotagmin [58••]. Synaptotagmin binds to the carboxyl terminus of SNAP-25, which is of great functional significance because this region of SNAP-25 is cleaved by botulinum toxins that inhibit exocytosis. In fact, after toxin treatment there is a good correlation between inhibition of exocytosis and loss of synaptotagmin binding activity [58••]. This suggests that exocytosis may require Ca^{2+}-dependent binding of synaptotagmin to SNAP-25 — a concept that merits further experimental analyses.

Other calcium-regulated interactions of synaptotagmin

In addition to these interactions, synaptotagmin has been reported to undergo other Ca^{2+}-regulated interactions. For example, Ca^{2+} inhibits the binding of synaptotagmin to SV2, another synaptic vesicle protein [59], and also promotes the intramolecular association of the C2A and C2B domains of synaptotagmin [60•]. A systematic screen has revealed Ca^{2+}-dependent binding of synaptotagmin to several other proteins, including the ATPase VCP [35•]. The functional significance of these interactions remain to be evaluated. Because interaction between the C2 domains of synaptotagmin is half-maximal at 3 mM Ca^{2+}, synaptotagmin's Ca^{2+} sensitivity may be too low to account for the Ca^{2+} dependence of transmitter release (Figure 2a).

Conclusions

It now seems clear that different geometrical arrangements between Ca^{2+} channels and secretory proteins allow a diverse range of presynaptic Ca^{2+} signals to trigger transmitter release. The remaining challenge is to identify the molecular mediators of Ca^{2+} action in presynaptic terminals. There are close similarities in the Ca^{2+} requirements for neurotransmitter release (Figure 2a) and those for the binding of synaptotagmin I to membranes and to several proteins (Figure 2b). These similarities are especially strong in the case of synapses, such as the bipolar cell and squid giant synapse, that exhibit high Ca^{2+} requirements for exocytosis. Synaptotagmin isoforms differ in some of their Ca^{2+}-dependent properties [37], which might account for transmitter release from synapses that have more modest Ca^{2+} requirements.

Together with early experiments demonstrating that synaptotagmin has an important role in neurotransmitter release, the case for synaptotagmin serving as a molecular transducer for Ca^{2+} action in neurotransmitter release now seems quite strong. Nonetheless, it still is not clear whether Ca^{2+} acts by causing synaptotagmin to bind to one (or more) of the binding partners shown in Figure 2b, or by binding to some other molecular entity. Clearly, the next goal is to perform experimental tests that discriminate among these possibilities, and thereby define precisely the role of synaptotagmin in the calcium-triggered release of neurotransmitters.

Update

In the few weeks that intervened between when this article was written and when the proofs arrived, a large number of new papers appeared on the topic of synaptotagmin and neurotransmitter release. Two of these are of special note. One is the paper by Fernandez-Chacon *et al.* [61], who show that mutating the C2A domain of synaptotagmin I produces a parallel reduction in neurotransmitter release and the Ca^{2+}-dependent binding of synaptotagmin to membrane lipids. This parallel provides another line of support for the idea that Ca^{2+}-dependent binding of synaptotagmin to membrane lipids may be involved in triggering neurotransmitter release. The other noteworthy paper was the report of Littleton *et al.* [62], which examined the consequences of mutating the C2B domain of *Drosophila* synaptotagmin I. This paper shows that mutating locations in the C2B domain reduced binding of synaptotagmin to the clathrin adapter protein AP-2 and appeared to cause a selective impairment of synaptic vesicle endocytosis. Another mutation reduced Ca^{2+}-dependent dimerization of synaptotagmin and yielded a reduced ability of docked synaptic vesicles to fuse. The latter finding adds to the evidence that synaptotagmin dimerization might also be a late action of Ca^{2+} in neurotransmitter release.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Katz B: *The Release of Neural Transmitter Substances*. Liverpool: Liverpool University Press; 1969.

2. Chad JE, Eckert R: Calcium domains associated with individual channels can account for anomalous voltage relations of Ca^{2+}-dependent responses. *Biophys J* 1984, 45:993-999.

3. Simon SM, Llinas RR: Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys J* 1985, 48:485-498.

4. Edmonds B, Reyes R, Schwaller B, Roberts WM: Calretinin modifies presynaptic calcium signaling in frog saccular hair cells. *Nat Neurosci* 2000, 3:786-790.

5. Kasai H: Cytosolic Ca^{2+} gradients, Ca^{2+} binding proteins and synaptic plasticity. *Neurosci Res* 1993, 16:1-7.

6. Schweizer FE, Betz H, Augustine GJ: From vesicle docking to endocytosis: intermediate reactions of exocytosis. *Neuron* 1995, 14:689-696.

7. Llinas R, Sugimori M, Silver RB: Microdomains of high calcium concentration in a presynaptic terminal. *Science* 1992, 256:677-679.

8. Roberts WM, Jacobs RA, Hudspeth AJ: Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *J Neurosci* 1990, 10:3664-3684.

9. Hernandez-Cruz A, Sala F, Adams PR: Subcellular calcium transients visualized by confocal microscopy in a voltage-clamped vertebrate neuron. *Science* 1990, 247:858-862.

10. Marengo FD, Monck JR: Development and dissipation of Ca^{2+} gradients in adrenal chromaffin cells. *Biophys J* 2000, 79:1800-1820.

11. Augustine GJ, Adler EM, Charlton MP: The calcium signal for transmitter secretion from presynaptic nerve terminals. *Ann NY Acad Sci* 1991, 635:365-381.

12. Stanley EF: The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci* 1997, 20:404-409.

13. Adler EM, Augustine GJ, Duffy SN, Charlton MP: Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *J Neurosci* 1991, 11:1496-1507.

14. Heidelberger R, Heinemann C, Neher E, Matthews G: Calcium dependence of the rate of exocytosis in a synaptic terminal. *Nature* 1994, 371:513-515.

15. Hsu SF, Augustine GJ, Jackson MB: Adaptation of Ca^{2+}-triggered exocytosis in presynaptic terminals. *Neuron* 1996, 17:501-512.

16. •• Yazejian B, Sun XP, Grinnell AD: Tracking presynaptic Ca^{2+} dynamics during neurotransmitter release with Ca^{2+}-activated K+ channels. *Nat Neurosci* 2000, 3:566-571.

By using Ca^{2+}-activated potassium channels as reporters, the authors provide the most reliable data available in the 30-year history of measurements of presynaptic Ca^{2+} signals.

17. Yoshikami D, Bagabaldo Z, Olivera BM: The inhibitory effects of omega-conotoxins on Ca channels and synapses. *Ann NY Acad Sci* 1989, 560:230-248.

18. Rieke F, Schwartz EA: Asynchronous transmitter release: control of exocytosis and endocytosis at the salamander rod synapse. *J Physiol (Lond)* 1996, 493:1-8.

19. Verhage M, McMahon HT, Ghijsen WE, Boomsma F, Scholten G, Wiegant VM, Nicholls DG: Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* 1991, 6:517-524.

20. Peng YY, Zucker RS: Release of LHRH is linearly related to the time integral of presynaptic Ca^{2+} elevation above a threshold level in bullfrog sympathetic ganglia. *Neuron* 1993, 10:465-473.

21. Borst JG, Sakmann B: Calcium influx and transmitter release in a fast CNS synapse. *Nature* 1996, 383:431-434.

22. •• Bollmann JH, Sakmann B, Borst JG: Calcium sensitivity of glutamate release in a calyx-type terminal. *Science* 2000, 289:953-957.

See annotation [23••].

23. •• Schneggenburger R, Neher E: Intracellular calcium dependence of transmitter release rates at a fast central synapse. *Nature* 2000, 406:889-893.

An important paper that establishes, beyond any doubt, that rapid transmitter release can occur at relatively low concentrations of Ca^{2+} within a presynaptic terminal. See also [22••].

24. Luebke JI, Dunlap K, Turner TJ: Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron* 1993, 11:895-902.

25. Takahashi T, Momiyama A: Different types of calcium channels mediate central synaptic transmission. *Nature* 1993, 366:156-158.

26. Sabatini BL, Regehr WG: Control of neurotransmitter release by presynaptic waveform at the granule cell to Purkinje cell synapse. *J Neurosci* 1997, 17:3425-3435.

27. Qian J, Saggau P: Modulation of transmitter release by action potential duration at the hippocampal CA3-CA1 synapse. *J Neurophysiol* 1999, 81:288-298.

28. Ravin R, Parnas H, Spira ME, Volfovsky N, Parnas I: Simultaneous measurement of evoked release and $[Ca^{2+}]_i$ in a crayfish release bouton reveals high affinity of release to Ca^{2+}. *J Neurophysiol* 1999, 81:634-642.

29. •• Kishimoto T, Liu T-T, Ninomiya Y, Takagi H, Yoshioka Y, Ellis-Davies GCR, Miyashita Y, Kasai H: Ion selectivities of the Ca^{2+} sensors for exocytosis in rat phaeochromocytoma cells. *J Physiol (Lond)* 2001, in press.

A tour-de-force that examines the divalent cation selectivity of exocytosis. Different types of secretory vesicles within single PC12 cells apparently differ in their ability to fuse in response to various divalent cations, leading to the intriguing conclusion that unique Ca^{2+}-binding proteins must be used by each vesicle type.

30. Rizo J, Sudhof TC: C2-domains, structure and function of a universal Ca^{2+} binding domain. *J Biol Chem* 1998, 273:15879-15882.

31. Mikoshiba K, Fukuda M, Ibata K, Kabayama H, Mizutani A: Role of synaptotagmin, a Ca^{2+} and inositol polyphosphate binding protein, in neurotransmitter release and neurite outgrowth. *Chem Phys Lipids* 1999, 98:59-67

32. Brunger AT: Structural insights into the molecular mechanism of Ca^{2+}-dependent exocytosis. *Curr Opin Neurobiol* 2000 10:293-302.

33. Shao X, Fernandez I, Sudhof TC, Rizo J: Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? *Biochemistry* 1998, 37:16106-16115.

34. • Sutton RB, Ernst JA, Brunger AT: Crystal structure of the cytosolic C2A-C2B domains of synaptotagmin III. Implications for Ca^{2+}-independent snare complex interaction. *J Cell Biol* 1999, 147:589-598.

The first image of the combined C2A and C2B domains of a synaptotagmin.

35. • Sugita S, Sudhof TC: Specificity of Ca^{2+}-dependent protein interactions mediated by the C2A domains of synaptotagmins. *Biochemistry* 2000, 39:2940-2949.

This paper questions the veracity of many of the reported *in vitro* interactions of synaptotagmins and also identifies some new interactions of this protein family.

36. Brose N, Petrenko AG, Sudhof TC, Jahn R: Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* 1992, 256:1021-1025.

37. Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Sudhof TC: Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* 1995, 375:594-599.

38. •• Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, Chapman ER: Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes. *Neuron* 1999, 24: 363-376.

The most important recent advance in our understanding of synaptotagmins. It provides reliable measurements of the time courses and Ca^{2+} dependencies of the interactions of synaptotagmin with lipids, SNARE proteins and other synaptotagmin-binding molecules.

39. Chapman ER, Desai RC, Davis AF, Tornehl CK: Delineation of the oligomerization, AP-2 binding, and synprint binding region of the

C2B domain of synaptotagmin. *J Biol Chem* 1998, 273: 32966-32972.

40. Littleton JT, Serano TL, Rubin GM, Ganetzky B, Chapman ER:
• Synaptic function modulated by changes in the ratio of synaptotagmin I and IV. *Nature* 1999, 400:757-760.
This paper indicates that synaptotagmin IV may function to scale down Ca^{2+}-dependent transmitter release by forming oligomers with other forms of synaptotagmin, such as synaptotagmin I, that promote transmitter release.

41. Osborne SL, Herreros J, Bastiaens PIH, Schiavo G: Calcium-
• dependent oligomerization of synaptotagmins I and II. *J Biol Chem* 1999, 274:59-66.
A nice study of the oligomerization properties of synaptotagmins. By forming oligomers that consist of synaptotagmins with different Ca^{2+}-binding properties, it may be possible to confer Ca^{2+} sensitivity over any dynamic range.

42. Thomas DM, Ferguson GD, Herschman HR, Elferink LA: Functional and biochemical analysis of the C2 domains of synaptotagmin IV. *Mol Biol Cell* 1999, 10:2285-2295.

43. Fukuda M, Mikoshiba K: Calcium-dependent and -independent
• hetero-oligomerization in the synaptotagmin family. *J Biochem* 2000, 128:637-645.
The authors compare the oligomerization properties of many members of the synaptotagmin family and come up with some general rules for oligomerization.

44. Desai RC, Vyas B, Earles CA, Littleton JT, Kowalchyck JA, Martin TF,
• Chapman ER: The C2B domain of synaptotagmin is a Ca^{2+}-sensing module essential for exocytosis. *J Cell Biol* 2000, 150:1125-1136.
One of the first papers to implicate the C2B domain of synaptotagmins in conferring Ca^{2+} sensitivity to neurotransmitter release.

45. Bommert K, Charlton MP, DeBello WM, Chin GJ, Betz H, Augustine GJ: Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* 1993, 363:163-165.

46. Fukuda M, Kabayama H, Mikoshiba K: ***Drosophila*** AD3 mutation of synaptotagmin impairs calcium-dependent self- oligomerization activity. *FEBS Lett* 2000, 482:269-272.

47. Mackler JM, Reist NE: Mutations in the C2B domain of
• synaptotagmin disrupt synaptic transmission at ***Drosophila*** neuromuscular junctions. *J Comp Neurol* 2001, in press.
This paper uses the power of *Drosophila* genetic approaches to show that the C2B domain of synaptotagmin plays a critical role in neurotransmitter release.

48. Fukuda M, Aruga J, Niinobe M, Aimoto S, Mikoshiba K: Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II. *J Biol Chem* 1994, 269:29206-29211.

49. Zhang JZ, Davletov BA, Sudhof TC, Anderson RG: Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* 78:751-760.

50. Rothman JE: Mechanisms of intracellular protein transport. *Nature* 1994, 372:55-63.

51. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE: SNAREpins: minimal machinery for membrane fusion. *Cell* 1998, 92:759-772.

52. Sutton RB, Fasshauer D, Jahn R, Brünger AT: Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 1998, 395:347-353.

53. Chapman ER, Hanson PI, An S, Jahn R: Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. *J Biol Chem* 1995, 270:23667-23671.

54. Wu MN, Fergestad T, Lloyd TE, He Y, Broadie K, Bellen HJ: Syntaxin
• 1A interacts with multiple exocytic proteins to regulate neurotransmitter release ***in vivo***. *Neuron* 1999, 23:593-605.
The first systematic genetic analysis of the significance of the interactions of various proteins with the SNARE protein, syntaxin.

55. Matos MF, Rizo J, Sudhof TC: The relation of protein binding to function: what is the significance of munc18 and synaptotagmin binding to syntaxin 1, and where are the corresponding binding sites? *Eur J Cell Biol* 2000, 79:377-382.

56. Leveque C, Boudier JA, Takahashi M, Seagar M: Calcium-dependent
• dissociation of synaptotagmin from synaptic SNARE complexes. *J Neurochem* 2000, 74:367-374.
Does Ca^{2+} act by influencing the interaction of synaptotagmin with the SNARE proteins? This paper reaches the conclusion that Ca^{2+} may remove synaptotagmin from the SNARE complex. This comes as a surprise because of many other reports that Ca^{2+} increases the binding of recombinant fragments of synaptotagmin to SNARE proteins, such as syntaxin.

57. Schiavo G, Stenbeck G, Rothman JE, Sollner TH: 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* 1997, 94:997-1001.

58. Gerona RR, Larsen EC, Kowalchyk JA, Martin TF: The C terminus of
•• SNAP25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes. *J Biol Chem* 2000, 275:6328-6336.
Excellent biochemical and functional data suggest that Ca^{2+} may trigger transmitter release by allowing synaptotagmin to bind to the SNARE protein, SNAP-25.

59. Schivell AE, Batchelor RH, Bajjalieh SM: Isoform-specific, calcium-regulated interaction of the synaptic vesicle proteins SV2 and synaptotagmin. *J Biol Chem* 1996, 271:27770-27775.

60. Garcia RA, Forde CE, Godwin HA: Calcium triggers an
• intramolecular association of the C2 domains in synaptotagmin. *Proc Natl Acad Sci USA* 2000, 97:5883-5888.
This paper reports the novel observation of an interaction between the C2A and C2B domains of synaptotagmin. Also noteworthy is its use of fluorescence resonance energy transfer, a technique that could be very useful for examining the interactions of presynaptic proteins.

61. Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC : Synaptotagmin I functions as a calcium regulator of release probability. *Nature* 2001, 410:41-49.

62. Littleton JT, Bai J, Vyas B, Desai R, Baltus AE, Garment MB, Carlson SD, Ganetzky B, Chapman ER: Synaptotagmin mutants reveal essential functions for the C2B domain in Ca^{2+}-triggered fusion and recycling of synaptic vesicles ***in vivo***. *J Neurosci* 2001, 21:1421-1433.