The C Terminus of SNAP25 Is Essential for Ca\textsuperscript{2+}-dependent Binding of Synaptotagmin to SNARE Complexes*  

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The plasma membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin and synaptosome-associated protein of 25 kDa (SNAP25) and the vesicle SNARE protein vesicle-associated membrane protein (VAMP) are essential for a late Ca\textsuperscript{2+}-dependent step in regulated exocytosis, but their precise roles and regulation by Ca\textsuperscript{2+} are poorly understood. Botulinum neurotoxin (BoNT) E, a protease that cleaves SNAP25 at Arg\textsuperscript{280}-Ile\textsuperscript{281}, completely inhibits this late step in PC12 cell membranes, whereas BoNT A, which cleaves SNAP25 at Gln\textsuperscript{197}-Arg\textsuperscript{198}, is only partially inhibitory. The difference in toxin effectiveness was found to result from a reversal of BoNT A but not BoNT E inhibition by elevated Ca\textsuperscript{2+} concentrations. BoNT A treatment essentially increased the Ca\textsuperscript{2+} concentration required to activate exocytosis, which suggested a role for the C terminus of SNAP25 in the Ca\textsuperscript{2+} regulation of exocytosis. Synaptotagmin, a proposed Ca\textsuperscript{2+} sensor for exocytosis, was found to bind SNAP25 in a Ca\textsuperscript{2+}-stimulated manner. Ca\textsuperscript{2+}-dependent binding was abolished by BoNT E treatment, whereas BoNT A treatment increased the Ca\textsuperscript{2+} concentration required for binding. The C terminus of SNAP25 was also essential for Ca\textsuperscript{2+}-dependent synaptotagmin binding to SNAP25-syntaxin and SNAP25-syntaxin/VAMP SNARE complexes. These results clarify classical observations on the Ca\textsuperscript{2+} reversal of BoNT A inhibition of neurosecretion, and they suggest that an essential role for the C terminus of SNAP25 in regulated exocytosis is to mediate Ca\textsuperscript{2+}-dependent interactions between synaptotagmin and SNARE protein complexes.

Regulated neurotransmitter secretion is a specialized version of a general membrane fusion mechanism in which exocytotic fusion is strictly Ca\textsuperscript{2+}-regulated. Studies of this process have yielded insights into universal mechanisms for intracellular membrane fusion and the identity of core components of the fusion machinery (1–3). VAMP,\textsuperscript{1} syntaxin, and SNAP25 are the neural protein substrates for clastriolar neurotoxins, a family of highly selective proteases that potently inhibits neurosecretion (4, 5). These proteins are soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that mediate the membrane association of N-ethylmaleimide-sensitive factor, a protein required for membrane fusion (6). The SNARE proteins are capable of assembling into extremely stable heterotrimeric complexes (7–9) that consist of a four-helix bundle in parallel alignment (10–13). A current hypothesis suggests that the formation of SNARE complexes in trans across apposing membranes promotes intimate bilayer interactions and provides the energy to drive membrane fusion (10, 13–16). Studies with donor and acceptor proteoliposomes containing VAMP or syntaxin/SNAP25 indicate that trans-SNARE complexes can mediate bilayer phospholipid mixing and possibly fusion (16). In contrast to the slow fusion mediated by reconstituted SNAREs in vitro (16), neurosecretion is rapid and Ca\textsuperscript{2+}-dependent suggesting that Ca\textsuperscript{2+} may regulate SNARE complex formation (1, 17). Genetic studies have established an important role for the Ca\textsuperscript{2+}-binding vesicle protein synaptotagmin I in rapid neurosecretion (18–21). Synaptotagmin I exhibits Ca\textsuperscript{2+}-dependent interactions with acidic phospholipids as well as with syntaxin (22–27) leading to its suggested role as a Ca\textsuperscript{2+} sensor for exocytosis (1, 18, 22, 23). The precise role of synaptotagmin and its Ca\textsuperscript{2+}-dependent interactions with phospholipids or syntaxin in regulated neurotranscretion remains to be determined.

Studies of regulated exocytosis in membrane preparations from neuroendocrine cells demonstrated that SNAREs are required for a late Ca\textsuperscript{2+}-dependent step that occurs after vesicle docking and ATP-dependent priming and immediately before fusion (28, 29). Tetanus toxin and botulinum neurotoxin (BoNT) B, C1, and E completely inhibited the Ca\textsuperscript{2+}-dependent triggering of exocytosis, which implied that VAMP, syntaxin, and SNAP25 participate in steps close to or at fusion. Paradoxically BoNT A, which like BoNT E cleaves SNAP25, was only partially inhibitory for triggered fusion despite proteolysis of SNAP25 (28). Because these toxins cleave SNAP25 at distinct C-terminal sites (Arg\textsuperscript{280}-Ile\textsuperscript{281} for BoNT E and Gln\textsuperscript{197}-Arg\textsuperscript{198} for BoNT A; see Refs. 30 and 31), it was inferred that the domain within the C terminus of SNAP25 between the toxin cleavage sites plays a distinct role in Ca\textsuperscript{2+}-dependent membrane fusion events (28). In the present study, this domain of SNAP25 is revealed to be essential for Ca\textsuperscript{2+}-dependent interactions with synaptotagmin. This finding clarifies classical observations that BoNT A inhibition of neurosecretion is partially reversed by elevating neuronal Ca\textsuperscript{2+} levels (32). Moreover, the results suggest that an important role for the C terminus of SNAP25 in regulated exocytosis is to mediate Ca\textsuperscript{2+}-dependent interactions between synaptotagmin and SNARE protein complexes.

EXPERIMENTAL PROCEDURES  
Assays for Ca\textsuperscript{2+}-activated Exocytosis—PC12 cells grown in Dulbecco’s modified culture medium supplemented with 5% horse serum and...
The effectiveness of toxins in inhibiting Ca$^{2+}$-dependent norepinephrine secretion at high Ca$^{2+}$ concentrations. The effectiveness of toxins in inhibiting Ca$^{2+}$-activated norepinephrine secretion was tested in PC12 cell membrane preparations as described under “Experimental Procedures.” Upper, comparison of inhibition by BoNT A ($10^{-7}$ M) and BoNT E ($10^{-7}$ M). Inhibition by BoNT A decreased at higher Ca$^{2+}$ concentrations, whereas that by BoNT E was substantial over the full range of Ca$^{2+}$. In effect, BoNT A treatment increased the Ca$^{2+}$ concentration required to activate secretion. The EC$_{50}$ for Ca$^{2+}$ in untreated membranes was 2.3 ± 0.3 μM, whereas it shifted to 4.4 ± 1.1 μM in BoNT A-treated membranes (mean ± S.D.; n = 6). Middle, BoNT A inhibition of secretion at different Ca$^{2+}$ concentrations. Indicated concentrations of BoNT A were tested for inhibition of norepinephrine secretion stimulated at 3, 10, and 30 μM Ca$^{2+}$. Lower, comparison of inhibition of norepinephrine secretion by BoNT B (10$^{-7}$ M) and BoNT C1 (10$^{-7}$ M) in the membrane preparation. Inhibition observed with these toxins was reversed to only a small extent at higher Ca$^{2+}$ concentrations. Each data point shown represents the mean of duplicate determinations that differed by less than 3%.
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RESULTS

Ca\(^{2+}\) Reversal of Toxin Inhibition Is Restricted to BoNT A—Studies of the late Ca\(^{2+}\)-dependent step of exocytosis in PC12 cells showed that BoNT E completely inhibited Ca\(^{2+}\)-triggered fusion, whereas BoNT A was only partially, if at all, inhibitory despite effective cleavage of SNAP25 (28, 33). An explanation for this difference between toxins was suggested by earlier studies demonstrating that BoNT A inhibition of neurotransmission was unique in being reversed by elevating Ca\(^{2+}\) levels in synapses (32). We therefore examined the effects of Ca\(^{2+}\) on the BoNT inhibition of regulated norepinephrine secretion in a cell-free assay that utilizes purified PC12 cell plasma membranes containing docked dense-core vesicles (29).

BoNT E treatment strongly inhibited Ca\(^{2+}\)-dependent norepinephrine release at all Ca\(^{2+}\) concentrations tested (Fig. 1, upper panel). In contrast, BoNT A treatment inhibited Ca\(^{2+}\)-dependent norepinephrine release at intermediate Ca\(^{2+}\) concentrations, but inhibition was strongly reduced at high Ca\(^{2+}\) concentrations. BoNT E and BoNT A were present at maximally effective concentrations and catalyzed similar extents of SNAP25 proteolysis (−80%, not shown). Treatment with BoNT A essentially increased the apparent EC\(_{50}\) for Ca\(^{2+}\) in triggering exocytosis (from 2.3 ± 0.3 to 4.4 ± 1.1 μM; mean ± S.D. for six experiments). This increase was not simply the result of a partial attenuation of exocytosis at all Ca\(^{2+}\) concentrations since the percent inhibition by BoNT A decreased with increasing Ca\(^{2+}\) concentrations unlike that for other BoNTs (Fig. 1, middle panel).

The Ca\(^{2+}\) reversal of inhibition by toxin was unique for BoNT A and was not observed for BoNT B, which cleaves VAMP (39), or for BoNT C1, which preferentially cleaves syntaxin (40). These toxins strongly inhibited Ca\(^{2+}\)-dependent norepinephrine release at all Ca\(^{2+}\) concentrations. BoNT E and BoNT A were present at maximally effective concentrations and catalyzed similar extents of SNAP25 proteolysis (−80%, not shown). Treatment with BoNT A essentially increased the apparent EC\(_{50}\) for Ca\(^{2+}\) in triggering exocytosis (from 2.3 ± 0.3 to 4.4 ± 1.1 μM; mean ± S.D. for six experiments). This increase was not simply the result of a partial attenuation of exocytosis at all Ca\(^{2+}\) concentrations since the percent inhibition by BoNT A decreased with increasing Ca\(^{2+}\) concentrations unlike that for other BoNTs (Fig. 1, middle panel).

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Synaptotagmin Interacts with the C Terminus of SNAP25 in a Ca\(^{2+}\)-dependent Manner—Because cleavage of SNAP25 by BoNT A increased the EC\(_{50}\) for Ca\(^{2+}\) activation of exocytosis, we considered the possibility that SNAP25 was involved in the Ca\(^{2+}\) regulation of exocytosis. The Ca\(^{2+}\)-binding vesicle protein synaptotagmin has been proposed to function as a Ca\(^{2+}\) sensor for exocytosis (1, 18, 22), so we determined whether synaptotagmin interacts directly with SNAP25. In the absence of Ca\(^{2+}\), recombinant SNAP25 was found to bind an immobilized recombinant synaptotagmin I cytoplasmic domain (Fig. 2A, upper panel) as was previously reported (44). However, the inclusion of Ca\(^{2+}\) in the binding reaction markedly enhanced SNAP25 binding to synaptotagmin I (Fig. 2A, upper panel). In the reverse orientation, binding of the synaptotagmin I cytoplasmic domain to immobilized SNAP25 was also observed, and the binding was strongly stimulated by inclusion of Ca\(^{2+}\) (Fig. 2A, lower panel). Quantitation of several binding experiments in

![Figure 2](https://www.jbc.org/)

**Fig. 2.** Distinct Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent interactions between synaptotagmin I (Syt I) and SNAP25. A, Upper, binding of SNAP25 to immobilized synaptotagmin I. Glutathione (GSSG)-agarose beads containing 5 μg of GST-cytoplasmic domain fusion protein of synaptotagmin I or without bound protein were incubated with 5 μg of SNAP25 either in the absence or presence of 1 mM Ca\(^{2+}\) as indicated. Beads were washed and solubilized in sample buffer for analysis of the bound fraction by Western blotting and quantified by densitometry. Inclusion of 1 mM Ca\(^{2+}\) increased binding of synaptotagmin I cytoplasmic domain to immobilized SNAP25. B, summary of synaptotagmin I-SNAP25 binding studies. Binding studies similar to those of A and B were analyzed by Western blotting and quantified by densitometry. Inclusion of 1 mM Ca\(^{2+}\) in the incubations stimulated binding 2.5–4-fold above that observed with 2 mM EGTA. C, synaptotagmin I binding to SNAP25 exhibits saturation for Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent interactions. Binding of the indicated concentrations of synaptotagmin I cytoplasmic domain to 5 μg of GST-SNAP25 immobilized on GSSG-agarose beads was determined. In four similar experiments, the stoichiometry of binding at saturation (± Ca\(^{2+}\)) was 0.3–0.8 mol of synaptotagmin I bound per mol of SNAP25.
SNAP25 require the C terminus of SNAP25 and the C2A domain (Fig. 3). Interactions between synaptotagmin I (Syt I) and SNAP25 were investigated. Cleavage of SNAP25 with botulinum neurotoxins was quantitative. Binding in the absence of Ca\(^{2+}\) with immobilized intact GST-SNAP25 was virtually no SNAP25 binding in the presence or absence of Ca\(^{2+}\). High Ca\(^{2+}\) concentrations were required to stimulate synaptotagmin I binding to SNAP25 with half-maximal stimulation observed at \(-200 \mu M\) Ca\(^{2+}\) (Fig. 3A). When tested at 1 mM, Sr\(^{2+}\), Ba\(^{2+}\), and Mg\(^{2+}\) did not stimulate the interaction (not shown). Synaptotagmin I interactions with syntaxin were previously reported to exhibit a very similar Ca\(^{2+}\) dependence and cation specificity (24–27). Synaptotagmin contains two C2 domains that mediate Ca\(^{2+}\)-dependent activities of the protein (45). To define the region of synaptotagmin I required for the Ca\(^{2+}\)-stimulated interaction with SNAP25, recombinant proteins representing the full cytoplasmic domain (C2AB), a membrane-proximal region containing the C2A domain (C2A), or a membrane-distal region containing the C2B domain (C2B) were used as immobilized ligands. The C2B protein exhibited virtually no SNAP25 binding in the presence or absence of Ca\(^{2+}\) (Fig. 3B). In contrast, the C2A fusion protein exhibited Ca\(^{2+}\)-stimulated interactions with SNAP25 although it did not possess the full activity of the complete cytoplasmic domain (C2AB). These results for SNAP25 were similar to those for synaptotagmin I interactions with syntaxin where the C2A domain exhibited partial Ca\(^{2+}\)-dependent binding activity (24–27). The syntaxin binding domain of synaptotagmin I has recently been shown to consist of the C2A domain with a linker region plus a portion of the C2B domain (62).

To define the region of SNAP25 essential for synaptotagmin I binding, immobilized SNAP25 was quantitatively cleaved with BoNT A or BoNT E. SNAP25 truncated by cleavage with either toxin retained its capacity to interact with synaptotagmin I in the absence of Ca\(^{2+}\) (Fig. 3C, closed bars) as previously reported (44). In contrast, the Ca\(^{2+}\)-dependent interaction of SNAP25 with synaptotagmin I was completely abolished by treatment with BoNT E and partially inhibited by treatment with BoNT A (Fig. 3C, open bars). The extent of inhibition of synaptotagmin I binding observed with BoNT A-treated SNAP25 was dependent upon the Ca\(^{2+}\) concentration in the binding reaction with the inhibition strongly reduced at high Ca\(^{2+}\) concentrations (Fig. 3A). BoNT A treatment essentially increased the EC\(_{50}\) for Ca\(^{2+}\) stimulation of synaptotagmin I binding to SNAP25.

Fig. 3. Interactions between synaptotagmin I (Syt I) and SNAP25 require the C terminus of SNAP25 and the C2A domain of synaptotagmin I. A, Ca\(^{2+}\) dependence of synaptotagmin-I-SNAP25 interaction. Binding studies were conducted at the indicated Ca\(^{2+}\) concentrations with 5 \(\mu M\) of synaptotagmin I cytoplasmic domain and 10 \(\mu M\) of GST-SNAP25 (control) or BoNT A-treated GST-SNAP25 (BoNT A) immobilized on GSSG-agarose beads. SNAP25 was quantitatively cleaved by BoNT A treatment. Cleavage by BoNT A shifted the Ca\(^{2+}\) dependence of the binding to the right. B, domains of synaptotagmin I required for SNAP25 binding. GST fusion proteins consisting of the complete synaptotagmin I cytoplasmic domain (C2AB), the membrane-proximal domain (C2A), or the membrane-distal domain (C2B) were immobilized in equimolar amounts on GSSG-agarose beads and incubated with 1 mM SNAP25 in the absence (−) or presence (+) of 1 mM Ca\(^{2+}\). SNAP25 binding to the C2B fusion protein was not different from binding to protein-free agarose beads. The C2A fusion protein exhibited significant SNAP25 binding, but it was quantitatively less than that for the C2AB fusion protein. C, effect of cleavage by BoNT A and BoNT E on SNAP25 interactions with synaptotagmin I. Binding reactions were conducted with immobilized intact GST-SNAP25 or with GST-SNAP25 preparations that were cleaved by treatment with BoNT A or BoNT E. Gel electrophoresis and Western blotting confirmed that cleavage by toxins was quantitative. Binding in the absence of Ca\(^{2+}\) (filled histograms) was not affected by BoNT treatment, whereas binding stimulated by Ca\(^{2+}\) (open histograms) was inhibited by BoNT treatment.
Additional studies on native synaptotagmin I and SNAP25 in brain detergent extracts revealed Ca\(^{2+}\)-dependent interactions between these proteins similar to those observed with recombinant proteins (Fig. 4). The specific immunoprecipitation of SNAP25 from rat brain detergent extracts resulted in the co-isolation of synaptotagmin I (Fig. 4A, lane 2), which was markedly enhanced by inclusion of Ca\(^{2+}\) (lane 3 versus 2). The Ca\(^{2+}\)-stimulated retention of synaptotagmin I in the SNAP25 immunoprecipitates was largely eliminated by treatment with BoNT E (lane 4). Neither condition altered the recovery of syntaxin in the SNAP25 immunoprecipitates, and neither condition promoted the retention of CAPS, an abundant cytosolic protein. In a similar manner, synaptotagmin I in brain detergent extracts exhibited Ca\(^{2+}\)-dependent retention on immobilized GST-SNAP25 (Fig. 4B): Ca\(^{2+}\) promoted synaptotagmin I binding over the same concentration range (EC\(_{50}\) ~ 300 \(\mu\)M) as was observed in binary protein binding studies with the recombinant proteins (Fig. 4B, lanes 3–6; compare with Fig. 3A). Cleavage of the C terminus of SNAP25 with BoNT A or E markedly reduced the Ca\(^{2+}\)-dependent binding of native synaptotagmin I to SNAP25 (Fig. 4B, lane 6 versus 7 and 8).

Neither Ca\(^{2+}\) nor toxin treatment markedly affected the binding of native syntaxin to SNAP25.

Ca\(^{2+}\)-Dependence of the Interaction with SNAP25 Varies with the Synaptotagmin Isoform—There were considerable similarities between the effects of Ca\(^{2+}\) in reversing BoNT A inhibition of exocytosis (Fig. 1, upper panel) and in reversing BoNT A inhibition of synaptotagmin I-SNAP25 interactions (Fig. 3A) except that the effective Ca\(^{2+}\) concentrations differed. Several synaptotagmin isoforms that differ in Ca\(^{2+}\) sensitivity have been characterized (26, 27, 45). Ca\(^{2+}\)-dependent interactions of isoforms such as synaptotagmin I with syntaxin occur at high (~200 \(\mu\)M) Ca\(^{2+}\) concentrations, whereas isoforms such as synaptotagmin III exhibit Ca\(^{2+}\)-dependent interactions with syntaxin at lower (~10 \(\mu\)M) Ca\(^{2+}\) concentrations (26, 27). Synaptotagmin III was found to exhibit Ca\(^{2+}\)-stimulated interactions with SNAP25 at much lower Ca\(^{2+}\) concentrations than those required for synaptotagmin I (Fig. 5A). The stimulation of binding exhibited an EC\(_{50}\) for Ca\(^{2+}\) of ~8 \(\mu\)M, which is closer to the EC\(_{50}\) for Ca\(^{2+}\)-activated dense-core vesicle exocytosis (2.3 \(\mu\M, Fig. 1). The Ca\(^{2+}\)-dependent binding of synaptotagmin III to SNAP25 required C-terminal regions of SNAP25 since cleavage by BoNT A and E inhibited the binding (Fig. 5B). High Ca\(^{2+}\) concentrations were partially effective in restoring synaptotagmin III binding to BoNT A-cleaved SNAP25 and to a lesser extent to BoNT E-cleaved SNAP25 (data not shown).

C-terminal Regions of SNAP25 Are Required for Ca\(^{2+}\)-dependent Synaptotagmin Interactions with SNARE Complexes—SNAP25 and syntaxin form relatively stable heterodimeric complexes consisting of a 2:1 molar ratio of syntaxin to SNAP25 (9). Such binary complexes may be the prevalent form of SNAP25 in cellular membranes, so we examined SNAP25 binding to synaptotagmin I in the presence of syntaxin under con-
drive bilayer fusion (14). To determine whether synaptotagmin in the absence of ternary complex formation) (Fig. 7A). Binding incubations similar to those of SNAP25, BoNT A-treated SNAP25, or BoNT E-treated SNAP25 in the absence or presence of 1 mM Ca\(^{2+}\) were conducted with 1 mM SNAP25, BoNT A-treated SNAP25, or BoNT E-treated SNAP25 in the absence or presence of 1 mM Ca\(^{2+}\). Bound SNAREs were detected by Western blotting with syntaxin and SNAP25 antibodies. Syntaxin binding to synaptotagmin I (upper panel), mediated through formation of binary syntaxin-SNAP25 complexes, was inhibited by treatment of SNAP25 with BoNT A or BoNT E. The 15% SDS-acrylamide gels resolved SNAP25 and co-migrating BoNT A-cleaved SNAP25 from higher mobility BoNT E-cleaved SNAP25 (lower panel). An artifact migrated immediately behind SNAP25 (see 11th and 12th lanes). BoNT E-cleaved SNAP25 bound to synaptotagmin I was equivalent to SNAP25 bound in the absence of Ca\(^{2+}\). BoNT A-cleaved SNAP25 bound to synaptotagmin I was equivalent to that observed in Fig. 3A at 1 mM Ca\(^{2+}\). The inhibition of VAMP binding could result either from destabilization of ternary complexes by BoNT treatment (8) or from inhibition of the binding of ternary complexes to synaptotagmin I by the removal of essential portions of SNAP25 by toxin cleavage. Under our conditions, BoNT E treatment was found to destabilize ternary SNARE complexes (Fig. 7D) as previously reported (8), and this likely accounts for the inhibition of Ca\(^{2+}\)-dependent VAMP binding to synaptotagmin I (Fig. 7C). In contrast, BoNT A treatment had virtually no effect on the stability of SDS-resistant ternary SNARE complexes (Fig. 7D) as previously reported (8). Therefore, the reduction in SNARE complex binding to synaptotagmin I resulting from BoNT A treatment (Fig. 7C) implies that SNAP25 C-terminal residues removed by BoNT A cleavage are essential for the Ca\(^{2+}\)-dependent interaction of synaptotagmin I with ternary SNARE complexes.

DISCUSSION

The data in this study indicate that synaptotagmin exhibits a high affinity Ca\(^{2+}\)-dependent interaction with SNAP25 monomers, with syntaxin/SNAP25 heterodimers, and with syntaxin/SNAP25/VAMP heterotrimers. In each case, an essential determinant for Ca\(^{2+}\)-dependent synaptotagmin binding is the C terminus of SNAP25. The results provide an explanation for the reversal of BoNT A inhibition of neurosecretion at elevated Ca\(^{2+}\) concentrations observed in classical studies. They also suggest a plausible basis for the Ca\(^{2+}\) regulation of exocytosis.

In 1977 Thesleff and co-workers (32) reported that treatment with a K\(^+\) channel blocker that increases synaptic accumulation of Ca\(^{2+}\) could partially overcome the paralytic effects of BoNT A in the neuromuscular junction. Similarly Simpson (47) reported that BoNT A decreased the Ca\(^{2+}\) cooperativity of neurotransmitter release and suggested that BoNT A acted close to the site of Ca\(^{2+}\) action in neurotransmitter release to cause a decreased affinity or efficacy of Ca\(^{2+}\). Reversal of inhibition by Ca\(^{2+}\) is relatively unique to BoNT A and has not been observed for tetanus toxin, BoNT B, BoNT F, or BoNT D (41, 48–50) and only to a lesser extent for BoNT E (51, 52). These classical studies, conducted before the molecular targets of BoNT action were known, foreshadowed the conclusion of the present study that the target of BoNT A action, SNAP25, is closely linked to the Ca\(^{2+}\) regulation of neurotransmitter release.

Studies of toxin action in permeable cell assays have found BoNT A to be far less effective in inhibiting Ca\(^{2+}\)-dependent secretion than BoNT E or other toxins in contrast to its effectiveness in intact nerve cells (28, 33, 53). The present results clarify this distinctive feature of BoNT A by finding that the high Ca\(^{2+}\) concentrations used to optimally activate exocytosis in permeable cells results in a reversal of BoNT A inhibition. Because such relatively high Ca\(^{2+}\) concentrations are rarely achieved in intact cell studies, these findings readily account...
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FIG. 7. Synaptotagmin I binds ternary SNARE complexes in a Ca$^{2+}$-dependent manner. A, VAMP binding to synaptotagmin I is mediated by binding of ternary SNARE complexes. Overnight incubations were conducted with 1 μM VAMP and 1 μM syntaxin and/or 1 μM SNAP25 as indicated. Preassembled complexes were incubated in the presence or absence of 1 mM Ca$^{2+}$ with 5 μg of GST-synaptotagmin I immobilized on GSSG-agarose beads. Bound VAMP was assessed by Western blotting with a VAMP monoclonal antibody. Ca$^{2+}$-mediated by binding of ternary SNARE complexes. Syntaxin and SNAP25 were preincubated to form heterodimers or preincubated with VAMP to form heterotrimers. Binding of the SYRINX complexes to synaptotagmin I in the presence or absence of 1 mM Ca$^{2+}$ was assessed. Similar amounts of SNAP25 were bound in a Ca$^{2+}$-dependent manner for binary and ternary complex binding. Ternary complex binding resulted in reduced Ca$^{2+}$-dependent binding in accord with the reduced molar content of syntaxin in heterotrimeric compared with heterodimeric complexes. 

B, comparison of synaptotagmin I binding by binary and ternary SNARE complexes. Syntaxin and SNAP25 were preincubated to form heterodimers or preincubated with VAMP to form heterotrimers. Binding to synaptotagmin I require the C terminus of SNAP25. Heterotrimeric complexes were assembled in overnight incubations with full-length, BoNT A-, or BoNT E-treated SNAP25. Binding to synaptotagmin I was determined in the absence (2 mM EGTA) or presence of Ca$^{2+}$ at 0.1, 0.3, and 1.0 mM as indicated. BoNT A treatment partially inhibited (by ~56%) and BoNT E treatment strongly inhibited (by ~72%) Ca$^{2+}$-dependent ternary complex binding to synaptotagmin I. D, BoNT E treatment inhibits ternary SNARE complex assembly, whereas BoNT A has no effect. SDS-resistant SNARE complexes were detected following assembly of complexes with intact, BoNT A-, and BoNT E-treated SNAP25. Samples were split for gel electrophoresis following incubation in sample buffer at 30 °C (−) or boiling (+). SDS-resistant SNAP25 complexes of ~60 and 90 kDa, similar to those previously reported (9), were detected by Western blotting with syntaxin monoclonal antibodies. BoNT A treatment had little if any effect (<10%) on SDS-resistant complexes in three similar experiments. Residual complexes following BoNT E treatment exhibited an altered electrophoretic mobility.

for reported discrepancies between intact cell and permeable cell studies on the efficacy of BoNT A as an inhibitor of regulated secretion (54). Decreased inhibition by BoNT A has also been observed in intact cell studies when high Ca$^{2+}$ concentrations are used to elicit exocytosis (45).

Because BoNT A cleavage of SNAP25 decreased the Ca$^{2+}$ sensitivity of regulated exocytosis, it seemed possible that SNAP25 was involved in the Ca$^{2+}$ sensing reactions of exocytosis. This idea motivated direct binding studies of SNAP25 with synaptotagmin I, a proposed Ca$^{2+}$ sensor for exocytosis (1, 18, 22). A Ca$^{2+}$-independent interaction between these proteins, which does not require the C terminus of SNAP25, was described previously (44). The binding studies reported here, in contrast, revealed a novel Ca$^{2+}$-independent interaction between SNAP25 and synaptotagmin that requires the C terminus of SNAP25. Truncation of SNAP25 from Ile$^{241}$ to Gly$^{206}$ by BoNT E treatment abolished Ca$^{2+}$-dependent synaptotagmin I binding, which correlates with the strong inhibitory effect of this toxin on Ca$^{2+}$-activated exocytosis. Truncation of SNAP25 from Arg$^{128}$ to Gly$^{206}$ by BoNT A treatment, in contrast, modified the Ca$^{2+}$ dependence for the binding of synaptotagmin I to SNAP25 by shifting it to higher Ca$^{2+}$ concentrations, an effect that was similar to that of BoNT A on Ca$^{2+}$-activated exocytosis. The qualitatively similar inhibition by BoNT A and reversal at elevated Ca$^{2+}$ concentrations for both Ca$^{2+}$-dependent synaptotagmin-SNAP25 binding and for Ca$^{2+}$-dependent exocytosis provides a striking correlation. This correlation is consistent with a role for SNAP25 as an important effectors for synaptotagmin in the Ca$^{2+}$-triggering reactions of exocytosis. Another possible basis for the Ca$^{2+}$ reversal of BoNT A inhibition of exocytosis is the cation-binding site in the ternary SNARE complex located near the BoNT A cleavage site (13, 55); however, Ca$^{2+}$ binding to this site remains to be demonstrated.

Previous studies identified a number of Ca$^{2+}$-dependent interactions for synaptotagmin including with acidic phospholipids, SV2, synaptotagmin itself, and syntaxin (23, 24–27, 56, 57, 58), but it has been unclear which, if any, of these mediates the essential role of synaptotagmin in exocytosis. The characteristics of the synaptotagmin I-SNAP25 interaction reported here bear a striking resemblance to those described for the synaptotagmin I-syntaxin interaction (24–27). Both are stimulated at high Ca$^{2+}$ concentrations, are not supported by Sr$^{2+}$ or Ba$^{2+}$, and involve in part the membrane-proximal C2A domain of synaptotagmin I (45). Different synaptotagmin isoforms exhibit characteristic Ca$^{2+}$ dependencies for interactions with both syntaxin and SNAP25. Synaptotagmin I interacts with both SNAREs at Ca$^{2+}$ concentrations similar to those required for triggering synaptic vesicle exocytosis (~200 μM), whereas synaptotagmin III requires the lower Ca$^{2+}$ concentrations characteristic of triggering dense-core vesicle exocytosis (~10 μM) (1–3, 17). The similarity of Ca$^{2+}$-dependent synaptotagmin binding to both SNAREs suggests the possibility that both plasma membrane SNAREs or complexes containing them are the Ca$^{2+}$-dependent effectors for synaptotagmin.

Ca$^{2+}$-dependent synaptotagmin binding to SNAP25 appeared to be of higher affinity than the binding to syntaxin. The
apparent \( K_D \) reported for Ca\(^{2+}\)-dependent synaptotagmin I-syntaxin interactions is \(-0.5\)–2 \( \mu \)M (24, 25), whereas we estimated an apparent \( K_D \) \(-0.2 \mu \)M for Ca\(^{2+}\)-dependent synaptotagmin I-SNAP25 binding. More directly, when compared at the same concentration, Ca\(^{2+}\)-dependent SNAP25 binding to immobilized synaptotagmin I was readily detected, whereas that of syntaxin was not (Fig. 5A). Syntaxin binding to synaptotagmin I was, however, evident in the presence of SNAP25 because syntaxin-SNAP25 binary complexes formed and bound to synaptotagmin I in a Ca\(^{2+}\)-dependent manner. The C terminus of SNAP25 was required for optimal Ca\(^{2+}\)-dependent interactions between synaptotagmin I and binary SNARE complexes. A similar high affinity Ca\(^{2+}\)-dependent binding of ternary SNARE complexes to synaptotagmin I was observed, which also required the C terminus of SNAP25. These results indicate that Ca\(^{2+}\)-dependent interactions between synaptotagmin and SNAP25 or SNARE complexes are mediated in part through the C terminus of SNAP25.

It was surprising that Ca\(^{2+}\)-dependent synaptotagmin binding to SNAP25 or to binary or ternary SNARE complexes was so similar since SNAP25 may be largely unstructured as a monomer but acquires \( \alpha \)-helicity in binary and ternary SNARE complexes (9). Possibly monomeric SNAP25 becomes structured in complexes with synaptotagmin. The C-terminal region of SNAP25 likely represents a direct site for Ca\(^{2+}\)-dependent synaptotagmin binding since Ca\(^{2+}\)-dependent binding to a C-terminal SNAP25 fragment can be detected, and a C-terminal SNAP25 peptide inhibits binding.\(^2\) The region Ile\(^{181}\)Gln\(^{197}\) may constitute the core of this binding site since BoNT E cleavage abolishes Ca\(^{2+}\)-dependent interactions with synaptotagmin. The region Arg\(^{198}\)-Gly\(^{206}\) may indirectly affect binding since cleavage by BoNT A reduces synaptotagmin binding in a manner that is partially compensated by elevating the Ca\(^{2+}\) concentration.

The recent structural elucidation of a protease-resistant core of the ternary SNARE complex as consisting of a four-helix bundle in parallel orientation (13) reveals a potential site for Ca\(^{2+}\)-dependent interactions of synaptotagmin. The C-terminal membrane-proximal region of syntaxin (residues 220–266), reported to mediate Ca\(^{2+}\)-dependent interactions with synaptotagmin I (Refs. 24, 25, and 62 but also see Ref. 59), is juxtaposed to the BoNT-sensitive C terminus of SNAP25 (residues 181–206) (e.g. syntaxin Ile\(^{230}\) aligns with SNAP25 Ile\(^{179}\) of SNAP25; Ref. 13). Residues from these regions contributed by SNAP25 and syntaxin in binary or ternary SNARE complexes may constitute the essential Ca\(^{2+}\)-dependent binding site for synaptotagmin.

The Ca\(^{2+}\) triggering of membrane fusion could be mediated by the Ca\(^{2+}\)-dependent binding of synaptotagmin on the vesicle to syntaxin and SNAP25 on the plasma membrane. Ca\(^{2+}\) could promote synaptotagmin binding to syntaxin/SNAP25 heterodimers, which would bring VAMP on the vesicle into proximity with syntaxin/SNAP25 heterodimers to form ternary SNARE complexes that could initiate fusion (16). Alternatively, Ca\(^{2+}\) could stimulate the binding of synaptotagmin to partially “zipped” pre-assembled ternary SNARE complexes bridging the vesicle and plasma membrane to promote full zipping and the initiation of fusion (10, 14, 15, 60). Finally, Ca\(^{2+}\)-independent interactions might drive the assembly of pre-fusion complexes between synaptotagmin and SNARE proteins (7). The Ca\(^{2+}\)-dependent interaction between synaptotagmin and SNAP25 described here could mediate molecular interactions within the complex that promote a fusion-competent conformation.

\(^2\) E. C. Larsen, unpublished data.
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