Importance of Two Adjacent C-terminal Sequences of SNAP-25 in Exocytosis from Intact and Permeabilized Chromaffin Cells Revealed by Inhibition with Botulinum Neurotoxins A and E†

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ABSTRACT: Types A and E botulinum neurotoxin (BoNT) are Zn^{2+}-requiring endoproteases which cleave nine and twenty-six residues, respectively, from the C-terminus of synaptosomal-associated protein of M_r = 25 kDa (SNAP-25). Involvement of SNAP-25 in the exocytosis of large dense-core vesicles in bovine adrenochromaffin cells was examined by measuring cleavage of SNAP-25 in relation to the levels of Ca^{2+}-evoked catecholamine release from cells exposed to BoNT/A or /E, either before or after permeabilization. The dose-dependency of inhibition of exocytosis correlated closely with the extents of SNAP-25 cleavage in cells permeabilized and then treated with BoNT/E. In intact cells exposed to 66 nM BoNT/A, virtually all of the SNAP-25 was truncated, accompanied by a near-complete inhibition of exocytosis; however, after their permeabilization a significant level of secretion was recorded upon Ca^{2+}-stimulation. Importantly, this BoNT/A-resistant release from the permeabilized cells was dramatically lowered by subsequently adding BoNT/E, which further truncated the SNAP-25 fragment (lacking the C-terminal nine residues) that had been produced earlier by BoNT/A. Moreover, anti-SNAP-25 IgG decreased the BoNT/A-insensitive exocytosis. When permeabilized cells were exposed to either neurotoxin, both blocked MgATP-dependent secretion but only BoNT/E attenuated the energy-independent phase. These distinct inhibitory effects of the two neurotoxins demonstrate that residues 197−205 at the C-terminus of SNAP-25 are absolutely essential for exocytosis from intact cells whereas even after their removal a significant proportion of the exocytotic response can be elicited from permeabilized cells, but this is reliant on amino acids 180−196. Moreover, the latter but not residues 197−205 are implicated in a late, MgATP-independent step of exocytosis, which is blocked by BoNT/E but nonsusceptible to BoNT/A.

Seven immunologically-distinct serotypes of botulinum neurotoxin (BoNT; A−G),[1] produced by *Clostridium botulinum*, inhibit the secretion of all fast-acting neurotransmitters (Ashton *et al*., 1988) and the release of neuropeptides from synaptosomes (McMahon *et al*., 1992) if delivered intracellularly. Each BoNT type is a large protein ($M_r \approx 150$ kDa) composed of a ∼100 kDa HC linked to a ∼50 kDa LC by a disulfide bond and noncovalent interactions [reviewed by DasGupta (1989)]. Whereas domains in the HC contribute to the binding of BoNT to ectoacceptors on cholinergic nerve endings (Poulain *et al*., 1990) and subsequent internalization (Poulain *et al*., 1988), LC alone intracellularly blocks transmitter release (de Paiva & Dolly, 1990), as well as secretion from permeabilized neuroendocrine cells (Bittner *et al*., 1989; McInnes & Dolly, 1990). Primary sequence determination revealed the presence of a highly-conserved Zn^{2+}-binding motif, characteristic of neutral metalloproteases (Jongeneel *et al*., 1989) in the central region of each LC (Neimann *et al*., 1994). Their selective endoproteolytic activities were first demonstrated using synaptic preparations; BoNT/B, D, F, and G cleave synaptobrevin (also known as vesicle-associated membrane-protein) and a homologue called cellubrevin; A and E truncate synaptosomal-associated protein of M_r = 25 kDa (SNAP-25)[2] and C proteolyses syntaxin [for review, see Montecucco and Schiavo (1995); Dolly *et al*. (1994)].

Expression of the neurotoxins' substrates in neuroendocrine cells was subsequently detected (Hodel *et al*., 1994; Roth & Burgoyne, 1994); moreover, it was shown that the dose-dependency of blockade by BoNT/A or /B of evoked catecholamine secretion from intact chromaffin cells correlated closely with the dimunition of immunoreactivity of SNAP-25 or synaptobrevin/cellubrevin, respectively (Foran

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[1] Abbreviations: BoNT/A, B, C, and E, botulinum neurotoxins type A, B, C, and E; HC, heavy chain; LC, light chain; SNAP-25, synaptosomal-associated protein of M_r = 25 kDa; SNAP-25_A, N-terminal fragment of SNAP-25 produced by BoNT/A; SNAP-25_E, N-terminal fragment of SNAP-25 resulting from cleavage by BoNT/E; pAb rec S25, polyclonal antiserum raised against recombinant SNAP-25; BSA, bovine serum albumin; GAP-43, growth-associated protein of M_r = 43 kDa; MgATP, complex of Mg^{2+} and adenosine triphosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); buffer A, 5 mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM Hepes pH 7.4, 5.6 mM glucose, 220 mM sucrose and 0.5% BSA; buffer B, 145 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes pH 7.4; KGEP, 139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES pH 6.5 and 0.5% (w/v) BSA.

[2] The amino acids comprising SNAP-25 that has been post-translationally processed are numbered 1−205 due to omission of the initial methionine, shown previously in its nucleotide-derived sequence.

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et al., 1995; Lawrence *et al*., 1996). Both syntaxin and SNAP-25 were proteolyzed in BoNT/C-poisoned chromaffin cells, revealing an unexpected specificity for two distinct protein sequences (Foran *et al*., 1996). Near-complete cleavage of the requisite substrate(s) by each neurotoxin was invariably accompanied by the virtual abolition of depolarization-induced secretory responses from intact chromaffin cells. However, upon permeabilization of the plasmalemma of the chromaffin cells with digitonin, a significant level of exocytosis (30−50% of the control value) could be elicited by Ca^{2+} after BoNT/A poisoning but not following treatment with either BoNT/B or /C (Lawrence *et al*., 1994, 1996; Foran *et al*., 1996). Such BoNT/A-resistant release, albeit seen only in permeabilized neuroendocrine cells (Bittner *et al*., 1989; Lomneth *et al*., 1991), raised the intriguing possibilities that this component of secretion either occurs independently of SNAP-25 or is mediated by the latter even after its cleavage (Schiavo *et al*., 1993) by BoNT/A. To distinguish between these alternatives, BoNT/A-intoxicated cells were permeabilized and then exposed to various concentrations of BoNT/E; the latter is known to cleave the R^{179}−I^{180} peptide bond in neuronal SNAP-25, 17 residues upstream from the cleavage site of BoNT/A (Q^{196}−R^{197}). The observed production of a further, smaller N-terminal SNAP-25 fragment (SNAP-25_E) by BoNT/E, accompanied by the loss of BoNT/A-resistant secretion from permeabilized chromaffin cells, demonstrates that this component of exocytosis relies on residues 180−196 of SNAP-25, a region also apparently concerned with an energy-independent step of secretion.

EXPERIMENTAL PROCEDURES

Materials. High purity digitonin was purchased from Novabiochem (U.K.); tissue culture reagents were from GIBCO BRL (Paisley, Scotland) and Immobilin-P membrane was from Millipore (Bedford, MA). Anti-rabbit or -mouse antibodies conjugated to alkaline phosphatase, BSA fraction V, a monoclonal antibody to growth-associated-protein of M_r = 43 kDa (GAP-43, also known as neuromodulin), affinity-purified polyclonal antibodies to actin and all other reagents were obtained from Sigma Chemical Co. Ltd. (Dorset, U.K.). Rabbit polyclonal antibodies against synaptobrevin and a C-terminal 12-residue peptide of SNAP-25 (ANQRATKMLGSG) were generated as previously described (Lawrence *et al*., 1996). A monoclonal antibody reactive with SNAP-25 (clone SMI 81) was purchased from Affinity Research Products Ltd (Nottingham). A polyclonal antibody preparation to full-length SNAP-25 (pAb recS25) was generated in guinea pigs using recombinant glutathione-*S*-transferase tagged SNAP-25 and affinity purified on immobilized antigen. A novel antibody engineered to be specifically reactive toward the C-terminus of BoNT/A-truncated SNAP-25, but not the full-length protein, was kindly provided by Drs. T.A.N. Ekong and D. Sesardic. Immunoglobulins reactive with syntaxin 1A and 1B were raised in rabbits, using soluble recombinant His^6-tagged syntaxin 1A lacking 27 residues from the C-terminal, and affinity-purified on immobilized antigen prior to use (Foran *et al*., 1996). BoNT/A and /E were purified as specified (Shone *et al*., 1993; Gimenez & Sugiyama, 1987). Aliquots of stock solutions of BoNT/A and /E (1.4 and 0.8 mg/mL, respectively, in 25 mM Hepes pH 7.4 containing 150 mM NaCl) were stored at −20 °C. BoNT/E was nicked prior to use by incubation with 10 μg/mL trypsin (TPCK-treated) in the latter buffer for 30 min at 37 °C; the reaction was stopped by the addition of 0.1 mM PMSF followed 15 min later by 50 μg/mL soybean trypsin inhibitor. If applied by themselves, these reagents had no effect on either the amount of secretion elicited from chromaffin cells or their SNAP-25 content.

Culture of Adrenochromaffin Cells and Intoxication with BoNT/A without Membrane Permeabilization. Chromaffin cells were prepared from bovine adrenal glands and maintained as primary cultures, as described (Lawrence *et al*., 1994). Within 2−3 days after preparation, the cells were poisoned by 24 h exposure (at 37 °C in a 5% CO_2 atmosphere) to 66 nM BoNT/A in a low ionic strength solution (buffer A). The cells were then returned to Dulbecco's modified Eagle's medium [supplemented with growth factors and anti-microbial agents as listed in Lawrence *et al*. (1994)] and maintained at 37 °C in a 5% CO_2 incubator for an additional 24 h before performing secretion experiments.

Permeabilization of the Cells with Digitonin and Quantification of Catecholamine Secretion. BoNT/A-treated and neurotoxin-free control cells were washed with buffer B then permeabilized by exposure to 20 μM digitonin in KGEP buffer, which included 2 mM ATP and 4.2 mM $MgCl_2$. After 15 min, the latter medium was aspirated and replaced with fresh KGEP now including 20 μM Ca^{2+} to elicit secretion; some cells were exposed instead to Ca^{2+}-free KGEP in order to determine the basal level of release. Aliquots were removed 15 min later from each sample and assayed for catecholamine content using a fluorimetric procedure (von Euler & Floding, 1959). The amount of catecholamine in the Ca^{2+}-free KGEP sample was subtracted from the level in Ca^{2+}-containing KGEP to yield the Ca^{2+}-dependent secretion. Representative values for the total amount of catecholamine originally present in the cells were obtained by their solubilization in buffer B containing 1% (v/v) Triton X-100, measurement of catecholamine content, and summing the values obtained with the quantities that had been released from the same cells. Ca^{2+}-dependent secretion was calculated as a percentage of these total catecholamine values.

In some experiments, control and BoNT/A pretreated cells were exposed to BoNT/E, anti-SNAP-25 IgG (pAb recS25), during permeabilization with 20 μM digitonin in KGEP (containing 50 μM $ZnSO_4$ when BoNTs were present); note that ATP and $MgCl_2$ were omitted in certain cases. BoNT/A and BoNT/E were reduced with 20 mM dithiothreitol in 0.15 M NaCl, 50 mM Tris·HCl, pH 7.4 for 40 min at 37 °C before dilution into KGEP and application to cells; neurotoxin-free control cells were exposed to the same final dithiothreitol concentration which never exceeded 1 mM and, in any case, had minimal effect on secretion (<5% inhibition). Except where stated otherwise, cells were exposed to neurotoxin for 15 min and the Ca^{2+}-dependent release of catecholamines measured over a subsequent 15 min period (as above, except the values were sometimes expressed as a percentage of the amount obtained for neurotoxin-free control cells). All plotted data are representative of the results from quadruplicate experiments (±SD) performed at least three times.

Quantification of the Proteolytic Activities of BoNT/A and /E in Chromaffin Cell Cultures. Initially, difficulties were experienced in the immunological detection of the neuro-

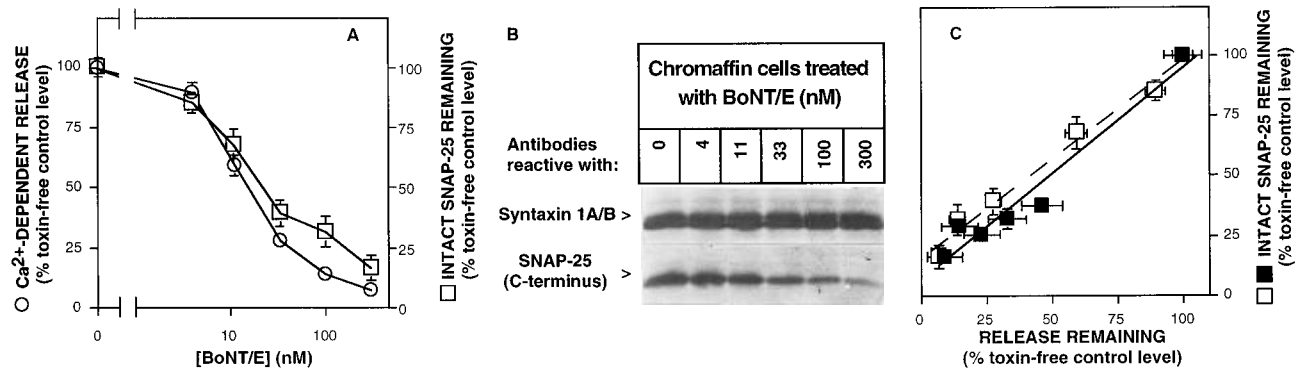

FIGURE 1: BoNT/E-induced inhibition of secretion from permeabilized chromaffin cells correlates with its proteolysis of SNAP-25. Bovine adrenochromaffin cells were rinsed with buffer B, then permeabilized by a 15 min exposure at 25 °C to 20 μM digitonin-containing KGEP in the absence or presence of BoNT/E (activated by trypsinization and reduced with dithiothreitol). After removal of this solution, the cells were placed in digitonin/KGEP with or without 20 μM free Ca^{2+} to allow evoked and basal secretion to be monitored; after 15 min, aliquots were removed and assayed for catecholamine content (±SD; $n = 4$). Ca^{2+}-dependent secretion (i.e., the difference between the amount recorded in 20 μM Ca^{2+}-containing buffer and digitonin/KGEP alone) was calculated as a percentage of the total catecholamine originally present in the cells. The levels of secretion from cells poisoned with each BoNT/E concentration were plotted as a percentage of the amount released from neurotoxin-free cells (A; ○). Immediately after Ca^{2+}-stimulation, the cells were harvested and P2 membrane fractions isolated. Equal amounts of each P2 sample (60 μg of protein) were subjected to SDS−PAGE, transferred to Immobilon-P membranes, and blotted overnight with the requisite antibodies at the following dilutions; anti-syntaxin 1A, 1:200; anti-SNAP-25 (C-terminal residues 194−205), 1:100. Each primary antibody was blotted individually and detected using alkaline phosphatase-conjugated secondary antibodies (B). For every sample, the Western blotting was performed twice; the amounts of SNAP-25 were quantified by densitometric scanning, calculated as percentages of the requisite value for toxin-free cells and the mean level for each sample from the two determinations (±difference) was plotted in panel A (□). Panel C shows the relationship between SNAP-25 proteolysis (±difference; $n = 2$) and the level of secretion from BoNT/E-poisoned chromaffin cells (±SD; $n = 4$); best-fitting lines through data from this (□; broken line) and a separate experiment (■; unbroken line) are shown. Error bars encompassed by the symbols are omitted. For details, see Experimental Procedures.

toxins' targets; thus, a differential centrifugation method was employed to enrich plasmalemma and granule membranes from small quantities of BoNT-treated cultured chromaffin cells. A minimum of eight wells (~10^6 cells/well), identically treated without or with various concentrations of neurotoxin (by the protocols described above), were washed twice with buffer B to remove proteinaceous growth medium. Cells were harvested and lysed in 1 mL of 50 mM $NaHCO_3$ pH 8.5 containing numerous protease inhibitors, and membrane fractions isolated as detailed by Foran *et al*. (1996). The resultant P2 pellets were resuspended in 80 μL of 0.1 M Tris·HCl pH 6.8 containing 2% (w/v) SDS and 1 mM EDTA and solubilized with heating at 90 °C for 10 min. Insoluble material was removed by centrifugation at $10000g_{max}$ for 10 min, and protein concentrations of the supernatants were determined from absorbancies at 280 nm. Equal amounts of protein from the supernatants (from cells treated with or without neurotoxin) were subjected to SDS−PAGE and electrophoretically transferred to Immobilon-P membranes, incubated with blocking solution [150 mM NaCl, 100 mM Tris·HCl pH 7.4, 1% (w/v) BSA and 3% (w/v) skimmed milk powder] for 20 min and then immunoblotted overnight at 25 °C with primary antibodies in blocking solution (see legend to Figure 1). After repeated washing, the membranes were incubated in blocking solution containing a 1:1000 (v/v) dilution of either anti-rabbit or -mouse immunoglobin alkaline phosphatase-conjugated secondary antibody. Primary Ig bound on Western blots was visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium. Immunoblots were quantified by scanning with a Hirshmann 400 densitometer; background readings were subtracted. Additional controls were run to ensure that the developed bands (for SNAP-25) yielded values in the linear range by relating them to the signals recorded for known quantities of membrane proteins from nontoxin-treated chromaffin cells.

RESULTS

BoNT/E Cleaves SNAP-25 and Inhibits Ca^{2+}-Triggered Secretion of Catecholamines from Digitonin-Permeabilized Chromaffin Cells. Although evoked catecholamine secretion from *intact* chromaffin cells is blocked following the cells' exposure to BoNT/A, /B, or /C in low ionic strength medium (Foran *et al*., 1996; Lawrence *et al*., 1996), it is not as susceptible to nicked BoNT/E (Foran *et al*., 1996). This low sensitivity seems to result from inefficient uptake because Ca^{2+}-triggered secretion was potently inhibited when BoNT/E was added to digitonin-permeabilized chromaffin cells (Figure 1A), as observed by Bittner *et al*. (1989). To confirm that this inhibition of release from permeabilized cells by BoNT/E is attributable to its known proteolytic activity (see introduction), membrane fractions prepared from cells poisoned with a range of BoNT/E concentrations were subjected to Western blotting and probed using an antibody reactive to the last twelve C-terminal residues of SNAP-25. A diminution in immunoreactivity was observed in these poisoned cells (Figure 1B), which is consistent with cleavage near the C-terminus of SNAP-25 and consequential loss of the antigenic fragment; moreover, the dose-dependency of this change closely matches the observed extents of inhibition of secretion (Figure 1A and C). Evoked catecholamine release from permeabilized cells exposed to 300 nM BoNT/E dropped to 9 ± 7% of the amount recorded from neurotoxin-free cells whereas SNAP-25 immunoreactivity was depleted to 16 ± 5% of the control level. Immunoblots of a BoNT/E-insensitive protein, syntaxin 1A/B, used as an internal marker confirmed that equivalent amounts of membrane protein were loaded for each sample (Figure 1B). No significant changes in the abundancies of other intracellular proteins tested (synaptotagmin, actin, GAP-43, and synaptobrevin/cellubrevin) were observed (see below), consistent with the proposed selectivity of its proteolytic action (Mon-

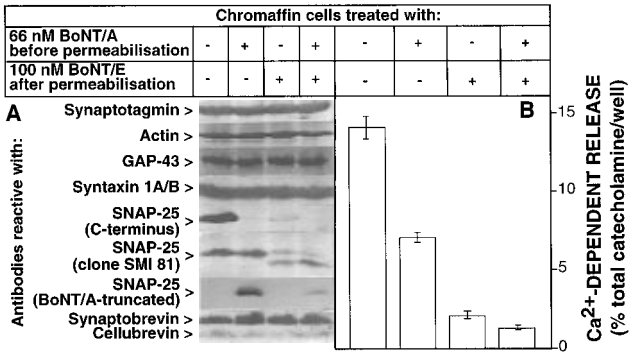

FIGURE 2: BoNT/E cleaves BoNT/A-truncated SNAP-25 and blocks BoNT/A-resistant secretion. Intact chromaffin cells were treated for 24 h in the absence or presence of 66 nM BoNT/A, in a low ionic strength buffer (buffer A) that facilitates neurotoxin uptake, before being incubated for another 24 h in Dulbecco's modified Eagle's medium (see Experimental Procedures). They were then quickly rinsed with buffer B before permeabilization for 15 min in 20 μM digitonin/KGEP, with and without the inclusion of 100 nM BoNT/E (trypsinized and reduced with dithiothreitol). The latter was then removed and the cells exposed for a subsequent 15 min to KGEP alone or KGEP containing 20 μM Ca^{2+} to allow measurement of basal and evoked secretion; Ca^{2+}-dependent release (B) was calculated ($\pm$SD; $n = 4$) as previously described. The cells were then harvested, membrane fractions prepared and subjected to Western blotting (A) as described in Figure 1 using the following dilutions of antibodies reactive with synaptotagmin, 1:400; actin, 1:100; GAP-43, 1:500; syntaxin 1A/B, 1:200; SNAP-25 C-terminus, 1:100; SNAP-25 (SMI 81), 1:1000; BoNT/A-truncated SNAP-25, 1:500; and synaptobrevin/cellubrevin, 1:100. The treatments with either or both neurotoxins are shown schematically.

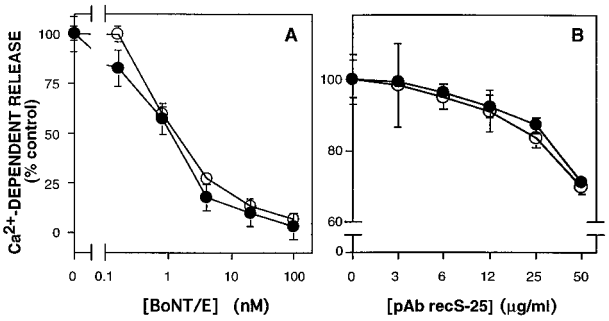

FIGURE 3: Dose-dependency for the inhibition of Ca^{2+}-triggered catecholamine release from chromaffin cells by BoNT/E or an antibody to SNAP-25. Intact control chromaffin cells (○) and those pre-treated with 66 nM BoNT/A (●) were permeabilized by either (A) a 15 min exposure to 20 μM digitonin/KGEP in the absence or presence of various concentrations of activated BoNT/E, as detailed in Figure 1, or (B) treatment with the detergent for 30 min in the presence of purified IgG raised against recombinant SNAP-25. In each case, Ca^{2+}-dependent secretion ($\pm$SD; $n = 4$) was quantified over a subsequent 15 min period; the values were expressed relative to that for the requisite control. IgG isolated from guinea pig preimmune serum by protein A chromatography did not inhibit release at concentrations up to 50 μg/mL.

tecucco & Schiavo, 1995; Neimann *et al*., 1994). Finally, pretreatment of BoNT/E with a Zn^{2+}-selective complexing agent 1,10-phenanthroline (2 mM) or a nonspecific divalent cation chelator EDTA (5 mM) nearly prevented both its proteolysis of SNAP-25 and its ability to block Ca^{2+}-elicited secretion (not shown). Clearly, the near-complete blockade of secretion reported here must arise from the extensive, Zn^{2+}-dependent cleavage of SNAP-25 by BoNT/E.

Different Effects of BoNT/A and /E on Ca^{2+}-Evoked Secretion from Permeabilized Chromaffin Cells Are Due to Their Cleavage of SNAP-25 at Separate Sites. Overnight exposure of intact chromaffin cells to 66 nM BoNT/A, in low ionic strength medium (buffer A) which facilitates its uptake (see Experimental Procedures), decreased the SNAP-25 C-terminus immunoreactivity of their membranes to 3.6 $\pm$ 1.7% ($n = 2$) of the level found in neurotoxin-free control cells (Figure 2A). However, there was no reduction in the staining with the monoclonal antibody SMI 81 against SNAP-25 (Figure 2A) because this reacts with a different epitope located N-terminally to the neurotoxin's cleavage site. Moreover, the electrophoretic mobility of BoNT/A-truncated SNAP-25 fragment (SNAP-25_A) detected by the latter antibody was not easily distinguished from that for native SNAP-25, confirming that only a small number of C-terminal amino acids had been removed. SNAP-25 cleavage at the established site (Schiavo *et al*., 1993) was reaffirmed by blotting with an antibody raised against a peptide designed to mimic the newly-created C-terminus of SNAP-25 (Ekong *et al*., 1997). Importantly, this antibody was unreactive with SNAP-25 in membranes from neurotoxin-free cells, but gave a strongly reactive band with the membranes of BoNT/A-poisoned cells (Figure 2A). No changes were detected in the labeling observed with several other antibodies that recognize BoNT/A-insensitive proteins (synaptotagmin, actin, GAP-43, syntaxin, and synaptobrevin/cellubrevin). This SNAP-25 cleavage was accompanied by a reduction in the Ba^{2+}-elicited secretory response to 13.7 $\pm$ 6% of the control value observed for intact cells, as reported previously (Lawrence *et al*., 1996). Nevertheless, when permeabilized and challenged with 20 μM Ca^{2+}, these BoNT/A-poisoned cells gave a secretory response of 50 $\pm$ 6% of that from neurotoxin-free control cells (Figure 2B). This was nearly abolished by adding BoNT/E during the permeabilization, an effect attributed to its cleavage of SNAP-25_A as reflected in the production of a smaller fragment (SNAP-25_E) recognized by SMI 81 antibody (Figure 2A). Moreover, BoNT/E must have cleaved SNAP-25_A at its C-terminus because the antibody which selectively recognizes the latter, but not full-length SNAP-25, failed to detect the BoNT/E-truncated molecule. As either neurotoxin alone produced near-complete cleavage of SNAP-25 (Figure 2A), the incomplete inhibition of secretion from permeabilized cells produced by BoNT/A is not simply due to an uncleaved pool of SNAP-25. Accordingly, it has been reported that the BoNT/A-resistant release is not decreased further by re-exposure to BoNT/A after the cells were permeabilized (Lawrence *et al*., 1996). Although it remains unclear how the nature of the BoNT/A-insensitive secretion differs from the susceptible component, both were blocked by BoNT/E (Figure 2B) with similar dose-dependencies (Figure 3). Corroborating evidence implicating SNAP-25_A in the BoNT/A-resistant response was obtained using polyclonal IgG (pAb recS25) raised against a recombinant form of SNAP-25. Exposure of BoNT/A-treated permeabilized cells to the latter for 30 min before stimulation of exocytosis with Ca^{2+} caused a dose-dependent reduction in the amount of catecholamine release elicited (Figure 3B); notably, like BoNT/E, pAb recS25 was equally potent in inhibiting secretion from control and BoNT/A-intoxicated cells.

MgATP Is a Prerequisite for the BoNT/A-Resistant Release. The Ca^{2+}-evoked response from permeabilized cells can be dissected into a MgATP-independent step that follows one requiring MgATP (termed "priming") [see Holz *et al*.

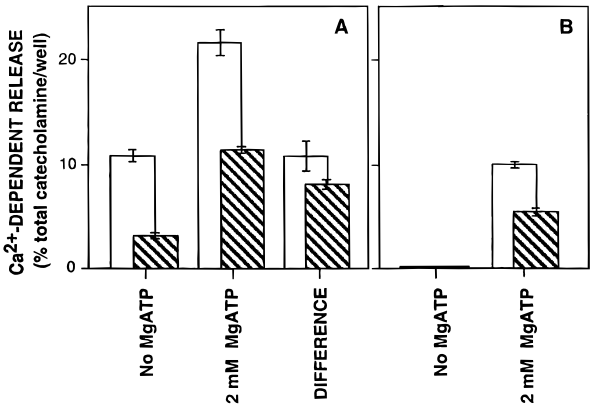

FIGURE 4: The BoNT/A-resistant secretion from permeabilized chromaffin cells is dependent on MgATP. Intact control (open bars) chromaffin cells or those pretreated with 66 nM BoNT/A (hatched bars) were permeabilized by a 15 min exposure to 20 μM digitonin in KGEP without or with 2 mM MgATP. Panel A shows Ca^{2+}-dependent release ($\pm$SD; $n = 4$) measured during that period by inclusion of 20 μM Ca^{2+} and the difference between the levels of release obtained in the presence and absence of the nucleotide ($\pm[(SD_{-MgATP})^2 + (SD_{+MgATP})^2]^{1/2}$). (B) Cells were permeabilized by a 15 min exposure to digitonin in Ca^{2+}-free KGEP, in the absence or presence of MgATP, before quantifying Ca^{2+}-dependent secretion ($\pm$SD; $n = 4$) elicited over a subsequent 15 min period.

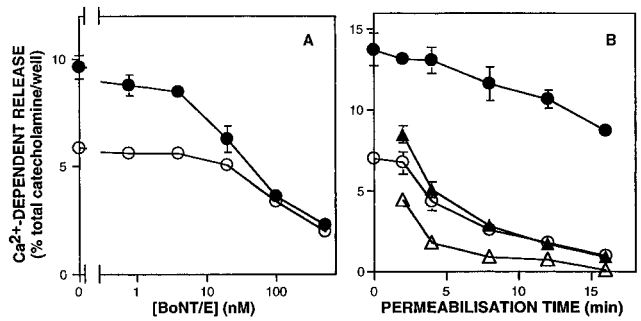

FIGURE 5: BoNT/E inhibits both MgATP-requiring and -independent phases of secretion. (A) Chromaffin cells were permeabilized by exposure for 5 min to 20 μM digitonin/KGEP in the absence (○) or presence (●) of 2 mM MgATP, but including the indicated concentration of activated BoNT/E. Secretion was then elicited over a subsequent 15 min with 20 μM Ca^{2+}, in MgATP-free or -containing digitonin/KGEP as for the prior stage; basal release in the absence of Ca^{2+} was also recorded. Ca^{2+}-dependent release ($\pm$SD; $n = 4$) was determined as, detailed before. (B) Cells were permeabilized in MgATP-free (open symbols) or MgATP-containing digitonin/KGEP (closed symbols), in the absence (○, ●) or presence (△, ▲) of 100 nM prereduced BoNT/E for the times indicated. The buffers were then replaced with neurotoxin-free, digitonin/KGEP (omitting or including MgATP as during permeabilization) with or without 20 μM Ca^{2+}. Ca^{2+}-dependent release ($\pm$SD; $n = 4$) after 15 min was measured and calculated as previously described. Error bars encompassed by symbols are omitted.

(1989)]. The former is revealed when chromaffin cells are permeabilized with digitonin in the absence of MgATP, whereas addition of MgATP to the permeabilized cells facilitates the priming step, thereby, increasing the size of the secretory response (Figure 4A). Most of the appreciable amount of MgATP-independent release elicited from control cells, when they were exposed to digitonin and Ca^{2+} simultaneously, was prevented by pretreatment with BoNT/A to deplete SNAP-25 before permeabilization (Figure 4A). However, a large BoNT/A-resistant response was recovered following inclusion of MgATP during the permeabilization step; in fact, subtraction of the release observed in the absence of MgATP from the level recorded in the presence of the nucleotide revealed only a slight decrease in the difference signal, as a result of the SNAP-25 truncation. When chromaffin cells were permeabilized for 15 min to allow rundown before eliciting secretion with Ca^{2+}, no response was detectable subsequently upon omission of MgATP but the majority of the MgATP-dependent fraction of release was retained (Figure 4B) and ~50% of this was resistant to BoNT/A. Hence, MgATP promotes the component of secretion that is not susceptible to BoNT/A.

Fusion of Primed Granules Is Inhibited by BoNT/E. Previous studies (Bittner & Holz, 1993; Lawrence *et al*., 1994) have demonstrated that BoNT/A does not inhibit MgATP-independent release when added to chromaffin cells *after* permeabilization, whereas the MgATP-requiring phase is partially blocked. Thus, the observed diminution of "MgATP-independent" secretion from cells treated with BoNT/A *before* permeabilization (Figure 4A) is most likely due to a blockade of the MgATP-requiring priming reaction. As the primed state is short-lived (Figure 4A and B), maintenance of a readily-releasable pool of primed granules in the cells must involve continual cycles of depriming and repriming. The inability of BoNT/A to block the exocytosis of granules that have already been primed could be due to inaccesibility of its substrate, SNAP-25, or it may indicate that the nine C-terminal residues removed from the latter by the toxin are not involved in the exocytotic process at any step after priming. To distinguish between these possibilities, we investigated whether the MgATP-independent release that was resistant to BoNT/A displayed any sensitivity to BoNT/E. It was found that BoNT/E inhibited both the MgATP-requiring and -independent phases of Ca^{2+}-evoked catecholamine release (Figure 5). In the case of cells permeabilized for 5 min in the absence of MgATP, the significant level of secretion elicited by Ca^{2+} (~5% of their catecholamine content) was considerably lessened when >30 nM BoNT/E was included during the permeabilization procedure. The larger secretory response seen in the presence of MgATP was also reduced by BoNT/E but at concentrations >3 nM; indeed, the MgATP-dependent increment was abolished by >100 nM BoNT/E (Figure 5A). Clearly, the MgATP-requiring steps are more susceptible to blockade by BoNT/E than the energy-independent phase. Nevertheless, BoNT/E inhibited the MgATP-independent release within 2 min, well before this component decayed due to rundown (Figure 5B). Thus, residues 180−196 of SNAP-25, between the cleavage sites of BoNT/A and BoNT/E, seem to be necessary for primed granule fusion whereas this was not found to be affected following removal of the last nine amino acids from SNAP-25 (197−205) by BoNT/A (Lawrence *et al*., 1996).

DISCUSSION

The combined findings of this and previous studies (Gutierrez *et al*., 1995; Lawrence *et al*., 1996) provide convincing evidence that SNAP-25 is essential for exocytosis from intact and detergent-permeabilized chromaffin cells; moreover, the results presented herein indicate that different regions of this protein are required at distinguishable stages of the exocytotic process. Exposure of intact chromaffin cells to BoNT/A truncated nearly all the SNAP-25 present and abolished the exocytotic responses to K^+-depolarization,

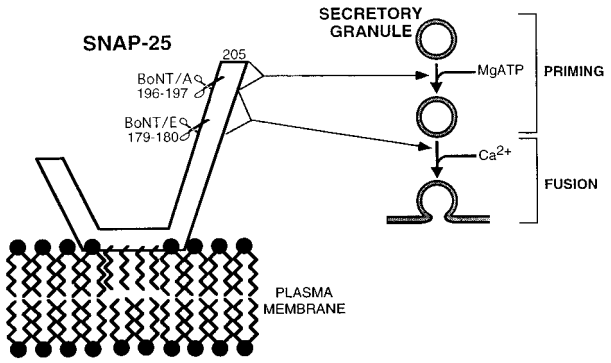

FIGURE 6: Blockade by BoNT/A and /E of two distinct steps in exocytosis from chromaffin cells. The removal by BoNT/A of nine C-terminal residues of SNAP-25 results in the inhibition of MgATP-dependent priming but does not perturb the Ca^{2+}-stimulated fusion of primed granules. Priming is abolished after BoNT/A-treatment of intact chromaffin cells, but partially recovers following digitonin-permeabilization of their plasma-membrane. In contrast, the secretion elicited from the permeabilized, BoNT/A-treated cells is eradicated by BoNT/E which removes a further 17 amino acids from the C-terminus of SNAP-25. Moreover, the latter neurotoxin inhibits significantly the MgATP-independent exocytosis of primed granules.

nicotinic stimulation, or exposure to 2 mM Ba^{2+} (Lawrence *et al*., 1996). Nevertheless, unlike the intact cells, when permeabilized in the presence of MgATP and challenged with 20 μM Ca^{2+}, these same cells secreted a significant amount of catecholamine. Thus, it became apparent that intact SNAP-25 is not essential for an appreciable component of evoked secretion from digitonin-permeabilized cells. There have been reports that SNAP-25_A, like the full-length protein, can participate with syntaxin and synaptobrevin in the formation of a complex whose disassembly is facilitated by *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment protein (α-SNAP) *in vitro* (Hayashi *et al*., 1994, 1995; Pellegrini *et al*., 1995) and in BoNT/A-poisoned synaptosomes (Otto *et al*., 1995). These raised the possibility of SNAP-25_A retaining some functionality. This postulation is substantiated herein by demonstrating that the BoNT/A-resistant secretion from permeabilized cells can be abolished by BoNT/E. The latter neurotoxin specifically proteolyses SNAP-25 at residues 179−180 (Schiavo *et al*., 1993); thus, it removes 17 C-terminal residues from SNAP-25_A. Also, BoNT/E alone prevented secretion from control permeabilized cells; notably, the dose-dependencies for its inhibition of the latter and SNAP-25 proteolysis were essentially identical to that obtained for its blockade of secretion from BoNT/A-pretreated cells. Thus, intact- and BoNT/A-truncated SNAP-25 appear to be equally susceptible to BoNT/E. The importance of SNAP-25_A for BoNT/A-resistant release is corroborated by the inhibition obtained with pAb recS25.

The distinguishable effects of BoNT/A and /E reveal some clues to the functional importance of two adjacent C-terminal regions of SNAP-25 in the exocytotic process; a scheme illustrating a likely interpretation of the data is shown in Figure 6. Residues 180−196, but not 197−205, of SNAP-25 are essential for a significant component of Ca^{2+}-evoked secretion from digitonin-permeabilized chromaffin cells, but the latter region is clearly vital for all of the secretory response from intact cells. This information may explain why no inhibition of Ca^{2+}-evoked secretion from permeabilized chromaffin cells was produced by a peptide (ANQRATKMLGSG; see Experimental Procedures) corresponding to residues 194−205 of SNAP-25 (this study, data not shown) or to the region 194−200 whereas a larger, 20 residue C-terminal peptide (186−205) caused a significant blockade (Gutiérrez *et al*., 1995). Importantly, 20 μM Ca^{2+} alone proved almost incapable of eliciting secretion after permeabilization of the BoNT/A-poisoned cells; inclusion of 2 mM MgATP was essential to reveal a significant BoNT/A-resistant response. This means that there were much less primed granules, at the time of permeabilization, in the BoNT/A-poisoned compared to control cells [because, by definition, primed granules can be triggered to fuse by Ca^{2+} alone (see Holz *et al*., 1989)]. Thus, it can be deduced that residues 197−205 of SNAP-25 are indispensible for the preparation and/or stability of primed granules in intact chromaffin cells (Figure 6). Such a proposal is supported by the report that SNAP-25_A can interact in ternary complexes with syntaxin and synaptobrevin *in vitro* as readily as intact SNAP-25, but those generated with the native form display much greater resistance to disassociation in SDS (Hayashi *et al*., 1994). SNAP-25_E forms a ternary complex too but this is even less stable, being completely dissociated in SDS. Even so, ternary complexes of syntaxin, synaptobrevin, and SNAP-25_E or SNAP-25_A can bind α-SNAP *in vitro* to form a larger complex which can be dissociated by NSF in the presence of MgATP (Hayashi *et al*., 1995).

It has previously been shown that MgATP-independent release persists transiently following chromaffin cell permeabilization with digitonin; even though such secretion is virtually abolished if cells are exposed overnight to BoNT/A before the detergent treatment, it cannot be inhibited by adding this neurotoxin after the creation of pores in the cell membrane (Bittner & Holz, 1993; Lawrence et al., 1994). In contrast, BoNT/E added to permeabilized cells is shown herein to rapidly block exocytosis regardless of the presence MgATP, suggesting that the failure of BoNT/A to attenuate the energy-independent phase is not due to inaccessibility of SNAP-25 to the neurotoxin after priming. This could not be confirmed with any of our SNAP-25 antibodies because the short incubation times necessary to retain MgATP-independent, Ca^{2+}-elicited release precluded their inhibitory effect (data not shown). As SNAP-25 is protected against cleavage by BoNT/E and /A when complexed with synaptobrevin and syntaxin *in vitro* (Hayashi *et al*., 1994), the BoNT/E-sensitivity of MgATP-independent release implies that formation of this complex occurs after priming and does not require exogenous MgATP. Moreover, blockade by BoNT/E of the exocytosis of primed granules implicates SNAP-25 residues 180−196, whereas the lack of inhibition by BoNT/A suggests that amino acids 197−205 are not involved, at this late stage of the secretory process (Figure 6). A similar conclusion was reached in a paper (Banarjee *et al*., 1996), published since our submission, in which it was demonstrated that BoNT/E prevented the exocytosis of ATP-primed granules from PC 12 cells whereas BoNT/A gave only a partial inhibition.

It remains unexplained why it is only after membrane-permeabilization that the secretory machinery of chromaffin cells becomes partially resistant to BoNT/A-poisoning; the same is true for mechanically permeabilized (cracked) PC12 cells (Lomneth *et al*., 1991) but not when the PC12 cells are NGF-differentiated; their secretory machinery is completely susceptible to BoNT/A after permeabilization as is

also the case for BoNT/E (Bannerjee *et al*., 1993). Leakage of proteins and/or small molecules from the cell cytoplasm and/or digitonin-induced changes in the structure of the plasma membrane could all be factors which allow the functionality of SNAP-25_A to be revealed after cell permeabilization.

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