SNAP-25 Is Required for a Late Postdocking Step in Ca\(^{2+}\) -dependent Exocytosis*

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Abhijit Banerjee, J. udith A. Kovalchyk, Bibhuti R. DasGupta, and Thomas F. J. Martin†
From the Departments of Biochemistry and Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin 53706

The Ca\(^{2+}\)-activated fusion of large dense core vesicles (LDCVs) with the plasma membrane is reconstituted in mechanically permeabilized PC12 cells by provision of millimolar MgATP and cytosolic proteins. Ca\(^{2+}\)-activated LDCV exocytosis was inhibited completely by the type E but not by the type A botulinum neurotoxin (BoNT) even though both BoNTs were equally effective in proteolytically cleaving the synaptosome-associated protein of 25 kDa (SNAP-25). The greater inhibition of exocytosis by BoNT E correlated with a greater destabilization of detergent-extracted complexes consisting of SNAP-25, synaptobrevin, and syntaxin. LDCVs in permeable PC12 cells can be poised at a late postdocking, prefusion stage by MgATP-dependent priming processes catalyzed by N-ethylmaleimide sensitive factor and priming in exocytosis proteins. BoNT E completely blocked Ca\(^{2+}\)-activated LDCV exocytosis in ATP-primed cells, whereas BoNT A was only slightly inhibitory, implying that the C-terminal region of SNAP-25 (Ile\(^{181}\)-Gln\(^{197}\)) between the cleavage sites for BoNT E and BoNT A is essential for late postdocking steps. A required role for SNAP-25 at this stage was also indicated by inhibition of Ca\(^{2+}\)-activated LDCV fusion in ATP-primed cells by a C-terminal peptide antibody. We conclude that plasma membrane SNAP-25, particularly residues 181-197, is required for Ca\(^{2+}\)-regulated membrane fusion at a step beyond LDCV docking and ATP utilization.

Neurotransmitter and peptide hormone secretion requires fusion between secretory vesicles and the plasma membrane, an exocytotic process activated by cytoplasmic Ca\(^{2+}\) elevation. Understanding regulated secretion requires identification of molecular components that mediate docking and fusion reactions and delineation of rate-limiting steps that are Ca\(^{2+}\)-regulated (1). The synaptic vesicle protein synaptobrevin and the presynaptic membrane proteins syntaxin and SNAP-25 are required components of the exocytotic apparatus as indicated by the inhibitory action of BoNTs and Ttx, which involves the specific endoproteolytic cleavage of these protein substrates (2, 3). The three toxin substrates were independently identified as receptors for SNAPs, proteins required for the membrane binding of NSF, a late acting component in constitutive membrane fusion reactions (4). A characterized ternary complex containing synaptobrevin, syntaxin, and SNAP-25 was suggested to represent a docking complex that mediates the targeting, docking, or fusion of secretory vesicles (5, 6). Identification of an ATP-dependent catalytic activity of NSF/SNAP that promotes the disassembly of ternary complexes in vitro led to the suggestion that a similar reaction in vivo was responsible for late steps in membrane fusion (7).

Ca\(^{2+}\)-triggered secretion in permeable neuroendocrine cells requires ATP; however, the requirement for ATP precedes that for Ca\(^{2+}\) (8, 9). ATP hydrolysis is required for prefusion events that prime the exocytotic apparatus, whereas Ca\(^{2+}\)-activated fusion proceeds in the absence of ATP (8-11). In priming, ATP serves as a substrate for polyphosphoinositide synthesis (10, 11) and as a substrate for the SNAP-dependent ATPase activity of NSF that catalyzes rearrangement of docking protein complexes. These studies experimentally identify a late step in the exocytotic pathway beyond LDCV docking and ATP utilization that is proximal to Ca\(^{2+}\)-dependent fusion reactions. To characterize events that lead to or are directly involved in membrane fusion, it is important to identify molecular components that act at this late Ca\(^{2+}\)-dependent step. The studies presented here describe a requirement for SNAP-25 at this and specify a role for the C-terminal region of SNAP-25 (Ile\(^{181}\)-Gln\(^{197}\)) between the cleavage sites for BoNT E and BoNT A.

**MATERIALS AND METHODS**

**PC12 Cell [\(^{3}\)H]Norepinephrine Loading, Permeabilization, and Secretion Incubations—PC12 cells were incubated 16 h with 0.5 μCi/ml \(^{3}\)H]NE (Amersham Corp.) plus 0.5 mM sodium ascorbate in cold KGlu-BSA buffer (0.02 M HEPES, pH 7.2, 0.12 M potassium glutamate, 0.02 M potassium acetate, 0.002 M EGTA, 0.1% bovine serum albumin) were permeabilized by passage through a ball homogenizer (12). Secretion assays were conducted either as single stage or as two-stage assays (9, 14). For the former, permeable cells were incubated for 15 min at 30°C in KGlu-BSA buffer supplemented with CaCl\(_2\) (to achieve 10 μM Ca\(^{2+}\)). 0.02 M MgATP, and 0.5 mg/ml rat brain cytosol. Two-stage assays were conducted as separate priming incubations (30 min at 30°C in KGlu-BSA buffer supplemented with 0.002 M MgATP plus 0.5 mg/ml rat brain cytosol followed by triggering incubations (1-3 min at 30°C in KGlu-BSA buffer supplemented with 10 μM Ca\(^{2+}\) plus 0.5 mg/ml rat brain cytosol) with extensive washing between incubations. \(^{3}\)H]NE release was determined by centrifugation of permeable cells at 800 x g for 30 min and scintillation counting of \(^{3}\)H in supernatants and in cell pellets to express NE release as percent of the total \(^{3}\)H. The SNAP-25 antibody used to inhibit secretion is a rabbit polyclonal antibody raised against the C-terminal peptide ANQRATKMLGSG generously provided by M. Takahashi (Tokyo). IgGs were purified by chromatography on protein A-Sepharose.

**Immunoprecipitation and Immunoblotting—Permeable PC12 cells were recovered by centrifugation (800 x g for 15 min) and resuspended in lysis buffer (0.02 M HEPES, 0.1% KCI, 0.002 M EDTA, 0.0005 M ATP, 0.001 M dithiothreitol, 1% Triton X-100, and 0.4 mM phenylmethylsulfonyl fluoride, pH 7.3). Docking complexes isolated from dilute detergent extracts were not formed following detergent extraction since their...
Release of the neurotransmitter NE from LDCVs in permeable PC12 cells is triggered by 10 μM calcium in the presence of ATP and cytosolic proteins (14). Ca²⁺-activated release was inhibited by BoNT E and BoNT A (Fig. 1A), confirming previous results (18). The inhibition of Ca²⁺-dependent NE secretion by BoNT E was complete whereas that by BoNT A was incomplete even at high concentrations. Since SNAP-25 is a specific substrate for endoproteolysis by both BoNT A and BoNT E (19–22), we determined the extent of SNAP-25 proteolysis and found that both neurotoxins were equally effective in cleaving >75% of the SNAP-25 in permeable PC12 cells (Fig. 1B). Since the sites of proteolysis for each toxin (Arg³⁸⁰–Ile³⁸¹ and Gin³⁸⁷–Arg³⁸⁸ for BoNT E and BoNT A, respectively (21, 22)) are near the C terminus of SNAP-25 but 17 residues apart, the preferential inhibition by BoNT E implies that the Ile³⁸¹–Gln³⁸⁷ domain of SNAP-25 is particularly important for regulated LDCV exocytosis in PC12 cells.

SNAP-25 forms binary complexes with the cytoplasmic domains of both recombinant synaptobrevin and syntaxin (23, 24) and participates in stable ternary complex formation with these proteins (23–25). The C terminus of SNAP-25 appears to be required for interactions with synaptobrevin but not with syntaxin and not for ternary complex formation as determined with BoNT A-treated SNAP-25 (23, 24). However, the effect of BoNT-catalyzed SNAP-25 cleavage on in situ docking complexes in cells has not previously been determined. We isolated complexes containing SNAP-25, syntaxin, and synaptobrevin from detergent extracts of permeable PC12 cells by co-immunoprecipitation with SNAP-25 or syntaxin antibodies (Fig. 2A). Treatment of permeable cells with BoNT E and A resulted in extensive cleavage of SNAP-25 with retention of SNAP-25 fragments in the immune complexes. BoNT E treatment reduced the recovery of synaptobrevin in syntaxin and SNAP-25 immunoprecipitates whereas BoNT A treatment had little effect (Fig. 2, A and B). BoNT E treatment also reduced the recovery of syntaxin in SNAP-25 immunoprecipitates and the recovery of SNAP-25 in syntaxin immunoprecipitates, whereas BoNT A treatment did not. The results indicate that BoNT E treatment markedly destabilized docking complexes whereas BoNT A treatment had little effect. Hence, the degree of destabilization correlated with the severity of inhibition of regulated secretion (see Fig. 1A).

Since the cleavage sites for BoNTs and Tetx in syntaxin, synaptobrevin, and SNAP-25 reside in domains predicted to form coiled-coils that are potentially involved in protein-protein interactions (2), it has been suggested that uncomplexed proteins are the preferred substrates for neurotoxin proteases (2, 24). Ternary complexes formed from recombinant protein cytoplasmic domains were found to be resistant to neurotoxin proteolysis (24, 26). However, it is unclear that proteolysis in secretory cells is restricted to a point prior to vesicle docking and docking complex formation, since treatment of susceptible neural cells with BoNTs does not alter the number of vesicles docked in an active zone (27). In recent studies, it was shown that LDCVs in permeable PC12 cells are docked but undergo a presynaptic, ATP-dependent priming step during which there is NSF-catalyzed disassembly of docking complexes. Hence, priming might allow a postdocking exposure of proteins to neurotoxin cleavage. As shown in Fig. 3, BoNT E treatment following the ATP-dependent priming reaction (P) exerted a greater destabilizing effect on protein complexes compared with treatment without priming (D). These results confirmed the partial disassembly of docking complexes during ATP-dependent priming prior to fusion and imply that BoNT E can act at a late postdocking, prefusion step.

To further address BoNT sensitivity at a late step, the effects of BoNTs on Ca²⁺-activated secretion from ATP-primed permeable PC12 cells were examined. LDCVs in ATP-primed cells are poised at a late stage and engage in the rapid release of NE upon addition of Ca²⁺ and a cytosolic protein in the absence of

Figure 1: BoNTs cleave SNAP-25 and inhibit Ca²⁺-activated NE secretion. A, inhibition of Ca²⁺-stimulated NE secretion by BoNT E and A. Permeable PC12 cells were incubated with the indicated concentrations of BoNTs (●, BoNT E; ○, BoNT A) for 5 min at 30°C prior to conducting a 15-min secretion incubation. Inhibition by 10 μM BoNT A was atypically greater in this experiment than in three similar experiments as well as in others (see Ref. 18). B, proteolysis of SNAP-25 by BoNTs. In parallel with the experiment of panel A, permeable PC12 cells were incubated with the indicated concentrations of BoNTs, and SNAP-25 proteolysis was determined by immunoblotting.

Composition was unaffected by extraction volume over a 10-fold range; however, their composition was affected by cell pretreatment conditions. For instance, similar complexes were detected by direct SDS extraction. More recently, their composition was unaffected by extraction volume over a 10-fold range; however, their composition was affected by cell pretreatment conditions.

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BoNT E treatment completely inhibited Ca$^{2+}$-triggered neurotransmitter release (Fig. 4A) indicating that SNAP-25 is required for regulated fusion at a late, post-ATP utilization step. This conclusion was also indicated by finding that IgGs from a SNAP-25 C-terminal peptide-specific antiserum inhibited Ca$^{2+}$-triggered secretion from ATP-primed permeable cells whereas nonimmune IgGs were without effect (Fig. 4C). In contrast, BoNT A exerted only weak partial inhibitory effects (Fig. 4A), implying that the C-terminal region of SNAP-25 between BoNT E and BoNT A cleavage sites, but not the most C-terminal region, was required for Ca$^{2+}$-triggered fusion. The uniqueness of the incomplete inhibition by BoNT A was indicated by the finding that Tetx or BoNT B and BoNT C, which extensively proteolyzed synaptobrevin and syntaxin, respectively (not shown), completely inhibited Ca$^{2+}$-activated secretion in ATP-primed cells (Fig. 4B).

There is a Ca$^{2+}$-independent pathway for stimulating NE secretion from permeable PC12 cells that is activated by non-hydrolyzable GTP analogs (e.g. GMPPNP) in the absence of Ca$^{2+}$, ATP, and cytosolic proteins (28). Although the mechanism of stimulation by GMPPNP remains to be elucidated, this pathway appears to converge with the Ca$^{2+}$-activated pathway at a point prior to or at SNAP-25 since GMPPNP-stimulated NE secretion was effectively inhibited by BoNT E and to a much lesser extent by BoNT A (Fig. 4D). In addition, the C-terminal SNAP-25 peptide-specific antibody completely inhibited GMPPNP-stimulated secretion (Fig. 4E). Complete inhibition of GMPPNP-stimulated secretion was also observed with Tetx, BoNT B, and BoNT C, indicating requirements for synaptobrevin and syntaxin as well.

**Discussion**

An unanticipated role for SNAP-25 in regulated neurotransmitter secretion was suggested by its recovery on NSF/SNAP affinity columns in a search for membrane SNAP receptors (4) and by its identification as a specific substrate for proteolysis by BoNT E and BoNT A (2, 3). Although a role for SNAP-25 in regulated exocytosis is implied by its discovery as a substrate for BoNTs, its precise role remains unknown. As a component of heteroligomeric complexes with syntaxin and synaptobrevin, it has been suggested to be a presynaptic membrane docking component for the specific targeting of synaptic vesicles to the active zone (7). While our studies did not address the role of SNAP-25 in docking, they did provide evidence for a postdocking role for SNAP-25 in regulated exocytosis.

The majority of the LDCVs in PC12 cells are anchored close to or docked at plasma membrane sites. Ca$^{2+}$ elevations prompt a single rapid phase of NE release involving most of the LDCVs (9, 14). In permeable PC12 cells, putative docking interactions between secretory vesicles and plasma membrane can be detected as detergent-soluble complexes containing synaptobrevin, SNAP-25, and syntaxin. While the majority of LDCVs are docked, Ca$^{2+}$-dependent NE secretion still requires ATP-dependent reactions that consist of prefusion activation processes catalyzed by NSF/SNAP and priming in exocytosis proteins (9–11). ATP-dependent priming activates LDCVs and poises them for Ca$^{2+}$-triggered fusion at a step beyond docking and ATP utilization, and Ca$^{2+}$-triggered fusion proceeds in the absence of ATP utilization (9). The inhibition of Ca$^{2+}$-triggered fusion of LDCVs in ATP-primed cells by BoNT E catalyzed proteolysis of SNAP-25 and by SNAP-25 antibody implies a postdocking, post-ATP utilization role for SNAP-25 in steps that immediately precede or directly cause membrane fusion.

The paradox that BoNT A and BoNT E are equally effective in proteolyzing SNAP-25 whereas only the latter substantially inhibits Ca$^{2+}$-activated exocytosis in permeable PC12 cells can be resolved by the suggestion that the most C-terminal residues Arg$^{198}$–Gly$^{206}$ of SNAP-25 removed by BoNT A are less

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3 T. F. J. Martin and J. A. Kowalchyk, unpublished results.
SNAP-25 Requirement for Regulated Exocytosis

SNAP-25 fragment generated by BoNT E cleavage, which is retained in ternary complexes, interferes with fusion events seems unlikely since direct tests of this and related C-terminal peptides failed to reveal inhibition (not shown).

Prior studies of recombinant protein interactions indicated an essential role for C-terminal SNAP-25 residues in synaptobrevin/SNAP-25 but not syntaxin/SNAP-25 interactions (23, 24). However, BoNT A-treated SNAP-25 was as impaired in binary interactions with synaptobrevin as was BoNT E-treated SNAP-25 (24), indicating that the role for Ile181-Gln197 in a late postdockng step cannot reside with synaptobrevin interactions alone. BoNT E-treated SNAP-25 was much more strongly impaired than BoNT A-treated SNAP-25 in assembling into a highly stable, SDS-resistant ternary complex (24) whereas BoNT A-deaved SNAP-25 functioned normally in the formation and NSF-catalyzed disassembly of docking complexes (13, 26). We found a correlation between toxin efficacy in inhibiting secretion and the destabilization of heteromeric complexes isolated by co-immunoprecipitation. This correlation between toxicity efficacy in inhibition of secretion and docking complex stability is intriguing and likely indicates that Ile181-Gln197, located in a region of SNAP-25 predicted to form coiled-coils (23), is required for interactions in a multiprotein complex.

Since the C terminus of SNAP-25 is not required for interactions with syntaxin (23, 24) and since NSF acts to promote the dissociation of SNAP-25 from synaptobrevin at a prefusion step (7), it is possible that the Ile181-Gln197 region of SNAP-25 is required for interactions with proteins other than syntaxin or synaptobrevin to form complexes that are directly involved in catalyzing membrane fusion.

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