Rescue of Exocytosis in Botulinum Toxin A-poisoned Chromaffin Cells by Expression of Cleavage-resistant SNAP-25

IDENTIFICATION OF THE MINIMAL ESSENTIAL C-TERMINAL RESIDUES

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Botulinum neurotoxin (BoNT/A) types A and B selectively block exocytosis by cleavage of SNAP-25 and synaptobrevin, respectively; in humans, many months are required for full recovery from the resultant neuromuscular paralysis. To decipher the molecular basis for such prolonged poisoning, intoxication in adreno-chromaffin cells was monitored over 2 months. Exocytosis from BoNT/B-treated cells resumed after 56 days because of the appearance of intact synaptobrevin. However, inhibition continued in BoNT/A-treated cells, throughout the same interval, with a continued predominance of cleaved SNAP-25-(1–197) over the intact protein. When recovery from poisoning was attempted by transfection of the latter cells with the gene encoding full-length SNAP-25-(1–206), no restoration of exocytosis ensued even after 3 weeks. To ascertain if this failure was because of the persistence of the toxin’s protease activity, the cells were transfected with BoNT/A-resistant SNAP-25 constructs; importantly, exocytosis was rescued. C-terminal truncation of the toxin-insensitive SNAP-25 revealed that residues 1–201, 1–202, 1–203 afforded a significant return of exocytosis, unlike shorter forms 1–197, 189–199, or 200; accordingly, mutants M202A or L203A of full-length SNAP-25 rescued secretion. These findings give insights into the C-terminal functional domain of SNAP-25, demonstrate the longevity of BoNT/A protease, and provide the prospect of a therapy for botulism.

BoNT/A type A is being employed successfully for the therapy of dystonias and dysphonias because it potently and selectively inhibits acetylcholine release at the neuromuscular junction (NMJ), thereby resulting in paralysis that lasts for several months (Refs. 1 and 2; and reviewed in Ref. 3). Seven serotypes of BoNTs (A to G) are produced by Clostridium botulinum, whereas only a single form of tetanus toxin (TeTX) is synthesized by Clostridium tetani; the latter blocks transmitter release at central inhibitory nerve terminals (4). These homologous, but immunologically distinct, proteins share many properties; each active neurotoxin consists of a heavy chain and a light chain (LC) linked by a disulfide bond and non-covalent interactions. The heavy chain is required for high affinity binding to specific neuronal ecto-acceptors, subsequent internalization, and translocation of the LC into the cytosol, where the latter blocks synaptic vesicle exocytosis (reviewed in Ref. 4). The LCs of BoNTs and TeTX are Zn2+-dependent endoproteases that exhibit remarkable substrate selectivities, targeting single bonds (except for BoNT/C, see below) in one of three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins proposed to constitute the core components of the vesicle docking/fusion complex that mediates the regulated exocytosis of neurotransmitters (5). Synaptosomal-associated protein with a molecular mass of 25 kDa (SNAP-25) is proteolysed by BoNT/A, /C, and /E at separate sites near the C terminus: Gln197–Arg198 Arg198–Ala199, and Arg198–Ile199 (6–8), respectively. Syntaxin 1A/B is also cleaved by BoNT/C and synaptobrevin (Sbr) by BoNT/B, /D, /F, /G, and TeTX (reviewed in Ref. 4). Unlike syntaxin 1A/B and Sbr, which each possess a transmembrane anchor (9), SNAP-25 is attached to the membrane through thioester-linked palmitate modifications of one or more of its four centrally located cysteines (10). BoNT/A- or /E-truncated SNAP-25 remain membrane-bound, but release is inhibited because of the destabilization of the ternary complex even though assembly and disassembly can still occur (11, 12). The precise basis of their blockade of neuroexocytosis has yet to be elucidated.

While the mechanism of BoNT-induced inhibition has received much attention, the molecular processes involved in recovery of the NMJ from poisoning, which can take up to 4 months depending on the serotype used, remains poorly understood. In humans, the extent of recovery from the neuromuscular paralysis caused by BoNT/A or /B was found to be 6–66%, respectively, at 49 days post-injection (2). Recently, the sequence of events involved in the proracted resumption of neurotransmission in BoNT/A poisoned motor endplates has been revealed by monitoring synaptic function in individual, identified nerve endings of living animals (13). This showed that the transient appearance of functional nerve sprouts mediates a partial return of neuromuscular function, with full recovery relying on the originally poisoned nerve terminals reacquiring synaptic activity. However, it is still unclear how
the lifetime of the SNAREs and/or persistence of toxin activity influences the time course of the recovery.

To gain insights into the molecular basis of the extended but varied recovery periods, this process was studied in adrenergic chromaffin cells. Evoked exocytosis from the latter shares many characteristics with synaptic vesicle-mediated neurotransmitter release from neurons; in particular, SNAP-25, Sbr, and syntaxin 1A/B are present and have all been shown to be required for release of their large dense core vesicles (LDCVs) (14, 15) because cleavage of these targets by BoNT/A, /B, or/C blocks exocytosis. Previous studies performed on chromaffin cells suggested that persistence of BoNT/A or TeTx activity was responsible for prolonged inhibition of catecholamine release (16). Thus, it was pertinent to compare the time course of recovery of exocytosis in chromaffin cells to the different durations of paralysis of NMJ in humans poisoned with BoNT/A and B (2). The changing amounts of the respective BoNT-truncated and full-length SNARE could be quantified throughout the periods of intoxication in these cells, a feat that cannot be easily achieved at the NMJ. Further, this cell model allowed rescue of BoNT/A poisoning to be attempted through the introduction of vectors encoding various forms of SNAP-25. Failure to recover exocytosis in this way with the wild-type molecule, up to 3 weeks post-intoxication, suggested continued activity of the toxin. This was shown conclusively by the successful rescue with SNAP-25 mutants that were engineered to be highly resistant to proteolysis. This important achievement facilitated experimentation which established that substituting residues in SNAP-25 at positions 197, 198, 202, or 203 does not affect its role in regulated secretion, whereas truncating SNAP-25 past residue 201 inhibits the process.

EXPERIMENTAL PROCEDURES

Materials—Highly purified BoNT/A was isolated as described previously (17). BoNT/B and /E were supplied by Drs. C. C. Shone (Centre for Applied Microbiology and Research, UK) and B. DasGupta (Madison, WI) and activated prior to use (18). Antiserum specific for SNAP-25 (1–197) was a gift from Drs. T. A. N. Ekong and D. Sesardic (National Institute for Biological Standards and Control, UK). Mouse cDNA encoding SNAP-25 from Syrian hamster was obtained from Dr. L. Zhou of this laboratory. Urografin (Schering Healthcare, Germany), radioimmunoassay (RIA) kit (Nichols Institute, CA, USA), QuickChange™ (Stratagene), calcium phosphate reagents (Life Technologies, Inc., UK), Superfect™ (QIAGEN, Inc.), pGEX-2T and enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, UK), and pcDNA1.1/Amp (Invitrogen) were purchased.

Culture of Chromaffin Cells and Intoxication with BoNT/A or/B—Chromaffin cells were prepared from bovine adrenal glands and maintained as primary cultures, as described previously (14). Cells required for transfection were further enriched using urografin density-gradient centrifugation (19). Within 2–3 days after preparation, the cells were incubated with a low ionic strength medium (LISM) at 37 °C for 24 h in the absence or presence of 6.6 nM BoNT/A or 66 nM BoNT/B; after washing, they were maintained at 37 °C (14).

Stimulation and Quantification of Catecholamine Secretion from Intact Chromaffin Cells—At various intervals after treatment with or without BoNT/A or/B, the cells were washed briefly with Locke’s solution (19) and incubated in quadruplicate at 22 °C with this buffer in the absence or presence of 2 mM Ba2+ (20). After 15 min, aliquots of the medium bathing the cells were removed and assayed for catecholamine content using a fluorometric procedure (14). Values for basal release of catecholamine were subtracted from the amounts recorded in the presence of Ba2+ to determine the evoked components, which were expressed as a percentage of the total catecholamine content within the cells.

Preparation of Bacterially Expressed Wild-type and Mutant SNAP-25 and Assessment of their Susceptibility to BoNT/A Cleavage Using a Novel ELISA—Full-length variants of glutathione S-transferase (GST)-SNAP-25 were expressed in Escherichia coli and purified by affinity chromatography (20). Each of the recombinant proteins was coated onto 96-well plates, rinsed, blocked with 2% (w/v) bovine serum albumin and exposed to various concentrations of DTT-reduced BoNT/A at 37 °C for 4 h. The wells were then aspirated, washed, and probed with anti-SNAP-25 antibodies (generated against the last 12 residues of SNAP-25) (14). Unbound IgG was removed by rapid washing, and the bound IgGs were detected indirectly using anti-species-specific IgG-conjugated to alkaline phosphatase; appearance of the colored product was quantified upon addition of p-nitrophenyl phosphate. The A405 nm values recorded from toxin-treated wells were expressed as percentages of those for toxin-free control and plotted against the toxin concentration used. Standard curves relating amounts of intact SNAP-25 remaining in wells were made, using defined mixtures of full-length and BoNT/A-truncated GST-SNAP-25. The A405 nm readings observed were expressed as percentages of that recorded for the 100% intact protein sample and plotted against the amounts of intact GST-SNAP-25 coated. This was used to extrapolate the actual amount of intact SNAP-25 remaining in each well; generally, ~70% of the maximal A405 nm signal represented 50% intact SNAP-25 in wells.

Demonstration of the Expression of SNAP-25 Mutants in Mammalian Cells and Measurement of the Proteolytic Activities of BoNT/A or/B—CHO cells, which lack SNAP-25, were transfected with pcDNA1.1/Amp-SNAP-25s using Superfect™ and 24 h later were incubated in LISM with and without 6.6 nM BoNT/A, as described for chromaffin cells. Membranes were isolated, subjected to immunoblotting with the following IgGs (14, 15); anti-SNAP-25 (full-length)-IgG (an antiserum solely reactive with SNAP-251–197), anti-SNAP-25A (an antiseraum solely reactive with SNAP-25, 15–197), anti-SNAP-25B (generated against the last 12 residues of SNAP-25 gene (wild-type or mutants) using the calcium phosphate precipitation method (21). Four to six days after transfection, hGH secretion from intact chromaffin cells was stimulated (as described for catecholamine release) and quantified using a RIA. In some experiments, transfected cells were permeabilized with 20 μM digitonin in a permeabilization buffer (18) and exposed for 15 min to reduced BoNT/E. Release over the subsequent 15 min was evoked by the addition of 20 μM free Ca2+ and was then assessed. Aliquots were removed and assayed for hGH content; Ca2+-evoked hGH secretion was calculated as outlined above for Ba2+-evoked hGH release.

RESULTS

RECOVERY OF EVOCATED SECRETION IN NEUROENDOCRINE CELLS AFTER POISONING WITH BoNT/A OR/B SHOWS DIFFERENT TIME COURSES, A PROCESS COINCIDENT WITH REAPPEARANCE OF INTACT SNARES—To investigate the important question of how BoNTs exert their inhibitory actions for prolonged periods, neuroendocrine chromaffin cells were treated for 24 h with BoNT/A (6.6 nM) or B (66 nM), using a LISM to overcome the absence of high affinity acceptors (14). Evoked secretion and cell component of SNAREs were assessed at 5, 19, 40, and 56 days post-intoxication (Fig. 1). Ba2+ was employed instead of other commonly used stimuli (e.g. 55 mM KCl and 10 mM caffeine) because it evokes more robust catecholamine release (up to 50% of the total cell complement compared with only ~20% by the latter stimuli) while still exhibiting the same requirements for SNAP-25 and Sbr/Cbr (14). After 5 days, cells that had been treated with BoNT/A or/B showed extensive inhibition of Ba2+-evoked catecholamine release of 84 ± 0.7 and 90 ± 0.4% (means ± S.E.; n = 4), respectively, relative to that for
Days after exposure to LISM ± BoNT/A

| Days | IgG reactive with: |
|------|-------------------|
| 5    | SNAP-25(A terminus) - | + |
| 19   | SNAP-25(A terminus) - SNAP-25(B terminus) | + |
| 40 | SNAP-25(A terminus) - SNAP-25(B terminus) | + |
| 56 | SNAP-25(A terminus) - SNAP-25(B terminus) | + |

Days after exposure to LISM ± BoNT/B

| Days | Sbr/Cbr |
|------|--------|
| 5    | -      |
| 19   | -      |
| 40 | -      |
| 56 | -      |

FIG. 1. Monitoring the duration of BoNT/A- and BoNT/B-induced inhibition of catecholamine release in chromaffin cells and assessment of their SNAP-25 and Sbr/Cbr contents. Intact chromaffin cells were incubated at 37 °C in LISM for 24 h in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A (A) or 66 nM BoNT/B (B). After replacement of LISM with the normal medium (14), the cells were maintained in culture for up to 56 days with weekly substitution of the medium. At the specified times, Ba^{2+}-evoked catecholamine release over 15 min was quantified fluorometrically (means ± S.E.; n = 4) by subtraction of basal release values measured in Locke's buffer from that induced by 2 mM Ba^{2+} in the same buffer. The resultant values were expressed as a percentage of the total cell content of catecholamines. Immediately after measuring secretion, membrane fractions were prepared, as detailed under “Experimental Procedures,” solubilized in SDS, boiled for 5 min, and then frozen at −80 °C until subjected to SDS-PAGE using 12.5% gels followed by immunoblotting with the indicated antibodies.

toxin-free controls (Fig. 1). Full-length SNAP-25 was monitored using anti-SNAP-25(C terminus)-IgG, whereas intact Sbr and cellubrevin (Cbr; a BoNT-sensitive homologue (22)) were quantified using anti-Sbr/Cbr-IgG; both of these antibodies exhibit no reactivity toward the membrane-retained portion of their antigens upon proteolytic cleavage by BoNT/A or /B. The presence of BoNT/A-truncated SNAP-25 in the membrane was monitored using IgG specific for SNAP-25(A), the latter does not recognize intact SNAP-25 but reacts strongly with the product of BoNT/A (18, 23). Western blotting of the requisite membrane fraction of the cells showed that the BoNT-mediated inhibition of secretion noted at day 5 was accompanied by total or near-complete disappearance of intact SNAP-25 or Sbr/Cbr (Fig. 1), with the BoNT/A-truncated SNAP-25 being readily detected at this time (Fig. 1A). At day 40 post-intoxication, only a trace of SNAP-25 C terminus immunoreactivity was observed in BoNT/A-poisoned cells relative to the amount in toxin-free control cells; accordingly, the level of evoked secretion was not significantly greater than that recorded on day 5 (Fig. 1A). However, at day 56 post-intoxication, slightly larger amounts of intact SNAP-25 were noted in the poisoned cells, enough to cause a statistically significant level of recovery of exocytosis (from 16 to 34% of control). Throughout this time course, SNAP-25_A persisted in the intoxicated cells (Fig. 1A). Difficulties inherent to primary cell cultures precluded studies longer than 8 weeks post-intoxication, periods that would appear to be necessary to attain complete recovery from BoNT/A poisoning.

In contrast, a much faster recovery from blockade by BoNT/B was observed. The high level of inhibition of evoked secretion in BoNT/B-poisoned cells, noted on day 5, of 90 ± 0.4% gradually subsided until only 10 ± 3.4% inhibition (means ± S.E.; n = 4) remained at day 56. This resumption of regulated exocytosis was coincident with reappearance of intact Sbr/Cbr to a level comparable with that observed in toxin-free cells (Fig. 1B).

SNAP-25 Expression Demonstrated in CHO Cells and Appraisal of the Ability of Wild-type to Rescue Evoked Exocytosis in BoNT/A-pretreated Chromaffin Cells—Based on the above findings, it was hypothesized that expression of an excess of the full-length SNAP-25 in BoNT/A-poisoned cells might restore regulated exocytosis by competing with the SNAP-25_A present. As a prerequisite to testing this possibility, expression of SNAP-25 in mammalian cells had to be demonstrated; this could be most readily achieved by transfecting pcDNA1.1/Amp-SNAP-25 into CHO cells that lack endogenous SNAP-25. SDS-PAGE and Western blotting of a Triton X-100 solubilized extract of the cells showed that SNAP-25 was expressed and exhibited the same Mr as the native species present in chromaffin cells (Fig. 2A) when probed with anti-SNAP-25(full-length)-IgG; it was also detectable with anti-SNAP-25(C terminus)-IgG but not by IgG specific for SNAP-25_A (Fig. 2A). Notably, exposure of these transfected cells to BoNT/A resulted in the loss of labeling with anti-SNAP-25(C terminus)-IgG and the appearance of SNAP-25_A reactivity (Fig. 2A), establishing the susceptibility of the recombinant protein to the toxin. To evaluate the prospect of rescuing release through the introduction of SNAP-25 into BoNT/A-poisoned chromaffin cells, the protein was transiently co-expressed with hGH. The latter, which is normally absent from these cells, co-localizes with catecholamines in LDCVs; thus, evoked hGH secretion serves as an excellent reporter for LDCV exocytosis (21). Control cells transfected with vectors encoding hGH and the non-toxic pro-drog SNAP-25 expressed an appreciable level of Ba^{2+}-evoked hGH release, and this was inhibited extensively in cells pretreated with BoNT/A (Fig. 2B). Notably, these two values approximate the respective levels of stimulated catecholamine secretion observed for non-transfected cells (Fig. 1A). Transfection with a plasmid encoding wild-type SNAP-25 increased slightly the amount of hGH secreted from toxin-free cells; significantly, such overexpression of SNAP-25 failed to overcome the extent of inhibition because of BoNT/A poisoning (Fig. 2B). These results suggested that persistence of BoNT/A protease for 5 days after intoxication maintained the blockade of release; to exclude other factors, SNAP-25 mutants insensitive to BoNT/A had to be constructed and tested.

Manufacture of Mutants of SNAP-25 Highly Resistant to Proteolysis by BoNT/A—To examine the likelihood of the enzymic activity of the toxin being sustained within poisoned cells, suitable forms of full-length SNAP-25 that were not susceptible to BoNT/A but might be able to mediate exocytosis were constructed and characterized. Amino acids at positions 197 and/or 198 in wild-type SNAP-25, located on either side of the susceptible bond (i.e., the P1 or P1′ sites, nomenclature of Schechter and Berger (24)), were altered because an investigation of SNAP-25 peptides had shown that the P1′ arginine residue is critical for cleavage by BoNT/A (25). Thus, the SNAP-25B gene was altered by PCR-based site-directed mutagenesis, and the mutant proteins were expressed as GST-linked products (see
“Experimental Procedures”) so that their susceptibility to BoNT-mediated proteolysis could be assessed in vitro. Seven different SNAP-25 mutants were generated containing either single or double substitutions at the P1 and/or P9 positions (listed in Fig. 3A). Each GST-SNAP-25 variant was purified by affinity chromatography; SDS-PAGE and Western blotting confirmed that they exhibited the appropriate mobilities and immunoreactivities (not shown). The cleavage of recombinant SNAP-25 by BoNT/A was assessed using an ELISA which measured the amount of full-length substrate remaining. No-rescued data are representative of two separate experiments.

proteins highly resistant to BoNT/A compared with the wild-type substrate; replacement of P9 arginine with either alanine (R198A) or threonine (R198T) reduced degradation of SNAP-25 by ~550- and ~16,000-fold, respectively (Table I). In contrast, alteration of the P1 glutamine to alanine caused little change (Q197A/R198A) or lysine (Q197A/R198K) greatly decreased sensitivity to proteolysis by type A toxin, with the latter proving to be the most resistant (~38,000-fold reduction in susceptibility). Other double-point mutations at the P1 and P9 positions (i.e. the wild-type Gln197/Arg198 sequence to either KH, naturally occurring in Torpedo SNAP-25 (26), or WW, caused far less resistance to BoNT/A (~440- and ~96-fold, respectively; Table I). It was hoped that, at least, some of these SNAP-25 variants could rescue evoked exocytosis from inhibition by BoNT/A.

For the initial rescue experiments, R198T was selected for the mutants identified by a code name used throughout the text. B, ELISA plate wells were coated with wild-type or specified SNAP-25 mutants before exposure for 4 h at 37 °C to various concentrations of DTT-reduced BoNT/A. Intact substrate remaining was labeled with anti-SNAP-25(C terminus)-IgG, probed with alkaline phosphatase-conjugated anti-species antibodies, and quantified using a colorimetric assay (as detailed under “Experimental Procedures”). Absorbances at 405 nm were measured with an ELISA plate reader and expressed (means ± S.D.; n > 6) as percentages of the maximal values recorded for SNAP-25-containing toxin-free wells. Symbols represent wild-type (●) or SNAP-25 mutants Q197A (○), Q197A/R198A (○); Q197A/R198K (△), Q197A/R198H (■), R198A (●), Q197A/R198A (●), R198T (◆), and Q197A/R198K (○).
product would be detectable with anti-SNAP-25A IgG because of the retention of Gln197 (which was altered in the double mutants). As a preliminary to the complicated rescue experiments, the expression of R198T and its lack of susceptibility to BoNT/A were first established in CHO cells. After transfection, as described above, R198T was found to be expressed at a level similar to that of wild-type SNAP-25, as judged from the labeling on blots using two antibodies reactive with full-length SNAP-25 or its C-terminal region (Fig. 2A). Exposure of the transfected cells to BoNT/A did not lower the R198T reactivity with anti-SNAP-25(C terminus)-IgG, and furthermore, no product could be observed with SNAP-25A-IgG (Fig. 2A). Having demonstrated that R198T mutant was expressed but not cleaved by BoNT/A in mammalian cells, plasmids encoding several such SNAP-25 mutants were introduced into chromaffin cells.

**Rescue of Evoked Exocytosis in BoNT/A-pretreated Chromaffin Cells by Expression of SNAP-25 Mutants Resistant to the Toxin and Validation of the Recovered Secretion by Its Inhibition with BoNT/E—**Expression of these mutants in toxin-free chromaffin cells did not appreciably reduce their abilities to secrete hGH, as compared with controls transfected with wild-type SNAP-25 or an unrelated protein which is not involved in exocytosis (CAT) (Fig. 2B). As hoped, expression of each protease-resistant SNAP-25 in BoNT/A-treated cells (in which catecholamine release was inhibited; data not shown) rescued secretory function, close to the levels recorded for toxin-free controls (Fig. 2B). Clearly, expression of BoNT/A protease-resistant SNAP-25 can give near-complete restoration of regulated secretion in cells poisoned 5 days earlier. Additional experiments were undertaken to demonstrate conclusively that the rescue of evoked hGH secretion in BoNT/A-pre-intoxicated cells was a direct result of the participation of expressed BoNT/A protease-resistant SNAP-25. Digitonin-permeabilization provided a means to introduce BoNT/E into cells (because the LISMV-based protocol is ineffective for this serotype) so that it could proteolyze the expressed BoNT/A protease-resistant R198T mutant and, thereby, negate its protective effect. As expected in cells lacking protease-resistant SNAP-25, prior poisoning with BoNT/A caused an 82 ± 4.3% inhibition of Ca2+-evoked hGH release relative to toxin-free CAT control. On the other hand, evoked hGH secretion in BoNT/A pre-intoxicated cells expressing the protease-resistant R198T mutant was largely rescued. Importantly, when BoNT/A-pre-treated cells expressing R198T mutant were permeabilized and exposed to reduced BoNT/E (using conditions known to proteolyze nearly all the SNAP-25 (18)), the above noted restoration of evoked secretion was largely abolished and replaced with an 88 ± 7.8% inhibition of evoked hGH secretion (Fig. 4).

**Only Protease-resistant SNAP-25 Rescues Regulated Exocytosis Three Weeks after Exposure to BoNT/A, Indirect Demonstration of the Presence of Active Toxin Protease—**The ability to rescue exocytosis upon transfection of a mutated, but not wild-type, SNAP-25 in BoNT/A poisoned cells suggested that the activity of the toxin persisted for up to a week (Fig. 2B). To determine whether BoNT/A remained active for longer, the cells were co-transfected with wild-type SNAP-25 and hGH 3 weeks after poisoning. As before, this failed to cause any restoration of evoked hGH secretion, the level of inhibition remaining unaltered relative to the normal values seen for non-toxin-treated cells transfected with CAT and hGH (Fig. 5). Hence, it seems that the protease of the toxin remained active and cleaved the newly expressed wild-type SNAP-25. Accordingly, the extent of evoked hGH secretion recorded from cells intoxicated 3 weeks earlier, but now expressing protease-resistant SNAP-25 R198T, was comparable with that occurring from toxin-free cells transfected with either the same mutant or CAT (Fig. 5).

**Identification of the C-terminal Amino Acids of SNAP-25 Necessary for Exocytosis—**When BoNT/A-truncated SNAP-25, which had been shown to be expressed in CHO cells (Fig. 2A), was introduced into chromaffin cells, it diminished evoked hGH release (Fig. 6A). Moreover, its expression in BoNT/A-pre-poisoned cells consistently diminished the residual evoked secretion of hGH that commonly remained following toxin treatment (Fig. 6A). Thus, it appears that the endogenous intact SNAP-25 can be displaced from the SNARE complex by SNAP-25

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**TABLE I**

Susceptibilities of wild-type and various SNAP-25 mutants to cleavage by BoNT/A

| SNAP-25 variant | Position of mutated residue(s) | Minimum BoNT/A concentration necessary to proteolyse 50% of SNAP-25 in the standard assay | Susceptibilities to proteolysis by BoNT/A relative to wild-type
|----------------|-------------------------------|---------------------------------|-------------------------------|
| Wild-type (Gln197/Arg198) | None | 0.0045 | 1.0 |
| Q197A | P1 | 0.0054 | 1.2 |
| R198A | P1 | 2.5 | 550 |
| R198T | P1, P1' | 72 | 16000 |
| Q197AR198A | P1, P1' | 20 | 4400 |
| Q197AR198K | P1, P1' | 170 | 38000 |
| Q197KR198H | P1, P1' | 2.0 | 440 |
| Q197WR198W | P1, P1' | 0.43 | 96 |

* Standard curves, relating increasing amounts of intact GST-SNAP-25 per well to the increasing A405 nm values recorded, were used to calculate the A405 nm reading which was equivalent to 50% intact SNAP-25 remaining in wells. This absorbance value was used to determine the concentrations of BoNT/A required to proteolyse 50% of either the wild-type or mutant SNAP-25 from Fig. 3B.

* Obtained by dividing the minimal BoNT/A concentration giving 50% cleavage of SNAP-25 mutant by the corresponding values for wild-type; larger values indicate greater resistance to proteolysis.

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**FIG. 4.** Exocytosis rescued by the introduction of BoNT/A-resistant SNAP-25 into BoNT/A-pre-poisoned cells is inhibited by BoNT/E. Control (open bars) and 6.6 nM BoNT/A pre-toxin chromaffin cells, (hatched bars and closed bar) were transfected with vectors encoding hGH and CAT or the BoNT/A-resistant SNAP-25 mutant (indicated). After culturing for an additional 5 days, the transfected cells were then permeabilized using a digitonin-containing buffer excluding (open and hatched bars) or containing 100 nM DTT-reduced BoNT/E (solid bar) for 15 min (18). The Ca2+-evoked component of hGH secretion, measured over a subsequent 15-min period, was quantified (means ± S.E.; n = 4) and expressed as a percentage of the total cell content. This experiment was repeated twice, with the pattern being the same in each case.
of truncated, toxin-resistant mutants illustrated in Fig. 6B. Recent structural data on the SNAP-25 C-terminal domain (27) suggests that, when it participates in the core complex, residues 141–206 form a helical structure in which two complete turns involve seven residues, labeled “a” to “g” (Fig. 6B). The identification of the maximum number of residues that can be removed from the C-terminal end of SNAP-25, without affecting its ability to rescue exocytosis, would indicate where this functional domain terminates. In this regard, it is noteworthy that the expression of SNAP-25Δ1–199 (the product of cleavage with BoNT/C1 and known to be resistant to BoNT/A (data not shown)), SNAP-25Δ1–199-R198T, or SNAP-25Δ1–202-R198T inhibited secretion in toxin-free or -treated cells (Fig. 6A) relative to that seen with full-length SNAP-25-R198T (Fig. 6A). In contrast, a normal level of secretion was observed with SNAP-25Δ1–202-R198T or SNAP-25Δ1–203-R198T in toxin-free cells; moreover, all of these BoNT/A-resistant mutants rescued release in BoNT/A pre-poisoned cells (Fig. 6A). The noted ability of SNAP-25Δ1–201-R198T to support exocytosis to a significant but lower level than SNAP-25Δ1–202-R198T suggested that the R198T mutation might compromise slightly the ability of SNAP-25 to function in exocytosis, which only becomes apparent in this shortened construct. Indeed, this seems to be the case because the wild-type Arg198 variant of SNAP-25Δ1–201 gave the control level of release, when introduced into non-poisoned chromaffin cells. To conclusively prove that residues 197 to 201 are sufficient for normal exocytosis, the adjoining Met202 was mutated to an alanine in full-length SNAP-25; significantly, SNAP-25Δ1–206-M202A preserved the full complement of hGH secretion (Fig. 6A). Further, expression of the BoNT/A-resistant SNAP-25Δ1–206-R198T/M202A not only supported exocytosis in non-intoxicated cells but it rescued secretion in poisoned chromaffin cells (Fig. 6A). Thus, it seems that wild-type SNAP-25 encompassing residues 197–201 can mediate exocytosis without Met202 or the last four residues. As a recent study (28) indicated that mutating Leu203 to alanine in wild-type SNAP-25 suppressed exocytosis in chromaffin cells, this point mutant was evaluated. Introduction of SNAP-25Δ1–206-L203A into chromaffin cells caused no inhibition of hGH secretion (Fig. 6A). Further, expression of its BoNT/A-resistant variant, SNAP-25Δ1–206-R198T/L203A, mediated exocytosis in non-intoxicated cells and afforded near-complete rescue of secretion after poisoning (Fig. 6A). This discrepancy may relate to the use of SNAP-25 mutants tagged with Green Fluorescent Protein (GFP) in the other study.

**DISCUSSION**

In this study, near-complete recovery of regulated catecholamine release was seen within 56 days after intoxication of chromaffin cells with BoNT/B, accompanied by the re-appearance of intact Sbr/Cbr. This time course approximates to the duration of paralysis caused by intramuscular injection of the toxin into humans (2) and also correlates with that found for chromaffin cells poisoned with TeTX (16), suggesting that either the lifetime of BoNT/B and TeTX are very similar and/or...
the rate of replenishment of intact Sbr/Cbr determines the speed of recovery. In the case of chromaffin cells poisoned with type A toxin, however, the resumption of evoked exocytosis was very much slower, with only a partial recovery being observed in 2 months; again, this is similar to published data for both chromaffin cells (16) and human NMJ (2). Moreover, it was demonstrated that this prolonged inhibition is because of the lack of intact SNAP-25 and persistence of its truncated product. Such differences between the two serotypes suggest that BoNT/A remains active for longer than BoNT/B and/or the turnover of SNAP-25 is significantly slower than that of Sbr/Cbr. Indeed, we show for the first time that BoNT/A is proteolytically active for 3 weeks after poisoning because expression of wild-type SNAP-25 in BoNT/A-poisoned cells failed to induce a recovery, whereas this was achieved using a toxin-resistant mutant. Thus, the continued action of BoNT/A-LC is a major contributory factor in the prolonged inhibition of release, at least in this model system, a conclusion supported by the observation that antibodies directed against BoNT/A-LC induced a fast recovery of secretion in chromaffin cells (16). However, it is not just the continued BoNT/A activity but, additionally, the persistence of SNAP-25A that limits recovery because injection into human muscles of BoNT/E, which exhibits a very short persistence of SNAP-25 that limits recovery because injection into human muscles of BoNT/E, which exhibits a very short duration of action, together with type A, yielded a time course of recovery typical of the C toxin (29). It has been proposed that the cleavage of 17 extra amino acids from the C-terminal end of SNAP-25A by BoNT/E accelerates removal of the product, thereby leading to a more rapid recovery of neurotransmission. The duration of poisoning, which is similar in both of these models, is determined by the ratio between the amount of toxin activity persisting and the rate of SNAP-25 synthesis. Although available information suggests that this ratio is lower in motor nerve endings than chromaffin cells, slow recycling of SNAP-25A in the nerve terminals (suggested by the accelerating effect of BoNT/E) could prolong the recovery period proposed in (29). In view of our demonstration that BoNT/A remained active for more than 21 days, a strategy was devised for rescuing release based on the use of SNAP-25 mutants that were resistant to BoNT/A but capable of mediating exocytosis. Analysis of a series of mutants constructed revealed that the P'1 residue is very important for efficient cleavage of full-length SNAP-25 by BoNT/A, as had been observed for C-terminal 17-mer peptide substrates (residues 187–203) of SNAP-25 (25). During the course of this study, substitutions at P'1 position were reported to create a similarly high resistance to scission by BoNT/A of SNAP-25 (7). Significantly, it is shown herein for first time that introduction of these BoNT/A-resistant forms of SNAP-25 into intact chromaffin cells rescues exocytosis; this successful “in vivo” recovery system provided a means for examining the importance of residues at the BoNT/A cleavage site. Notably, expression of wild-type SNAP-25 or BoNT/A-resistant mutants did not prove toxic to the cells, as reflected by the extent of evoked hGH release remaining unaltered. Accordingly, recombinant SNAP-25 has been shown to be targeted to the appropriate plasma membrane locations in PC12, as well as insulinoma cells (30, 31). As SNAP-25 must complex with the limited amounts of syntaxin 1A/B and Sbr present, to serve its essential role in exocytosis, it can reasonably be assumed that excess protease-resistant SNAP-25 would competitively antagonize the participation of native intact or BoNT/A-cleaved SNAP-25 in SNARE complex formation. The fact that BoNT/E was found to inhibit the rescue of exocytosis provides further evidence for the involvement of BoNT/A-resistant SNAP-25 in SNARE-mediated exocytosis. In light of these considerations, it is evident that Gln197 and Arg198 in SNAP-25, which were altered in the protease-resistant mutants, are not essential for evoked exocytosis. In fact, this study and others provide mounting evidence for there being a high degree of amino acid degeneracy in SNAP-25. For example, introduction of a BoNT/E-resistant SNAP-25 (residues 180–181) mutant exerted minimal effect on regulated exocytosis in insulinoma cells, but it prevented the anticipated inhibition of evoked secretion upon exposure of the permeabilized cells to BoNT/E (31). Further, a recent comprehensive site-directed mutagenesis study of SNAP-25 has revealed that mutations of “a” and/or “d” positions (Fig. 6B), hydrophobic residues predicted to be important for coil-coil interactions, lowered the stability of SNAP-25-syntaxin 1A/B-Sbr ternary complexes and diminished the ability of this region of SNAP-25 to perform its function in regulated vesicular fusion (32). The full extent of this degeneracy was seen when human SNAP-25 (a BoNT/E-resistant non-neuronal homologue which is only ∼60% identical to SNAP-25) was overexpressed in insulinoma cells; this allowed exocytosis which was not susceptible to BoNT/E when applied after permeabilization of the cells (33). The present study is the first to show the amino acid degeneracy of SNAP-25 by quantifying the ability of BoNT/A-resistant mutants to rescue exocytosis in intact prepoisoned cells.

As the expression system adapted here enabled accurate evaluation of the functionality of mutated and wild-type SNAP-25, it was used to determine the minimal essential domain for exocytosis within amino acids 198 to 206 (i.e. those excised by BoNT/A). This is an important issue because recent structural data on SNAP-25 show that the C-terminal core domain orients in such a way that it appears to make the initial contact with Sbr/Cbr (27). The observed inability of SNAP-25-(1–198) to cause rescue is significant because it proves that the cleavage product generated by BoNT/C does not support exocytosis; this result was expected but had not been proven because BoNT/C proteolizes both syntaxin 1A/B and SNAP-25 (7, 15, 34). Thus, the truncation of SNAP-25 by BoNT/C is inhibitory in its own right regardless of its cleavage of syntaxin 1A/B. This finding agrees with the structural data which reveals that residue 199, found in the C-terminal predicted helical domain of SNAP-25 and removed by BoNT/C, comes in close contact with the predicted helical domains of the N-terminal SNAP-25 and syntaxin 1A/B (27). The deletions studied herein established that only residues encompassing positions 1–201 are absolutely essential for the participation of SNAP-25 in exocytosis, with residues 1–200 failing to rescue release; and furthermore, SNAP-25 lacking the last five amino acids without the BoNT/A resistant R198T alteration proved as effective as the full-length mutant for rescuing evoked secretion in BoNT/A-poisoned cells. Further evidence was provided for 202–206 being redundant by demonstrating that point mutations at positions 202 or 203 did not affect their ability to support secretion. However, during the preparation of this manuscript, another study (28) showed that only three of these C-terminal amino acids are redundant for SNAP-25 functioning; thus, residues 1–203 are essential for exocytosis. This apparent discrepancy could be explained in one of several ways. First, a slower rise time for exocytosis in cells expressing SNAP-25-(1–202) might explain why a complete block of exocytosis was only observed after 10 s, with only a partial inhibition being recorded within 1 min in the latter study, compared with slight inhibition being apparent within 15 min in our experiments (Fig. 6A). Second, the R198T mutation might render the coiled-coil domain more stable, lessening the importance of Leu203 for regulated exocytosis. However, amino acids at the "g" position, such as Arg198, tend to be charged. Changing this amino acid might cause a reduction in the ability of the C-terminal end of
SNAP-25 to interact with syntaxin 1A/B (Fig. 6B), which is consistent with our data (Figs. 2B, 4, 5, and 6A). Third, the use of a GFP tag at the N-terminal end of SNAP-25 in the other investigation (28) might have slightly altered its ability to support exocytosis when this truncation was introduced. A scenario that would reconcile these apparently different findings would imply that there is not a single cut off point which determines its ability to support exocytosis. Our work (Fig. 6A) and theirs (28) suggest that the addition of each of three residues after 200 improves exocytosis to a small extent until the maximum is reached with 203. Thus, the differences between the abilities of constructs 1–201, 1–202, or 1–203 to support exocytosis is slight.

The majority of the outbreaks of human botulism are caused by intoxication with BoNT/A, /B, or /E (reviewed in Ref. 35), and at present, the only useful treatment relies on the prompt administration of neutralizing antibodies. Unfortunately, this treatment is ineffective once the toxins have been internalized by motor nerves and patients start displaying symptoms of botulism. Although effective toxoids exist for vaccination, they are becoming increasingly unpopular because of the widespread and successful use of BoNTs in the treatment of numerous muscle movement disorders (2). In view of this recently acquired attitude and the above-noted factors, there is clearly an urgent requirement to design novel treatments for botulism. One such approach that is being pursued with success is the development of small peptide-based inhibitors (25). An alternative and attractive approach involves the use of gene therapy to rescue neurotransmitter release by expression in motor neurons of BoNT/A protease-resistant SNAP-25. Our work clearly demonstrates that this is a viable proposition because the regulated exocytotic pathway is not required for the transfer of SNAP-25 to the plasma membrane. Clearly, the constitutive pathway which transfers palmitoylated SNAP-25 to the plasma membrane from the Golgi (36) is not blocked in chromaffin cells by BoNT/A.

Acknowledgments—We express our gratitude to the following people for assistance and invaluable advice during the course of this work: Drs. B. Livett, P. Marley, F. A. Meunier, D. T. O’Connor, and S. Wilson.

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