Calcium-dependent Regulation of SNARE-mediated Membrane Fusion by Calmodulin

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Neuroexocytosis requires SNARE proteins, which assemble into trans complexes at the synaptic vesicle/plasma membrane interface and mediate bilayer fusion. Ca\(^{2+}\) sensitivity is thought to be conferred by synaptotagmin, although the ubiquitous Ca\(^{2+}\)-effector calmodulin has also been implicated in SNARE-dependent membrane fusion. To examine the molecular mechanisms involved, we examined the direct action of calmodulin and synaptotagmin in vitro, using fluorescence resonance energy transfer to assay lipid mixing between target- and vesicle-SNARE liposomes. Ca\(^{2+}\)/calmodulin inhibited SNARE assembly and membrane fusion by binding to two distinct motifs located in the membrane-proximal regions of VAMP2 (\(K_D = 500 \text{ nM}\)) and syntaxin 1 (\(K_D = 2 \mu\text{m}\)). In contrast, fusion was increased by full-length synaptotagmin 1 anchored in vesicle-SNARE liposomes. When synaptotagmin and calmodulin were combined, synaptotagmin overcame the inhibitory effects of calmodulin. Furthermore, synaptotagmin displaced calmodulin binding to target-SNAREs. These findings suggest that two distinct Ca\(^{2+}\) sensors act antagonistically in SNARE-mediated fusion.

Neurotransmitter release at axonal terminals entails calcium-dependent exocytosis of synaptic vesicle contents into the synaptic cleft, a process involving several distinct steps (reviewed in Ref. 1). First, priming reactions fill a readily releasable pool in which vesicles are immediately available for triggering by Ca\(^{2+}\) entry. Triggering opens a fusion pore connecting the vesicle lumen to the extracellular medium, which then expands as the vesicle collapses into the plasma membrane with total release of contents. Alternatively, the pore may open and close without expanding, allowing transient (kiss-and-run) exocytosis, a mechanism conferring partial release of transmitter while avoiding incorporation of vesicles into the plasma membrane (2, 3). Interestingly, Ca\(^{2+}\) ions not only trigger fusion itself but also regulate pre- and post-fusion events, including priming and fusion pore closure (4, 5). Membrane fusion is mediated by the synaptic soluble NSF attachment protein receptors (SNAREs). VAMP2 (vesicle-associated membrane protein 2, a v-SNARE, also known as synaptobrevin) is anchored in the synaptic vesicle membrane via a C-terminal transmembrane region, whereas the t-SNAREs syntaxin 1 and SNAP-25 (synaptosome-associated protein of 25 kDa) are predominantly inserted in the plasma membrane via a C-terminal transmembrane region and palmitoylation of centrally located cysteine residues, respectively (1, 6–8). The key role of synaptic SNAREs is underlined by the fact that they are specifically cleaved by the endoprotease activity of botulinum and tetanus (TeNT) neurotoxins, which potently inhibit neurotransmitter release (9). Although the precise role of SNAREs is still debated, vesicle fusion requires the assembly of SNARE complexes at the interface between a docked synaptic vesicle and the target membrane (10). Bacterially expressed SNAREs reconstituted into liposomes mediate membrane fusion, and fluorimetric monitoring of lipid mixing provides an assay to analyze the molecular mechanisms that control trans-SNARE assembly (11).

SNAREs do not display intrinsic Ca\(^{2+}\) sensitivity. Ca\(^{2+}\)-dependence of fast neurotransmitter release is generally thought to be conferred by synaptotagmins, a family of Ca\(^{2+}\)-sensor proteins that interact with SNAREs (1, 12). Synaptotagmin 1 is a synaptic vesicle protein with tandem C2 domains that bind Ca\(^{2+}\), promoting the interactions of synaptotagmin with t-SNAREs and anionic phospholipids (1, 12). Electrophysiological analysis of neurotransmitter release from neurons of synaptotagmin null flies and mice suggests that synaptotagmin ensures the synchronization of Ca\(^{2+}\)-dependent exocytosis with the presynaptic action potential (13–15). Furthermore, in SNARE liposome fusion assays, addition of the soluble cytoplasmic domain of synaptotagmin (sytC2AB) or incorporation of full-length lipid-anchored synaptotagmin into v-SNARE liposomes promotes lipid mixing in a Ca\(^{2+}\)-dependent manner (16–20).

Synaptotagmin, however, is not the only Ca\(^{2+}\) effector that regulates release, and considerable evidence supports a role for calmodulin in SNARE-mediated membrane traffic throughout evolution, from yeast vacuoles to mammalian neuroexocytosis.

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\(^2\) The abbreviations used are: SNARE, SNAP (soluble NSF attachment protein) receptors; t-SNARE, target-SNARE; v-SNARE, vesicle-SNARE; TeNT, tetanus neurotoxin; sytC2AB, cytosolic domain of synaptotagmin 1; PC, 1-palmitoyl-
2-oleoylphosphatidylcholine; PS, 1,2-dioleoylphosphatidylserine; NBD, N-(7-nitro-2,1,3-benzoxadiazol); PE, 1,2-dipalmitoyl phosphatidyl-
ethanolamine; SPR, surface plasmon resonance; CaM, calmodulin; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay.
Unraveling the mode of action of calmodulin in cellular assays is a complex task, because multiple proteins implicated in exocytosis are targets of calmodulin (e.g., Ca²⁺ channels, Ca²⁺/calmodulin kinase II, Rab3A and Munc13). Our laboratory has previously identified direct molecular interactions between calmodulin and a v-SNARE machinery. Ca²⁺/calmodulin binds to VAMP2 via a conserved basic amphipathic motif overlapping the C-terminal end of its SNARE domain (29), and mutations of basic or hydrophobic residues strongly inhibit Ca²⁺-dependent exocytosis (30). This membrane-proximal region of VAMP2 also interacts with acidic phospholipids that limit trans-SNARE complex assembly (31–33).

Functional analysis of Ca²⁺-dependent exocytosis in neurons and neuroendocrine cells is consistent with the conclusion that calmodulin regulates events just before fusion is triggered (22–24, 26, 28), although it has also been suggested that calmodulin operates post-fusionally in endocytosis (34, 35).

To determine whether direct binding of calmodulin to the SNARE machinery can confer Ca²⁺-dependent regulation on membrane fusion, we have used a fluorimetric assay to monitor lipid mixing between v- and t-SNARE liposomes in the presence or absence of synaptotagmin. Our data indicate that Ca²⁺/calmodulin specifically restricts trans-SNARE complex assembly and inhibits membrane fusion by binding to VAMP2 and to a newly identified motif in syntaxin 1. Furthermore, they are consistent with the hypothesis that Ca²⁺ regulation of SNARE-mediated fusion involves the antagonistic action of calmodulin and synaptotagmin.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Protein Purification**—Human calmodulin and biotinylated bovine calmodulin were from Calbiochem. Plasmids used to generate VAMP2 constructs (residues 1–116 and 1–92 subcloned into pET28a; residues 1–96 in pGEX-2T) and the syntaxin 1a/SNAP25 heterodimer (co-expressed or individually expressed from pGEX-KG and pET28a respectively) were provided by G. Schiavo (London Research Institute, London, UK). Syntaxin 1a(186–265) (H3ΔTM, pGEX-4T) was generated from a 186–286 construct (36) provided by W. A. Catterall (University of Washington, Seattle), and point mutations were performed using a Stratagene PCR kit. cDNAs encoding calmodulin and calmodulin mutants (pGEX-4T), TeNT light chain (pET28a), and the C2AB fragment of synaptotagmin 1 (pGEX-2T) were obtained from A. Lee (Emory University, Atlanta, GA), H. Niemann (Medizinische Hochschule, Hannover, Germany), and J. Lang (Université de Bordeaux, Bordeaux, France), respectively. Proteins were purified using standard methods and were either eluted or cleaved from their tags by thrombin, and sytC2AB was purified as reported previously (37). Full-length synaptotagmin 1 was cloned into pET28a. SDS-PAGE of the purified recombinant proteins is illustrated in supplemental Fig. 1.

**Proteoliposomes**—Reconstitution of v- and t-SNARE liposomes (11) was carried out at a 1:100 (mol/mol) protein-to-lipid ratio. Full-length synaptotagmin was incorporated with VAMP2 at a ratio of 1:4.5 (mol/mol) using exactly the same procedure but in the presence of 1 mM EGTA (19). Lipid compositions (from Avanti Polar Lipids) were 85% (mol/mol) 1-palmitoyl,2-oleoylphosphatidylcholine (PC), 15% 1,2-dioleoylphosphatidylserine (PS) for the v-SNARE liposomes, and 83% PC, 15% PS, 1.5% (mol/mol) N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-PE), and 1.5% (mol/mol) N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-phosphatidylethanolamine (rhodamine-PE) for t-SNARE liposomes. Dried lipids were resuspended in VAMP2 or syntaxin 1/SNAP25 heterodimer solutions in 15% (w/v) sodium cholate (Sigma), at a 1:100 (mol/mol) protein-to-lipid ratio. Liposomes were obtained by rapid dilution and extensive dialysis in 25 mM HEPES-KOH, pH 7.4, 140 mM KCl, 1 mM dithiothreitol in the presence of Bio-Beads (Bio-Rad), and unincorporated proteins and aggregates were removed by a 4-h centrifugation (250,000 × g) on a discontinuous Optiprep (Abcys) gradient (31).

**Fusion Assays and Data Analysis**—In each fusion reaction, 2 µl of t-SNARE liposomes (100–150 nm full-length syntaxin 1a/SNAP25 dimer) were mixed on ice with 10 µl of v-SNARE liposomes (2–2.5 µM VAMP2) in 25 mM HEPES-KOH, pH 7.4, 140 mM KCl, 1 mM dithiothreitol, 3 mM EGTA, and the appropriate amount of Ca²⁺ in a total volume of 50 µl, and then fusion was monitored at 37 °C. Calmodulin was preincubated 30 min on ice with t-SNARE liposomes prior to v- and t-SNARE mixing. In control experiments, t-SNARE liposomes were preincubated with 10 µM VAMP4TM W89A, W90A for 2 h before mixing to define SNARE-independent fusion. This mutation in the calmodulin-binding site precludes interactions with calmodulin or anionic phospholipids (30). Each assay condition was carried out in triplicate. Fluorescence values obtained by maximal dequenching with excess sodium cholate at the end of each reaction were set to 100%. Representative experiments illustrating absolute amounts of fusion as dequenching (percentage total fluorescence) are illustrated in Fig. 1. In all other figures, data were normalized to the control fluorescence level at 60 min, and unless otherwise specified, means from at least three totally independent experiments using different liposome preparations are presented ± S.E. Experiments were performed in Nunclon fluorescence plates using a BioTek Sirius HT injector plate reader and KC4 software to monitor fluorescence at 525 nm with an excitation wavelength of 485 nm and data acquisition every 2 min. For data analysis, the lowest values obtained in controls, after the initial drop in fluorescence due to fluorophore warming (16), were set to t = 0, and all the conditions were analyzed from the same time point.

**SNARE Complex Assembly**—His-VAMP2(1–92) (10 pmol) was incubated with t-SNARE liposomes (35 pmol of the syntaxin 1/SNAP25 dimer) to form complexes in the presence or absence of recombinant calmodulin. At the indicated time, 60 pmol of TeNT was added, and samples were incubated for 2 h to cleave uncomplexed His-VAMP2(1–92). Samples were then boiled and processed for Western blotting. His-VAMP2(1–92) that had been protected from TeNT cleavage by SNARE complex assembly was revealed with a polyclonal antibody directed against the N terminus of VAMP2.

**Surface Plasmon Resonance**—SPR spectroscopy was performed at 25 °C on a Biacore 3000 system (GE Healthcare). Biotinylated bovine brain calmodulin (Calbiochem) or peptides were loaded onto a neutravidin-coated CM5 sensor chip to
yield 400–600 or 300 resonance units, respectively, and then both control and experimental flow cells were saturated with biotin. SNAREs or calmodulin were injected in the running buffer (25 mM HEPES-KOH, 140 mM KCl, pH 7.4, in presence of either 0.5 mM CaCl$_2$ or 5 mM EGTA). Sensorgrams illustrate the difference between signals from experimental and control cells. To monitor SNARE complex assembly, His-VAMP(1–92) (2000–3000 resonance units) was coupled

**FIGURE 1. Ca$^{2+}$/calmodulin inhibits SNARE-dependent fusion.**

A, v-SNARE liposomes, containing rhodamine-PE and NBD-PE, were preincubated for 60 min at 37 °C in the absence (black circles) or presence (gray circles) of 400 nM recombinant TeNT. Upon addition of t-SNARE liposomes, membrane fusion resulted in an increase in fluorescence, which was strongly inhibited when VAMP2 was cleaved by TeNT. B, t-SNARE liposomes, containing rhodamine-PE and NBD-PE, were mixed with v-SNARE liposomes in the absence (black circles) or presence (gray circles) of 10 μM VAMP$\Delta$TM. Fusion was inhibited when t-SNAREs were saturated with soluble VAMP2 cytosolic domain, blocking productive trans-SNARE complex assembly. C, membrane fusion was measured upon mixing t-SNARE and v-SNARE liposomes in buffer containing 50 μM Ca$^{2+}$, in the absence (black circles) or in the presence (gray circles) of 10 μM human brain calmodulin. SNARE-dependent fusion was defined by blocking trans-SNARE pairing with 10 μM VAMP$\Delta$TM (triangles). Mutant VAMP$\Delta$TM (NB His-VAMP2(1–92) W89A,W90A) was used as a control in all subsequent fusion experiments to preclude direct interactions with calmodulin or anionic phospholipids (30). Data were normalized by setting controls at 60 min to 100%, means ± S.E., n = 5. D, assays with 50 μM Ca$^{2+}$ (controls, black circles), 10 μM bacterially expressed wild-type calmodulin (gray circles) or mutant calmodulin (open triangles) with reduced Ca$^{2+}$-binding affinity, means ± S.E., n = 6. E, fusion assays were performed as in D but with 3 mM EGTA and no added Ca$^{2+}$, in the absence (black circles) or presence (gray circles) of 10 μM wild-type calmodulin, means ± S.E., n = 5. Inhibition of fusion by calmodulin was abolished in the absence of Ca$^{2+}$. F, dose-effect curve. Assays with 50 μM Ca$^{2+}$ and the indicated concentrations of human brain calmodulin, means ± S.E., n = 4. Fusion measured in the presence of 10 μM VAMP$\Delta$TM was subtracted from each trace to determine the % inhibition of SNARE-dependent fusion at 60 min with each calmodulin concentration.
to the sensor chip and syntaxin1/SNAP-25 dimer injected into the flow buffer.

**ELISA**—Glutathione-coated microwell plates (Pierce) were saturated with wild-type or mutant GST-calmodulin at 4 °C. After washing with 25 mM HEPES-KOH, pH 7.4, 140 mM KCl, 1 mM dithiothreitol, 0.1% bovine serum albumin, v- or t-SNARE liposomes were added (5 pmol of proteins) with 4 mM EGTA or 50 μM CaCl₂ and incubated for 90 min at 37 °C, with each assay condition in triplicate. Wells were then washed four times with CaCl₂ or EGTA buffers, and bound SNAREs were detected with either anti-VAMP2 polyclonal antibody (0.5 μg/ml) or the anti-syntaxin1 antibody (mAb10H5, 5 μg/ml) and peroxidase-coupled secondary antibody (1 h at 37 °C, followed by four washes). 3,3′,5,5′-Tetramethylbenzidine liquid peroxidase substrate (Sigma) was then added to each well; the reaction was stopped at 20 min with 1 M H₂SO₄, and absorbance was measured at 450 nm using the BioTek plate reader.

**Pulldown Experiments**—GST-H3ΔTM (5 pmol) was preincubated for 1 h in the presence or absence of sytC2AB and then incubated with calmodulin-agarose beads for 90 min at 37 °C with 0.3 mM CaCl₂ or 4 mM EGTA. Beads were washed four times with CaCl₂ or EGTA buffers, and samples were processed for Western blotting. Bound proteins were stained with an anti-GST antibody (GE Healthcare) and a monoclonal anti-synaptotagmin 1 antibody (mAb1D12).

**RESULTS**

**Ca²⁺/Calmodulin Inhibits SNARE-dependent Liposome Fusion**—Fusion between v-SNARE and t-SNARE liposomes was measured using a fluorescence (rhodamine/NBD) dequenching assay to monitor lipid mixing. Initial experiments were performed in the two possible liposome configurations (i.e. fluorophores integrated in either v- or t-SNARE liposomes). A predominant SNARE-dependent component of fusion was measured as the fraction inhibited either by treatment with TeNT light chain, which specifically cleaves VAMP2 (Fig. 1A), or by saturating t-SNAREs with an excess of soluble VAMP2 cytosolic domain (Fig. 1B). In all subsequent experiments, we integrated rhodamine-PE and NBD-PE in t-SNARE liposomes and used VAMPΔTM to define SNARE-driven fusion. In the presence of Ca²⁺, purified calmodulin from brain inhibited fusion by about 50% (Fig. 1C). Ca²⁺/calmodulin selectively reduced the SNARE-dependent component, as it had no effect in the presence of VAMPΔTM (Fig. 1C). Similar data were obtained with bacterially expressed wild-type calmodulin, which inhibited fusion in the presence of 50 μM Ca²⁺ (Fig. 1D), whereas mutant calmodulin (CaM₁₂₅₆) with alanine substitutions for aspartate residues that impair Ca²⁺ coordination in both N- and C-lobes of calmodulin was ineffective (38). Furthermore, inhibition by wild-type calmodulin was abolished in the presence of EGTA confirming that it is Ca²⁺-dependent (Fig. 1E). Finally, inhibition of the SNARE-dependent component of fusion displayed concentration dependence, with a threshold at 0.3 μM reaching close to 50% inhibition at 10 μM (Fig. 1F).

As the level of inhibition attained was about 50%, we asked whether Ca²⁺/calmodulin might stabilize a hemi-fused state in which outer phospholipid leaflets have merged, but inner leaflets are separate. Preincubation with sodium dithionite selectively extinguishes outer leaflet fluorescence and consequently reduced fluorescence by half (Fig. 2A). In these conditions, Ca²⁺/calmodulin still produced 50% inhibition of fusion (Fig. 2B). These data indicate that 10 μM calmodulin does not promote hemifusion but globally reduces lipid mixing by 50%.

**Ca²⁺/Calmodulin Inhibits SNARE Complex Assembly**—Is inhibition of fusion by calmodulin the consequence of a reduction in SNARE complex assembly? Assembly of VAMP2 into SNARE complexes protects it from the proteolytic action of tetanus neurotoxin (39). Thus, acquisition of resistance to TeNT can be used to monitor the formation of SNARE complexes. Preliminary experiments (data not shown) with liposomes containing full-length SNAREs yielded multiple high molecular weight SDS-resistant complexes that were poorly resolved by PAGE and difficult to quantify. Thus, as reported (19), SNARE assembly was achieved by incubating cytosolic VAMP2 domain with t-SNARE liposomes. Experiments were carried out in the presence or absence of Ca²⁺/calmodulin (Fig. 3), and then a large excess of TeNT light chain was added at the indicated times to digest unassembled VAMP2. Incubations were stopped by boiling in SDS, and TeNT-resistant mono-
meric VAMP was quantified by Western blotting yielding an index of SNARE assembly. In the absence of t-SNARE liposomes, VAMP2 was almost totally proteolyzed by TeNT, and proteolysis was not affected by calmodulin alone (Fig. 3A, right panel). When t-SNARE liposomes were added, an increase in TeNT-resistant VAMP2 occurred at 5 and 30 min, but this increase was clearly restricted in the presence of 1 or 10 μM calmodulin and Ca2+ (Fig. 3A, left panel, and B). Thus, SNARE assembly shielded VAMP2 from TeNT. Protection from cleavage was reduced in the presence of Ca2+/calmodulin (Fig. 3, A–C), but to a lesser extent with mutant CaM1234, which has a reduced affinity for Ca2+ (Fig. 3C). Furthermore, SPR (Biacore) measurements indicated that assembly of cytoplasmic SNARE domains into complexes was blocked by wild-type but not by mutant calmodulin (Fig. 3D). These data indicate that calmodulin inhibits SNARE complex formation in a Ca2+-dependent manner, which in turn attenuates liposome fusion.

Inhibition of Fusion Is Mediated by Distinct Calmodulin-binding Sites on VAMP2 and Syntaxin—Where are the calmodulin-binding sites located that account for inhibition of SNARE assembly and fusion? We have previously reported a Ca2+/calmodulin-binding motif, close to the transmembrane region of VAMP2, overlapping the C-terminal extremity of the SNARE domain (29). A VAMP(77–90) peptide bound Ca2+/calmodulin, and the interaction was strongly reduced by the W89A, W90A mutation (30). To determine whether this calmodulin-binding motif underlies inhibition of SNARE-mediated fusion, experiments were performed using v-SNARE liposomes (Fig. 4B). This result is close to the 1.4-fold increase expected (because of the increment in bound molecular mass) when 1:1 syntaxin/SNAP-25 heterodimers rather than syntaxin monomers interact with calmodulin. These data indicate that SNAP-25 does not interfere with calmodulin binding to syntaxin. They are consistent with binding of calmodulin to pre-assembled t-SNARE heterodimers via syntaxin. In contrast, when pre-assembled trimeric SNARE complexes were injected, no interaction with calmodulin was detected (Fig. 4B). These data suggest that calmodulin-binding sites on VAMP2 and syntaxin 1 are occluded when the SNARE complex is fully zipped.

The Ca2+/calmodulin binding affinity of syntaxin 1 H3 domain was determined by SPR, KD = 2.3 μM ± 0.6, n = 6 (Fig. 5A and supplemental Fig. 2). Analysis of the syntaxin 1 sequence in the calmodulin target data base strongly predicted a single calmodulin-binding motif in the C-terminal region (Asp250–Arg262), overlapping the SNARE domain. Thus, a peptide encompassing syntaxin246–265) was immobilized on an SPR sensor chip. Recombinant calmodulin at 2 μM, but not mutant CaM1234, displayed specific association (Fig. 5B). For comparison, a peptide containing the calmodulin-binding domain of VAMP (VAMP(77–94)) was analyzed in a parallel cell on the same sensor chip. VAMP(77–94) peptide bound significantly more calmodulin than syntaxin246–265) (Fig. 5B), consistent with the KD = 0.5 μM for GST-VAMP4TM (29), which is about 4-fold lower than GST-H3ATM. Thus, syntaxin 1 also carries a calmodulin-binding motif in a membrane-proximal position, topologically analogous to the calmodulin motif with mutant VAMP (W89A, W90A) and wild-type t-SNARE liposomes. Intriguingly, Ca2+/calmodulin still strongly inhibited fusion, although binding to VAMP was reduced (see below and Fig. 6D). These findings suggest that SNAREs must contain another previously undetected calmodulin-binding motif.

This hypothesis was explored using SPR. Biotinylated calmodulin was coupled to a neutravidin sensor chip, and cytosolic domains of SNARE proteins were injected into the flow cell. Injections at a single concentration (0.4 μM) did not reveal interactions of calmodulin with GST (not shown) or SNAP-25 (Fig. 4A). However, a strong SPR signal was generated by GST-VAMP and to a lesser degree by GST-syntaxin H3 domain (Fig. 4A). We next asked whether assembly of syntaxin/SNAP-25 heterodimers might influence calmodulin binding to syntaxin. SPR measurements indicated that preincubation of GST-syntaxinATM with a 10-fold molar excess of HisSNAP-25 led to a 1.6-fold increment in SPR signal.

**FIGURE 3. Ca2+/calmodulin inhibits SNARE complex assembly.** A, VAMP4TM (10 pmol) was incubated with (left panel) or without (right panel) t-SNARE liposomes (35 pmol) in 0.5 mM Ca2+ and in the presence or absence of calmodulin. In each condition, after 0, 5, and 30 min of SNARE assembly, TeNT light chain (2 μM) was added, and samples were then incubated for a further 2 h to allow VAMP cleavage. Following denaturation at 100 °C, VAMP2 that had been protected from TeNT by assembly into SNARE complexes was quantified by Western blotting. For representative Western blots, VAMP2 that had been protected from TeNT by assembly into SNARE complexes was quantified by Western blotting. For representative Western blots, VAMP2 that had been protected from TeNT by assembly into SNARE complexes was quantified by Western blotting. For representative Western blots, VAMP2 that had been protected from TeNT by assembly into SNARE complexes was quantified by Western blotting.


**Ca\(^{2+}\) Sensors in SNARE-dependent Membrane Fusion**

![Graph A](image1.png)

**Graph A**: Ca\(^{2+}\)/calmodulin binding to syntxin 1 and VAMP2. A, biotinylated calmodulin was immobilized on a neutravidin sensor chip in the flow cell of an SPR apparatus. GST, SNAP-25, and GST fused to syntaxin 1 H3 domain (residues 246–265) or VAMP2 cytoplasmic domain were injected into the running buffer. The nonspecific signal obtained with GST or buffer was subtracted, and the SPR signals generated by 0.4 μM GST-SNAREs in 0.5 mM Ca\(^{2+}\) was measured at the beginning of the dissociation phase, means from a representative experiment ± S.D., n = 3. B, full-length GST-syntaxin 1ΔTM (0.5 μM) was preincubated in the presence or absence of His-SNAP-25 (5 μM) and then injected over calmodulin as in A. The 1.6-fold increase in SPR signal measured when syntaxin was injected with SNAP-25 is consistent with the increment expected (1.4-fold) from the additional molecular mass bound when syntaxin/SNAP-25 heterodimers bind to calmodulin. In contrast, 0.5 μM SNAP25 complexes (syntaxin-SNAP-25-VAMP), which were pre-assembled and then isolated by gel filtration, did not interact with calmodulin.

in VAMP. Globally, \(K_D\) values are compatible with inhibition of SNARE liposome fusion at low micromolar calmodulin. To confirm the location of the calmodulin-binding motif and to ascertain whether interactions between Ca\(^{2+}\)/calmodulin and syntaxin 1 are involved in regulating SNARE-dependent fusion, we designed mutations that perturb binding. The K260E,K264E mutations in the basic C-terminal stretch of amino acids strongly reduced Ca\(^{2+}\)-dependent interaction with calmodulin (Fig. 5, C and D), leading to an estimated 10-fold increase in \(K_D\) (determined as in Fig. 5A, results not shown). Furthermore, calmodulin binding to H3ΔTM was eliminated when 4 mM EGTA was substituted for Ca\(^{2+}\) (Fig. 5D). These observations probably explain why we previously failed to detect calmodulin binding to syntaxin 1 using a GST-syntaxin(1–261) construct that lacked the critical C-terminal residues (29).

The calmodulin interaction sites of VAMP2 and syntaxin 1 are both close to the membrane, and interactions between these domains and phospholipids have been documented (32, 40, 41). Consequently, lipids may interfere with calmodulin binding to the juxtamembrane SNARE sequence. Hence, we checked whether calmodulin-binding motifs are accessible when full-length SNAREs are anchored in liposomes. GST-calmodulin was immobilized in glutathione-coated 96-well plates and then incubated with v- or t-SNARE liposomes. Liposome capture by calmodulin was quantified by ELISA, using antibodies against N-terminal regions of VAMP2 or syntaxin 1. Both v-SNARE (Fig. 6A) and t-SNARE (Fig. 6B) liposomes displayed Ca\(^{2+}\)-dependent binding to wild-type calmodulin. Interactions with mutant CaM1234, or with wild-type calmodulin in the presence of EGTA, were reduced, and both controls indicated a similar level of nonspecific binding. Mutations located close to the C-terminal ends of the calmodulin-binding motifs in VAMP2 (W89A,W90A) and in syntaxin 1 (K260E,K264E) significantly reduced the Ca\(^{2+}\)-dependent component of binding to calmodulin. These results demonstrate that full-length membrane-inserted VAMP and syntaxin both bind Ca\(^{2+}\)/calmodulin, and mutations within the defined motifs attenuate the Ca\(^{2+}\)-dependent component of the interaction by about 50%. Consequently, we examined the effects of individual and combined mutations on Ca\(^{2+}\)-dependent inhibition of fusion by calmodulin.

Neither individual nor combined mutations had significant effects on the basal rate of fusion. In assays with mutant t-SNARE (syntaxin K260E,K264E) plus wild-type v-SNARE liposomes, or with wild-type t-SNARE plus mutant v-SNARE (VAMP2 W89A,W90A) liposomes, inhibition of fusion by Ca\(^{2+}\)/calmodulin clearly persisted (Fig. 6D). However, when mutant t-SNARE and mutant v-SNARE liposomes were combined, inhibition by calmodulin was reduced from 50 to 10% at 60 min (Fig. 6, C and D). Note that the double mutation practically abolished the effect of calmodulin over the first 30 min (Fig. 6C). It is thus likely that interactions of calmodulin with sites on both VAMP2 and syntaxin 1 contribute to inhibition of SNARE assembly and lipid mixing.

Finally, SPR experiments with PS/PC bilayers immobilized on a hydrophobic L1 chip were performed to determine whether calmodulin binds to protein-free lipid bilayers. A positive control was provided by GST-VAMPΔTM, which interacts with acidic phospholipids (29, 30). GST-VAMP injected at 0.1 μM yielded an SPR signal = 74.3 ± 0.6 resonance units, whereas calmodulin at 2 μM = 0.6 ± 0.8 resonance units (data not shown). We conclude that calmodulin does not interact directly with phospholipids. Thus inhibition involves calmodulin binding to VAMP2 and to the novel interaction domain identified in syntaxin 1.

Calmodulin and Synaptotagmin 1 Have Antagonistic Effects on SNARE-dependent Liposome Fusion—As both calmodulin and synaptotagmin (42, 43) can bind to the membrane-proximal region of syntaxin 1, we examined whether synaptotagmin displaced interactions between t-SNAREs and calmodulin. Syntaxin (GST-H3ΔTM domain) binding to calmodulin was evaluated by pulldown experiments with calmodulin-agarose beads and revealed by Western blotting with anti-GST antibodies (Fig. 7A). Addition of the cytoplasmic domain of synaptotagmin 1 (syt2AB), at equimolar concentrations to syntaxin, inhibited binding to calmodulin. However, blots with anti-synaptotagmin antibodies indicated that synaptotagmin also binds...
**Ca**<sup>2+</sup> Sensors in SNARE-dependent Membrane Fusion

The aim of this study was to analyze the consequences of direct calmodulin binding to SNAREs by evaluating its action on SNARE-mediated liposome fusion *in vitro*. Calmodulin conferred **Ca**<sup>2+</sup>-dependent inhibition of liposome fusion. This effect of calmodulin required the presence of SNARE proteins because no inhibition occurred when trans-SNARE pairing was blocked by adding excess soluble VAMP2 cytoplasmic domain, which binds to liposomal t-SNAREs to form nonproductive complexes. Furthermore, SNARE mutations that reduce calmodulin binding practically abolished inhibition over the first 30 min of the assay. SPR experiments indicated that calmodulin does not bind to SNARE-free lipid bilayers. Thus, inhibition involved calmodulin binding to VAMP2 and to a novel interaction domain identified in syntaxin 1, rather than the direct action of calmodulin on lipids. Our data indicated that the reduction in liposome fusion was associated with inhibition of SNARE complex assembly, consistent with previous findings using SNARE domains in solution (29). A recent report (46) confirmed calmodulin binding to VAMP2 but did not detect an effect on SNARE-mediated liposome fusion, possibly because of the use of a 10-fold lower calmodulin concentration. Our calmodulin binding data indicated *K<sub>D</sub>* values for soluble SNAREs in the 1 μM range, placing them in the low affinity (≥100 nM) category of calmodulin targets. However, our results do not rule out the possibility that inhibition involves a single calmodulin molecule binding simultaneously to the v- and t-SNARE motifs yielding a ternary interaction with higher affinity than that detected by binary binding assays. Complexin 1, another SNARE-interacting protein, has also been shown to inhibit SNARE-dependent liposome fusion at similar concentrations (47). However, both inhibitory and stimulatory effects of complexin have been reported (47–49).

The **Ca**<sup>2+</sup>-dependent calmodulin-binding sequences of VAMP2 and syntaxin 1 are similar in terms of topology. They both terminate in membrane-flanking polybasic stretches but belong to distinct categories in terms of sequence

to calmodulin-agarose. Thus, displacement of calmodulin from t-SNAREs may involve synaptotagmin interacting with both syntaxin and calmodulin. ELISAs with liposomes were also carried out to determine whether calmodulin binding to full-length membrane-inserted t-SNAREs was displaced by sytC2AB. Experiments were performed with phosphatidylinerine-free liposomes to preclude synaptotagmin binding to lipids. Displacement of calmodulin by synaptotagmin was practically maximal at 25 nM sytC2AB (Fig. 7B).

We next asked whether mutually exclusive binding to syntaxin resulted in antagonistic action of synaptotagmin and calmodulin on fusion. Liposome fusion assays were performed using full-length synaptotagmin incorporated into v-SNARE liposomes. Membrane-anchored synaptotagmin promotes liposome fusion (44). However, **Ca**<sup>2+</sup>-dependent activation of full-length synaptotagmin requires abrogation of cis interactions between synaptotagmin and acidic phospholipid headgroups, achieved by omitting phosphatidylinerine from v-SNARE liposomes (19). No effect of removing PS in the absence of synaptotagmin was observed, in agreement with a previous report (45). In these conditions, v-SNARE liposomes with membrane-anchored synaptotagmin displayed a significant increment in fusion compared with control v-SNARE liposomes (Fig. 7C). Activation of fusion was also achieved by addition of soluble sytC2AB (supplemental Fig. 3). Inhibition of fusion by calmodulin was completely overridden when v-SNARE liposomes contained membrane anchored-synaptotagmin (Fig. 7C). Concentration dependence curves indicated
Ca\(^{2+}\) Sensors in SNARE-dependent Membrane Fusion

**FIGURE 6.** Ca\(^{2+}\)/calmodulin binding to membrane-proximal SNARE domains inhibits membrane fusion. v-SNARE liposomes with wild-type or W89A, W90A VAMP (A) or t-SNARE liposomes with full-length wild-type (wt) or K260E, K264E syntaxin 1 and SNAP-25 (B) were incubated with GST-calmodulin or GST-mutant calmodulin (CaMmut, coupled to microwells in 50 \(\mu M\) Ca\(^{2+}\) or 4 mM EGTA. Bound liposomes were then quantified by ELISA with anti-VAMP or anti-syntaxin 1 antibodies. Error bars are S.E. for the indicated n. Confidence intervals for the null hypothesis in unpaired Student’s t test are indicated. C, membrane fusion assays were performed with mutant v-SNARE (W89A, W90A VAMP2) liposomes and mutant t-SNARE (K260E, K264E syntaxin, wild-type SNAP-25) in 50 \(\mu M\) Ca\(^{2+}\) and in the absence (black circles) or presence (gray circles) of 10 \(\mu M\) recombinant calmodulin, means \(\pm\) S.E., n = 6. D, fusion assays were performed with the four possible combinations of wild-type and mutant t- and v-SNARE liposomes (wild-type syntaxin, wild-type + mutant v-SNARE liposomes, wild-type syntaxin + mutant t-SNARE, wild-type + wild-type). W89A, W90A VAMP + wild-type syntaxin, WAWA + wild-type; wild-type VAMP + K260E, K264E syntaxin, wild-type + KEKE; W89A, W90A VAMP + K260E, K264E syntaxin, WAWA + KEKE). The inhibition of fusion by 10 \(\mu M\) calmodulin was measured at 60 min, means \(\pm\) S.D., n = 3.

VAMP2 contains a typical calmodulin motif with hydrophobic residues in positions 1–5–8–14 and a net charge of 5+ (29). The calmodulin-binding site of syntaxin resembles a basic motif, with a net charge of 8+ in which lysine/arginine residues alternate or occur in tandem with alanines.

The SNARE complex is stabilized in its core by 16 interaction layers, which are hydrophobic with the exception of the ionic 0 layer. The last four (+5 to +8) overlap the predicted calmodulin-binding motifs on VAMP and syntaxin, consistent with Ca\(^{2+}\)/calmodulin preventing the SNARE complex from zipping up in an N- to C-terminal direction. A helical wheel projection of the two calmodulin-binding sites illustrates their amphipathic nature with basic or hydrophobic residues clustered on opposite sides of the helix (supplemental Fig. 4). Basic amphipathic peptides interact with membranes in a parallel orientation, with the positive charges at the surface and the hydrophobic side contacting the fatty acid chains. The membrane-proximal domains of both VAMP2 and syntaxin 1 interact with the cis membrane and may contribute to fusion (29–32, 40, 41, 50). As the SNARE complex zips up, it is likely that tangentially inserted amphipathic domains promote the membrane curvature required for fusion. Ca\(^{2+}\)/calmodulin binding to SNAREs could thus screen juxtamembrane regions from interactions with lipids and prevent lipid mixing. Regulatory mechanisms of this kind, involving mutually exclusive binding of Ca\(^{2+}\)/calmodulin or acidic membrane phospholipids, have been documented. Consequently, we suggest that Ca\(^{2+}\)/calmodulin inhibits fusion by both restricting SNARE complex assembly and reducing SNARE/lipid interactions at membrane interfaces. Furthermore, Ca\(^{2+}\) regulation at these sites may not be restricted to calmodulin, as other EF-hand sensor proteins such as NCS-1 can bind to calmodulin target sequences (51).

In our assays, full-length synaptotagmin was anchored in v-SNARE liposomes with a composition (10 copies synaptotagmin/45 copies VAMP2) approximating that of synaptic vesicles (52) and a calmodulin concentration close to that of the cytoplasm. Results indicated that synaptotagmin and calmodulin both confer Ca\(^{2+}\) sensitivity to the SNARE machinery, respectively enhancing and restricting fusion. Synaptotagmin binds to the C-terminal region of syntaxin (42, 43), at a site overlapping the calmodulin-binding motif we report here, and it displaced the interaction of calmodulin with t-SNAREs, consistent with antagonistic effects on lipid mixing. A direct interaction between synaptotagmin and calmodulin has also been reported (53), and our data (Fig. 7A) confirm this conclusion. Thus, displacement of the interaction between syntaxin and calmodulin by synaptotagmin may involve synaptotagmin binding to either or both proteins. However, the large molar excess of calmodulin in our fusion experiments makes it unlikely that synaptotagmin blocks the effect of calmodulin by complexing it. Nevertheless, detailed conclusions concerning the molecular mechanisms that underlie their antagonistic effects on fusion must await further analysis.

Patch clamp studies of Ca\(^{2+}\)-dependent transmitter release generally support the view that calmodulin activates the rate of release at a pre-fusion step. In chromaffin cells, dialysis with calmodulin increased the initial rate of exocytosis (26), whereas in nerve terminals calmodulin replenished readily releasable vesicle pools via Ca\(^{2+}\)-dependent priming (25, 28). Furthermore, mutations in the Ca\(^{2+}\)-binding domain of calmodulin prevent its ability to stimulate release in cracked PC12 cells,
Ca\textsuperscript{2+} Sensors in SNARE-dependent Membrane Fusion

indicating that direct Ca\textsuperscript{2+} binding to calmodulin is required to enhance exocytosis (54).

Our laboratory and others have also demonstrated that mutating calmodulin-binding sequences in the membrane-proximal domains of VAMP2 (Trp\textsuperscript{89,90}; see Refs 30, 54, 55) and syntaxin 1 (Lys\textsuperscript{260–265} (41)) reduce evoked exocytosis. Although mutations in these regions reduce calmodulin binding, consistent with the view that calmodulin activates exocytosis, they also inhibit SNARE interactions with acidic phospholipids (30, 41) and may affect SNARE complex dimerization (56). Consequently, data from cellular models cannot readily provide an insight into the molecular mechanisms involved.

The stimulatory effect of calmodulin was not mimicked in SNARE liposome fusion assays. In contrast, our results indicate that calmodulin restricts SNARE complex assembly and lipid mixing. How can we explain the apparent discrepancy between inhibition of fusion in vitro and activation of exocytosis in vivo? SNARE-liposome fusion provides a biochemical assay for mechanisms that regulate trans-SNARE pairing but does not recapitulate Ca\textsuperscript{2+}-dependent exocytosis. In fact, priming processes were not fully reconstituted in vitro because they require additional proteins upstream of SNARE pairing (25). We suggest that priming may include calmodulin binding to SNAREs to clamp membrane fusion. Calmodulin is a high affinity Ca\textsuperscript{2+} sensor that would predominate at low [Ca\textsuperscript{2+}] to prevent vesicle fusion. Modest elevations of [Ca\textsuperscript{2+}] could thus promote priming without triggering fusion and increment the pool of vesicles subsequently available for release. In support of the calmodulin clamp hypothesis, expression of the VAMP W89A,W90A mutant in VAMP knock-out neurons induced a 2–3-fold increase in spontaneous release and 2-fold decrease in evoked release compared with wild-type rescue (57). This mutation reduces calmodulin binding to VAMP2 (30), but it does not affect SNARE assembly nor binding of complexin or synaptotagmin to the SNARE complex (57). Furthermore, inhibition of calmodulin in intact PC12 cells has been shown to increase basal catecholamine release (58). Thus, like complexin, calmodulin seems to act as a fusion clamp that can be removed by synaptotagmin. A model has been suggested in which the complexin accessory helix interacts with the t-SNAREs near the membrane, forming an alternative four-helix bundle that prevents the v-SNARE from zipping up completely (59). Hence the juxtamembrane region of VAMP2 may remain accessible to regulatory factors in a partially zipped SNARE complex. An interesting future direction will be to determine whether complexin and calmodulin act in a mutually exclusive manner or in synergy.

Antagonism between calmodulin and synaptotagmin might also contribute to Ca\textsuperscript{2+} regulation of post-fusion events. In chromaffin cells, a moderate elevation of [Ca\textsuperscript{2+}], induces transient vesicle fusion events (60–63). Transient fusion (kiss-and-run) involves release through a pore, which then constricts, without vesicle collapse into the plasma membrane (2, 3).


Ca\textsuperscript{2+} Sensors in SNARE-dependent Membrane Fusion

Intriguingly, substituting other divalent cations for Ca\textsuperscript{2+} and the use of pharmacological antagonists have indicated that pore opening and closing involve two distinct Ca\textsuperscript{2+} sensors (57–59). Furthermore, it has been reported that calmodulin must be activated for fusion pore closing to proceed (34). We suggest that restriction of SNARE zipping and lipid mixing by calmodulin might also prevent the vesicle and target membranes from merging until the pore constriction machinery is activated.

In conclusion, the equilibrium between calmodulin versus synaptotagmin binding to SNAREs is presumably determined by cytoplasmic [Ca\textsuperscript{2+}] as calmodulin and synaptotagmin are activated in different but overlapping ranges of [Ca\textsuperscript{2+}]. Calmodulin and synaptotagmin might thus act in concert to define the window of [Ca\textsuperscript{2+}] in which release can occur.

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