Novel Chimeras of Botulinum Neurotoxins A and E Unveil Contributions from the Binding, Translocation, and Protease Domains to Their Functional Characteristics*

Jiafu Wang, Jianghui Meng, Gary W. Lawrence, Tomas H. Zurawski, Astrid Sasse, MacDara O. Bodeker, Marcella A. Gilmore, Ester Fernández-Salas, Joseph Francis, Lance E. Steward, K. Roger Aoki, and J. Oliver Dolly

From the International Centre for Neurotherapeutics, Dublin City University, Glasnevin, Dublin 9, Ireland and Allergan Incorporated, Irvine, California 92623

Hyperexcitability disorders of cholinergically innervated muscles are treatable with botulinum neurotoxin (BoNT) A. The seven serotypes (A–G) potently block neurotransmission by binding to presynaptic receptors, undergoing endocytosis, transferring to the cytosol, and inactivating proteins essential for vesicle fusion. Although BoNT/A and BoNT/E cleave SNAP-25, albeit at distinct sites, BoNT/E blocks neurotransmission faster and more potently. To identify the domains responsible for these characteristics, the C-terminal heavy chain portions of BoNT/A and BoNT/E were exchanged to create chimeras AE and EA. After high yield expression in Escherichia coli, these single chain chimeras were purified by two-step chromatography and activated by conversion to disulfide-linked dichains. In vitro, each entered neurons, cleaved SNAP-25, and blocked neuromuscular transmission while causing flaccid paralysis in vivo. Acidification-dependent translocation of the light chain to the cytosol occurred more rapidly for BoNT/E and EA than for BoNT/A and AE because the latter pair remained susceptible for longer to inhibitors of the vesicular proton pump, and BoNT/A proved less sensitive. The receptor-binding and protease domains do not seem to be responsible for the speeds of intoxication; rather the N-terminal halves of their heavy chains are implicated, with dissimilar rates of cytosolic transfer of the light chains being due to differences in pH sensitivity. AE produced the most persistent muscle weakening and therefore has therapeutic potential. Thus, proof of principle is provided for tailoring the pharmacological properties of these toxins by protein engineering.

Seven serotypes (A–G) of botulinum neurotoxin (BoNT),2 dichain (DC) proteins (~150 kDa) from Clostridium botulinum, selectively bind and enter certain nerve terminals. They potently block the exocytosis of neurotransmitters by cleaving proteins that are essential for the fusion of synaptic vesicles with the presynaptic membrane: SNAP-25 (synaptosome-associated protein of 25 kDa; BoNT/A, BoNT/E, and BoNT/C1), vesicle-associated membrane protein (BoNT/B, BoNT/D, BoNT/F, and BoNT/G), and syntaxin (a second substrate of BoNT/C1) (reviewed in Refs. 1 and 2). Potent and preferential blockade by the toxins of cholinergic synaptic transmission in the peripheral nervous system underlies the clinical symptoms of botulism, which include dry mouth, ptosis, difficulty in swallowing, and respiratory failure (3). However, these same properties have proven most advantageous for the successful treatment, particularly with the BoNT/A complex, of a wide variety of neurogenic hyperactivity disorders such as dystonias, dys phonias, spasticity, overactive bladder, hyperhidrosis, hyper salivation, and certain types of headache (4). BoNT/A gains access to overactive nerve endings by binding with high affinity, via the C-terminal half (HC) of its heavy chain (HC; ~100 kDa), to a luminal domain of SV2A/SV2B/SV2C (synaptic vesicle protein 2), which is exposed at the cell surface after exocytotic fusion of synaptic vesicles (5, 6). An intravesicular region of synaptotagmin (I and II), another synaptic vesicle protein, acts as an avid receptor for BoNT/B and BoNT/G (7–9). BoNTs also interact with gangliosides with lower affinity, and the latter stabilize binding of the toxins to their respective protein receptors (10, 11). The distinct protein receptors (2) for the other BoNT serotypes remain to be identified. As synaptic vesicle proteins are recovered from the plasmalemma, BoNTs are carried into the lumen of recycling vesicles, and acidification triggers the translocation of their light chain (LC; ~50 kDa) into the presynaptic cytosol. A basis for a disulfide link between the LC and HC being required for uptake (12) has been demonstrated; a membrane-spanning channel formed by the HC mediates translocation of the LC, which is released upon reduction in the cytosol (13, 14).

Notably, BoNT/E enters cultured neurons more quickly than BoNT/A (15), has a higher potency, and acts faster to inhibit transmission at the neuromuscular junction (16–18). Furthermore, BoNT/E totally inhibits vesicle fusion more akin to the...
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effects of vesicle-associated membrane protein- or syntaxin-cleaving BoNTs than the reduction in fusion rate induced by BoNT/A (19, 20). Moreover, the blockade of transmitter release by BoNT/E is relatively unaffected by treatments that elevate cytosolic [Ca$^{2+}$] and antagonize inhibition by BoNT/A (20–24). All these properties would be highly desirable for incorporation into new and improved therapeutic versions of BoNT, although BoNT/E produces transient muscle weakness compared with the long duration of action of BoNT/A (25). Thus, the feasibility of such an approach was assessed.

Herein, chimeric BoNTs were constructed by recombinant substitution of the HC$_{a}$ domains in BoNT/E and BoNT/A with their counterpart region from the other serotype and expressed as His$_{6}$-tagged single chain (SC) proteins in *Escherichia coli*. The resultant chimeras could be converted into their disulfide-linked DC form by controlled proteolysis, which also removed the tag. The EA chimera, containing the LC and the N-terminal half (His$_{6}$) of the HC of BoNT/E fused to the HC$_{a}$ domain of BoNT/A, was shown to enter cultured neurons almost as rapidly as BoNT/E and blocked neuromuscular transmission with equipotency, although paralysis was transient (as for BoNT/E). Its speedy internalization into neurons could be attributed to an elevated sensitivity to pH of the BoNT/E LC-HN$_{a}$ portion that facilitates expedient cytosolic transfer of the proteolytic LC rather than to the receptor utilized. On the other hand, chimera AE (the LC and HC$_{a}$ domain of BoNT/A fused to the HC$_{a}$ domain of BoNT/E) was less potent than EA or BoNT/E and much slower to enter neurons, but produced prolonged muscle weakness in mouse, matching that induced by clinical preparations of type A toxin-hemagglutinin complex. As AE should utilize a different receptor than BoNT/A for neuronal entry, it could be a useful alternative for clinical application in the minority of patients who are primary non-responders to the BoNT/A complex.

EXPERIMENTAL PROCEDURES

**Materials**—The following materials were obtained as indicated; the pET29a expression vector, Novagen; *E. coli* BL21(DE3), Stratagene; restriction enzymes, New England Biolabs; TALON Superflow resin, Clontech; and UNO S1 and Q1 columns, Bradford protein assay kit, Bio-Rad. AccuPrime™ Pfx DNA polymerase, precast gels, and reagents for SDS-PAGE were supplied by Invitrogen. PD-10 desalting columns were provided by GE Healthcare. Substrates for ECL detection of horseradish peroxidase were purchased from Millipore. Antibodies were from Sigma (rabbit anti-SNAP-25, anti-HPC-1, and anti-IgG conjugated to either horseradish peroxidase or alkaline phosphatase), Sternberger Monoclonals (SMI-81), and Novagen (anti-His$_{6}$). Custom antibodies specific for LC$_{a}$/LC$_{e}$/HC$_{a}$/HC$_{e}$, or BoNT/A were prepared by Zymed Laboratories Inc. Purified natural BoNTs were purchased from List Laboratories (BoNT/A, DC form) and Metabiologics (BoNT/E, SC form). The latter was proteolytically nicked to the DC (>95%) with TrypZean (8 µg/mg of BoNT) for 40 min at 27°C. Bafilomycin A$_{1}$ (BafA$_{1}$) was supplied by LC Laboratories (Woburn, MA). Concancamycin A (ConA) and all other reagents were from Sigma. Mice were obtained from Charles River Laboratories (C57) and Harlan UK (female Tyler’s Ordinary); Sprague-Dawley rats were bred in an approved animal unit at Dublin City University. All experimental procedures involving animals were approved by an Institutional Ethics Committee and licensed by the Irish Government, Department of Health and Children.

**Generation of Constructs for BoNT AE and EA Chimeras:** Their Expression, Purification, and Characterization—The cloning and expression of BoNTs were performed in accordance with European Union regulations, registered in Ireland with the Environmental Protection Agency, and notified to the Health and Safety Authority.

Genes encoding either BoNT/A or BoNT/E SCs were codon optimized for expression in *E. coli* and synthesized, and the sequences were verified. For constructing chimera AE (see Fig. 1), a fragment encoding the LC plus the translocation domain (His$_{6}$) of BoNT/A (LC-HN$_{a}$/A) and that encoding the binding domain of BoNT/E (HC$_{a}$/E) were amplified by standard PCR using a high fidelity polymerase. The products were cloned into the pET29a vector using suitable restrictions sites to generate the AE chimera (Fig. 1). Nucleotides encoding a nine-residue linker were inserted between the translocation and binding domains; also, modification of the original vector sequence introduced trypsin cleavage sites between H$_{c}$/E and the C-terminal six histidines (His$_{6}$) (see Fig. 1). For constructing chimera EA, a fragment encoding the binding domain of BoNT/A (H$_{a}$/A) was likewise generated, and nucleotides were added to encode a trypsin cleavage site at its C terminus. This fragment, as well as a separate DNA sequence encoding the LC plus the translocation domain of BoNT/E (HC$_{a}$/E), was amplified by PCR and cloned into pET29a to create an expression vector containing the EA insert (see Fig. 1).

After DNA sequence verification, each new SC gene was transformed into *E. coli* BL21, and expression was elicited by autoinduction (26); optimal incubation periods and temperatures were determined empirically. Cells were then pelleted, washed, and lyzed using lysozyme and several freeze/thaw cycles; insoluble material was removed by centrifugation. The AE SC was trapped by immobilized metal affinity chromatography (IMAC) on TALON resin, eluted with 500 mM imidazole, and transferred into 50 mM Tris-HCl buffer (pH 8.1) using Sephadex G-25. For further purification, the sample was loaded onto a UNO Q1 column, and after washing with 30 mM NaCl, a stepwise gradient up to 1 mM NaCl in 50 mM Tris-HCl buffer was applied. Fractions containing the eluted SC toxin were pooled and either stored at −80°C or proteolytically nicked by TrypZean (1 µg/mg of BoNT) for 1 h at 25°C before the addition of trypsin inhibitor (10 µg/mg of toxin) and storage of the DC (as for the SC). The expression and affinity purification of the chimera EA SC were performed similarly. The pooled eluate from IMAC was gel-filtered into 0.02 M sodium phosphate buffer (pH 6.5) and further purified by loading onto a UNO S1 column, followed by washing with 150 mM NaCl and elution with a stepwise gradient up to 1 mM NaCl in 0.02 M sodium phosphate buffer. After transfer of the eluted toxin into 50 mM

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3 K. R. Aoki, unpublished data.
were monitored on a daily basis, and the DAS was determined using a five-point scale (0 = normal to 4 = maximal reduction in digit abduction) as described (28). Toxin lethalities were determined by intraperitoneal injection into mice as described previously (29). The lowest amount of toxin that killed 50% of mice within 4 days was taken as one minimal lethal dose (mLD$_{50}$) and expressed as the number of mLD$_{50}$ units/mg of toxin.

**Isolation and Culturing of Mouse Spinal Cord, Rat Cerebellar Granule, and Superior Cervical Ganglion Neurons: Exposure to BoNTs and Assay of SNARE Cleavage—** All neuronal cultures were maintained at 37 °C in a 5% CO$_2$ atmosphere. Neurons were prepared from mouse spinal cords removed at gestation day 13, dissociated with trypsin, and seeded at a density of 10$^6$ cells/well on rat tail collagen-coated 24-well plates in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated horse serum and other factors (15). At 21 days in vitro, cultures were exposed for 20 min to each toxin in stimulation buffer (15), washed twice with toxin-free medium, and incubated for the periods specified in the figure legends before harvesting for analysis. In some experiments, an endosomal acidification inhibitor, ConA, was added (250 nM final concentration) at various times after toxin removal as described (15); the cells were harvested after an additional 20 h of culturing. Rat cerebellar granule neurons (CGNs) were prepared and maintained following established methodology (30). At 7 days in vitro, the cells were exposed for 24 h at 37 °C to a series of toxin concentrations. In some cases, cells were incubated for 5 min with 500 pm BoNTs in HEPES-buffered solution containing 70 mM K$^+$ (stimulation) with adjustment of the NaCl concentration (30); after being washed twice and incubated in medium at 37 °C for the periods specified in the figure legends, the cells were harvested. The isolation and culture of neurons from the superior cervical ganglia of 1–3-day-old rat pups were performed as described (31). The neurons were dispersed in L15 medium supplemented with vitamins, plated on rat tail collagen-coated 48-well plates, and maintained for 8–10 days before experimentation. The neurons were exposed to 10 nM toxin for the periods indicated in the figure legends before harvesting.

At the end of each experiment, all cell types were solubilized in lithium dodecyl sulfate sample buffer and heated to 80 °C for 5 min before SDS-PAGE and Western blotting; SNAP-25 was visualized with a monoclonal antibody (SMI-81, which recognizes intact and BoNT/A- or BoNT/E-cleaved products) and quantified as described (30, 32).

**Statistical Analysis—** Data were calculated and graphs generated using GraphPad Prism 4.0. $p$ values were calculated by Student’s $t$ test.

**RESULTS**

**Generation of BoNT Chimeras by Genetic Recombination of Functional Domains from BoNT/A and BoNT/E—** Based on sequence alignments and published crystal structures of BoNT/A and BoNT/B (33, 34), a portion of the gene deduced to encode the protease and translocation domains of BoNT/A was linked to that for the C-terminal receptor-binding moiety of BoNT/E plus 19 additional amino acids, including a His$_{15}$ tag, to generate chimera AE (LC-H$_{15}$/H$_{C}$/E-His$_{15}$) (Fig. 1). More-
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FIGURE 1. Schematic representation of engineered BoNT chimeras AE and EA. Fusion of the requisite synthetic DNA sequences (see “Experimental Procedures”) created the recombinant AE incorporating residues 1–874 of BoNT/A (LC/A and H2/A; white box), a linker of nine foreign residues, H2/E (residues 845–1252 of BoNT/E; gray box), and an additional 19 exogenous residues including trypsin cleavage sites and a C-terminal His6 tag (see “Experimental Procedures”). Chimera EA is composed of LC-H2/E (residues 1–1244 of BoNT/E; white box), two foreign amino acids, H2/A (residues 871–1296 of BoNT/A; white box), and 16 foreign residues including two trypsin cleavage sites before a His6 tag. Numbers refer to residue positions in the amino acid sequences (GenBankTM accession numbers AF488749 and X62683) of corresponding parental BoNTs. S–S denotes the disulfide bridge that spans a trypsin-nicking site. Letters between boxes specify the above-noted extra residues that were incorporated as linkers. Predicted exposed sites for cleavage by trypsin for tag removal are underlined.

FIGURE 2. Purification of BoNT chimeras. Chimeras AE (A) and EA (C) were purified from E. coli lysates (see “Experimental Procedures”) by IMAC. Aliquots were analyzed by SDS-PAGE under reducing conditions, followed by Coomassie Blue staining (A and C). Lane 1, protein standards; lane 2, cleared E. coli lysate; lane 3, flow-through from IMAC; lane 4, wash fraction; lanes 5–10, eluted fractions. Pooled fractions from A and C were subjected to anion-exchange (B) and cation-exchange (D) chromatography, respectively, as described under “Experimental Procedures.” The eluted peak of each chimera is marked (▼). mS, millisiemens.

However, AE possesses a linker of nine exogenous residues between the H2N/A and H2C/E domains to increase flexibility between these functional moieties (Fig. 1). This chimeric toxin was expressed in E. coli using Studier’s autoinduction medium (26) and isolated to ~80% purity from lysed bacteria by IMAC on a Co2+-charged resin. A protein of ~146 kDa was eluted by imidazole together with smaller amounts of lower molecular mass impurities as demonstrated by SDS-PAGE and Coomassie Blue staining (Fig. 2A); the major component co-migrated with natural BoNT/A and BoNT/E when lower quantities of proteins were run under nonreducing conditions (data not shown). All contaminants were removed by anion-exchange chromatography (Fig. 2B); the bound toxin was eluted by 70 mM NaCl (typically 10 mg of pure toxin (Fig. 3A) from a 1-liter culture). The resultant AE chimera gave a single band of ~146 kDa upon SDS-PAGE in either the absence or presence of DTT, confirming that its expression is in the SC form (Fig. 3A). Western blotting demonstrated the presence in the 146-kDa SC of the BoNT/A LC and epitopes from the receptor-binding domain of BoNT/E and confirmed the absence of truncated forms (Fig. 3A). Controlled nicking with TrypZean gave near-complete conversion of the SC to a disulfide-linked DC as revealed by the appearance of the HC and LC upon SDS-PAGE in the presence of DTT (Fig. 3A); continued migration at ~146 kDa in the absence of reducing agent indicates that the interchain disulfide was formed in virtually all of the DC (Fig. 3A). As expected, antibodies to H2C/E recognized the HC but not LC, whereas anti-LC/A antibody labeled the latter (Fig. 3A); the observed lack of reactivity with an antibody against His6 highlighted that all of the C-terminal tag was removed during nicking (Fig. 3A).

In a similar manner, DNA encoding the LC and H2N of BoNT/E was fused to the sequence for the H2C of BoNT/A to create a gene for chimera EA (Fig. 1), which was expressed in E. coli and affinity-purified as described for AE (Fig. 2C). Further purification was achieved by cation-exchange chromatography (Fig. 2D) on an UNO S1 column, with elution of the toxin by ~220 mM NaCl (~15 mg of pure EA from a 1-liter culture). Although the major protein band migrated upon SDS-PAGE at ~145 kDa, a minor amount of larger, aggregated material (which disappeared upon reduction) was detected by Coomassie Blue staining and Western blotting (Fig. 3B). Complete conversion of the EA SC to DC with TrypZean was observed as described above for AE; likewise, the interchain disulfide was found to have been formed in the vast majority of the toxin. The presence of the requisite moieties (LC/E and H2C/A) in EA and the successful removal of the His6 tag were confirmed by Western blotting (Fig. 3B). Staining of the nonreduced nicked toxin with IgGs specific for LC/E revealed a weak signal at ~97 kDa, which is assumed to correspond to...
LC-H$_N$ resulting from a small degree of cleavage midway along the HC; accordingly, this disappeared upon reduction. Again, immunovisualization of LC and HC bands in the nicked sample, run without reductant, confirmed that the interchain disulfide failed to form only in a minor proportion of the toxin (Fig. 3B).

Chimera EA Rapidly Enters Neurons, Cleaves SNAP-25, and Blocks Neuromuscular Transmission, whereas AE Is Relatively Slow—The biological activities of these chimeras were assessed in vitro, using mouse phrenic nerve diaphragms and cultured neurons, and in vivo following intraperitoneal injection into mice (see “Experimental Procedures”). Chimera EA readily paralyzed the hemidiaphragm (Fig. 4A). Time to neuromuscular paralysis relates to concentration by a power function, as shown by a linear plot on double log axes (Fig. 4A). There is little difference between chimera EA and the parental fast-acting BoNT/E neurotoxin, and both were more potent than BoNT/A (as reflected by its rightward-shifted concentration-paralysis time power relationship) (Fig. 4A) (18), suggesting that the more rapid paralysis is a function of the LC and/or H$_N$ region of BoNT/E and not influenced by binding of the HC to a distinct receptor. Moreover, chimera AE was significantly slower/less potent than EA or either of the natural toxins (Fig. 4A), indicative of this protein lacking features responsible for binding or faster translocation used by BoNT/E (see “Discussion”). The mLD$_{50}$ observed for chimera EA was 150 pg; this equates to 7 × 10$^6$ units/mg of protein. Notably, AE was only slightly less toxic, and both chimeras displayed lower potencies than natural BoNT/A and BoNT/E (Table 1). Notably, the values recorded for the SCs of EA and AE were at least 100-fold lower, showing that significant activation occurred upon conversion of each to the DC form (data not shown). Curiously, the order of lethality for the DC (BoNT/A > BoNT/E > EA > AE) differs from their relative order of neuroparalytic potency on excised diaphragm (BoNT/E > EA > BoNT/A > AE) (Fig. 4A). Clearly, speed of neuromuscular paralysis onset is not an accurate gauge of lethality; moreover, the rank order of potency on diaphragm is also altered if paralysis time is plotted against mLD$_{50}$ units of toxin (Fig. 4A, inset; see “Discussion”).

In cultured CGNs, AE proved to be less potent than BoNT/E, BoNT/A, and EA, which were similar (Fig. 4B). Using condi-
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TABLE 1
Proteolytic activities and mouse lethalities of DC chimeras and parental toxins

| Toxin   | EC_{50} for cleavage of GFP-SNAP-25-(134–206)-His_{6}\textsuperscript{a} | mL D_{50}/mg\textsuperscript{b} |
|---------|-------------------------------------------------|-------------------------------|
| Chimera AE | 0.11 ± 0.02 (n = 5) | 0.3 × 10\textsuperscript{7} |
| Chimera EA | 0.15 ± 0.02 (n = 9) | 0.7 × 10\textsuperscript{7} |
| BoNT/E  | 0.33 ± 0.02 (n = 6) | 10 × 10\textsuperscript{7} |
| BoNT/A  | 0.12 ± 0.01 (n = 7) | 30 × 10\textsuperscript{7} |

\textsuperscript{a} Proteolytic activities of parental and chimeric DC toxins were determined using a model substrate (13.5 μM GFP-SNAP-25-(134–206)-His_{6}\textsuperscript{a}). Values represent the amount of each toxin needed to cleave 10% of substrate within 30 min at 37 °C; n values indicate the number of independent experiments.

\textsuperscript{b} mL D_{50} is the lowest dose of toxin that killed 50% of a group of four mice within 4 days after intraperitoneal injection.

...tions that stimulate synaptic vesicle recycling (30) in CGNs, which may promote the activity-dependent uptake of BoNTs as occurs at the neuromuscular junction (5), the potency of all four toxins was increased, but to different extents (data not shown). However, the rates of cellular intoxication by BoNT/E and EA were clearly faster compared with BoNT/A and AE (Fig. 4C). Cleaved SNAP-25 was observed within 15 or 45 min of exposure to BoNT/E or chimera EA; furthermore, in both cases, 50% of the total target had been cleaved within 160 min. In contrast, no product was detectable at 80 min in BoNT/A-treated cells, of the total target had been cleaved within 160 min. In contrast, entry of BoNT/A remained largely blocked even after an additional 10 min, and AE remained sensitive for the longest period despite its 10-fold higher concentration. Clearly, chimera EA (like BoNT/E) proceeds beyond an acidification-dependent step more rapidly than BoNT/A. Thus, the BoNT/E translocation activity, but not receptor binding, is implicated in the faster translocation of its LC out of acidified endosomes.

Using superior cervical ganglion neurons (SCGNs), equivalent rates of SNAP-25 cleavage (and presumably entry) were recorded for BoNT/E and chimera EA, but BoNT/A proved much slower (Fig. 5C); AE produced minimal cleavage in SCGNs, even after 24 h (data not shown). The importance of endosomal acidification was established using BafA1, which inhibited dose-dependently SNAP-25 cleavage by BoNT/A, BoNT/E, and chimera EA (Fig. 5D). However, higher concentrations of this proton pump inhibitor were required to inhibit BoNT/A compared with BoNT/E or EA; whereas both BoNT/E and EA were attenuated by 3.3 mM BafA1, BoNT/A remained unaffected by this concentration (Fig. 5D, inset; \( p < 0.05 \)). Therefore, BoNT/A apparently differs from BoNT/E and EA in its ability to sense the changes in endosomal pH caused by blockade of the vesicular H\textsuperscript{+}-ATPase; notably, the LC-H\textsubscript{N} domain is implicated in this differential pH sensing because chimera EA displayed near-identical BafA1 sensitivity to BoNT/E, and their LCs have similar protease activities (see above).

Unlike EA, Chimera AE Causes Persistent SNAP-25 Cleavage in Vitro and Neuromuscular Paralysis in Vivo—Striking differences were unveiled upon monitoring the proportion of intact SNAP-25 over time in CGNs that had been exposed for 24 h to chimera EA or AE, BoNT/A, or BoNT/E. Whereas the ratio of intact to cleaved SNAP-25 remained constant for 30 days in cells treated with various concentrations of chimera AE or BoNT/A (Fig. 6A and inset), a relatively fast recovery of full-length SNAP-25 and loss of cleaved SNAP-25 were seen in those exposed to EA or BoNT/E (Fig. 6B and inset). To assess duration of paralytic action in vivo, toxins were injected into the right hind legs of mice, and muscle paralysis was measured using the DAS scale (Fig. 6C). For optimal performance, the maximal tolerated dose of each toxin was established empirically (Fig. 6C, inset; see “Experimental Procedures”). Notably, BoNT/E gave full inhibition (DAS = 4) within 3 h, whereas a similar reduction in mobility took >12 h to develop with BoNT/A; this concurs with the faster action of BoNT/E on excised diaphragm (see above). Chimera EA showed a faster onset (5 h to DAS = 4) than BoNT/A or AE, but slightly less rapid than BoNT/E. As found previously (23), full muscle activ-
ity (DAS = 0) was recovered within 5 days in mice injected with BoNT/E (Fig. 6C), but those treated with BoNT/A remained paralyzed fully (DAS = 4) for 5–6 days and took more than five times longer to recover completely (up to 28 days). Mice recovered rapidly from paralysis with chimera EA (5 days), which accords with the notion that the BoNT/E LC is less stable inside neurons than its counterpart in BoNT/A. Chimera AE outlasted (up to 37 days) all of the other toxins tested, matching published reports for clinically used type A complex (35).

Clearly, BoNT LCs determine the time course for recovery from neuromuscular paralysis rather than by the receptors involved or, by extrapolation, any possible difference in targeting (2, 5).

Collectively, these results show that effective BoNT chimeras can be constructed by recombination of fragments from the genes of two separate serotypes, expressed efficiently, purified to homogeneity by simple two-step chromatography, and readily converted to the much more active forms. Chimeras EA

FIGURE 5. Like BoNT/E, chimera EA enters cultured neurons, escapes from acidified endosomes, and cleaves SNAP-25 more rapidly than BoNT/A. Mouse spinal cord neurons (A) and rat SCGNs (C) were exposed to 0.4 and 10 nM toxins, respectively, as described under “Experimental Procedures.” At the indicated times, cells were subjected to SDS-PAGE and blotted with antibodies that recognize full-length and C-terminally truncated SNAP-25. The percent SNAP-25 cleaved was calculated from the relative signal intensities for the intact and truncated forms. Note that a log scale is used on the abscissa in C from a representative of three experiments. B, spinal cord neurons were treated with 0.4 nM BoNT/A, BoNT/E, or EA or 4 nM AE in stimulation buffer for 20 min; after washing with medium, ConA was added at the times indicated. Following culture for an additional 20 h, the extents of SNAP-25 cleavage were measured and normalized to the respective percentage cleaved in non-ConA-treated BoNT-intoxicated cultures, which were 62.1, 97.2, 74.7, and 68.1% for BoNT/A, BoNT/E, EA, and AE, respectively. D, SCGNs were exposed for 20 h to 10 nM toxin in the presence of BafA1 at the concentrations indicated before analysis of SNAP-25 cleavage. Inset, 3.3 nM BafA1, significantly (Student’s t test; *, p < 0.05) antagonized the action of EA (black bars) and BoNT/E (gray bars), but not BoNT/A (white bars). N.S., not significant. Values in A, B, and D are the means ± S.E. (n = 4). □, BoNT/A; ▲, BoNT/E; ◊, EA; △, AE. Error bars are not shown where they were smaller than the symbols.
and AE are potent neuroparalytic proteins, indicating that multiple functions of their parental neurotoxins have been utilized. Moreover, EA displays the BoNT/E-like property of rapid onset of paralysis, a feature apparently conferred by the translocation function of its LC-HN domain. AE exerts a neuroparalysis that is slower to develop but persistent and therefore is a potential candidate for clinical application in patients that do not respond well to BoNT/A.

**DISCUSSION**

Herein, it has been demonstrated that individually folded domains of BoNTs can be transferred between serotypes to generate fully functional, novel BoNT chimeras. Thereby, the most desirable characteristics of two distinct serotypes can be combined in one protein. By swapping the H\textsubscript{C} domains between BoNT/A and BoNT/E, chimeras EA (comprising the LC and H\textsubscript{C} of BoNT/E fused to the H\textsubscript{C} of BoNT/A) and AE (LC-H\textsubscript{N}/A plus H\textsubscript{C}/E) were expressed as SC proteins in *E. coli*, a bacterial host that offers several advantages over *C. botulinum*, e.g. faster growth, controlled and specific induction of transgene expression, and well characterized and optimized protocols for gene transfer and manipulation. Moreover, with the aid of codon optimization and incorporation of a His\textsubscript{6} tag, high yields were achieved, and the SCs were readily purified to homogeneity by affinity and ion-exchange chromatography; these procedures are much simpler and quicker than the multiple precipitation and chromatographic steps required for purification of natural BoNTs (36). Following conversion to the DC by controlled proteolysis, each chimera was activated by the respective domains from BoNT/A and BoNT/E: receptor recognition, channel formation, and translocation of an active protease. However, unique functional characteristics of each parent neurotoxin were found to be differentially combined in each of the chimeras, allowing identification of underlying structural moieties.

Chimera EA proved to be faster-acting and more potent than BoNT/A or AE at the neuromuscular junction; in fact, EA is as potent and acts as quickly as BoNT/E. Moreover, in several tissue culture models (spinal cord neurons, CGNs, and autonomic SCGNs), EA gained entry and cleaved SNAP-25 faster than BoNT/A or AE. These observations imply that fast action is an intrinsic characteristic of the LC-H\textsubscript{N}/E fragment rather than the H\textsubscript{C} domain of BoNT/E. As the H\textsubscript{C} is sufficient for recognition by BoNT/A of neuronal ectoreceptors, SV2C, and gangliosides (6), and as EA binds to the intralumenal domain of SV2C in *vitro*, it is reasonable to expect that the binding and endocytotic uptake route of EA do not differ from those of BoNT/A. Furthermore, no major differences were observed in *vitro* for the sensitivity of SNAP-25 proteolysis by EA, BoNT/A, and AE, indicating that their respective LCs do not influence either their speed of action in cultured neurons or the onset of neuromuscular paralysis; indeed, fast-acting BoNT/E displayed the lowest protease activity, although the reasons for this remain unclear. Thus, by a process of elimination, quicker translocation from the endosomal lumen to the cytosol is implicated in the more rapid action of EA. Such a notion is supported by the demonstration that its entry into the cytosol of cultured neurons rapidly became insensitive to inhibitors of endosomal acidification, whereas both AE and BoNT/A remained susceptible for a much longer time. Moreover, EA and BoNT/E displayed equivalent sensitivity to dose-dependent inhibition of the vesicular proton pump with Baf\textsubscript{a} in SCGNs, whereas cytosolic delivery of BoNT/A was less susceptible. This suggests that EA and BoNT/E enter cells more rapidly than BoNT/A due to a heightened sensitivity to pH changes imparted by their

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4. J. Meng, J. Wang, M. Pickering, A. Sasse, K. Murphy, G. W. Lawrence, K. R. Aoki, and J. O. Dolly, submitted for publication.
common LC-H\textsubscript{c}/E moiety. It is thought that acidification induces structural changes in the LC and/or HC to initiate the translocation of the former through a membrane-spanning channel formed by the latter (14). Poor translocation in motor nerve terminals may also underlie the anomalous slope of the AE dose-response (Fig. 4A), as BaF\textsubscript{2} is known to alter such slopes for BoNT/A, BoNT/B, and tetanus toxin (37). The active chimeras reported herein suggest either that the H\textsubscript{c} moieties are not involved in channel formation/LC translocation or that these respective domains from BoNT/A and BoNT/E are functionally interchangeable in any such role. The latter notion seems less likely, as (yet undefined) regions of the respective HCs of BoNT/A and BoNT/E are postulated to perform a chaperone role for the partially unfolded LC, stabilizing its passage via several unwound conformations through the HC channel (14). Moreover, the H\textsubscript{c} moiety of diphtheria toxin can be replaced with the binding domain from tetanus toxin without disruption of the acidification-dependent translocation of its enzymatic chain into cells (38). In contrast, the LC-H\textsubscript{N} moieties with an intact disulfide bridge are crucial for productive translocation (12, 39, 40). Noncovalent interactions are also involved in maintaining LC-H\textsubscript{N} interactions that appear to be important for structural stability, as a chimera consisting of the LC of BoNT/A linked to the HC of BoNT/E was poorly expressed, relatively insoluble, and susceptible to proteolysis (data not shown). The surface complementarity of the LC and H\textsubscript{N} domains is likely to be crucial for the successful formation of the channel through which the LC could be translocated. Further investigation into the structural basis for the faster transport of BoNT/E protease relative to BoNT/A is fully warranted, considering that development of the full therapeutic response to BoNT/A takes days to a week after injection (4), and faster onset of paralysis would be a highly desirable property. Although the relatively transient duration of EA activity precludes its replacement of long-lasting BoNT/A for many clinical applications, it would be a suitable addition for muscle immobilization for short periods.

Even though AE entered cultured neurons more slowly than EA and required a much longer time to block neuromuscular activity in vitro, the lethalities of these two proteins in vivo were similar (0.3 \times 10\textsuperscript{-7} and 0.7 \times 10\textsuperscript{-7} mL\textsubscript{50}/mg of protein, respectively, remarkably potent in view of the recently elucidated differences in the global structures of their parental BoNTs) (41). The difference noted in the neurotoxicities of these chimeras, relative to the natural parental BoNT/A and BoNT/E, may be attributable to imperfect complementarity of the interactive surfaces of the LC-H\textsubscript{N} and H\textsubscript{c} from the two toxin serotypes. Also, the rank order of activity in excised diaphragm (BoNT/E > EA > BoNT/A >> AE) does not match that for lethality in mice (BoNT/A > BoNT/E >> EA > AE). Clearly, the time taken for paralysis of diaphragm in vitro does not reliably reflect toxin potency in vivo. Obviously, time to paralysis is dependent on the amount of toxin applied, but it is also influenced by the rates of toxin binding, internalization, translocation, and protease. Hence, the excised diaphragm cannot measure the true potency, but rather gives an apparent value, which is a combination of true potency and intoxication rate. Toxin lethality in vivo is assayed over 96 h, in contrast to the much shorter time window used for measuring diaphragm paralysis (up to 5 h), allowing slower toxins time to induce full paralysis in vivo. Moreover, fast-acting BoNT/E and EA are clearly less stable than BoNT/A and AE, as demonstrated by their much more transient protease activity and immobilization of mouse hind leg. The fact that BoNT/A enters neurons more slowly than BoNT/E is a cogent explanation for the lower apparent potency of the former on diaphragm. The true potency (i.e. lethality) of BoNT/A is higher because it maintains activity over a longer period, whereas BoNT/E inactivates very rapidly. EA exhibits high apparent potency on excised diaphragm, but its true value (i.e. lethality) is similar to that for AE and lower than that for either BoNT/A or BoNT/E. On the other hand, AE enters nerve cells slowly and appears relatively impotent on diaphragm, but is quite effective in vivo and, moreover, produces a persistent muscle weakening in mice similar to that reported for type A toxin complex (35). BoNT/E \textsuperscript{4} and its H\textsubscript{c} domain (6) do not interact with SV\textsubscript{2}; therefore, BoNT/E and AE utilize a different receptor than BoNT/A to bind and enter neurons. Thus, AE could be useful for long-term therapy in primary non-responders to BoNT/A.

In summary, proof of principle is provided herein for new generations of toxins whose properties can be tailored for specific applications. This proven technology should allow the creation of hybrid toxins optimized for specific interaction with different populations of neurons or other secretory cells; for example, exogenous ligands have been inserted into BoNT/A to replace H\textsubscript{c} (42, 43).

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