Two Protein Trafficking Processes at Motor Nerve Endings Unveiled by Botulinum Neurotoxin E

Gary Lawrence, Jiafu Wang, C. K. N. Kwo Chion, K. Roger Aoki, and J. Oliver Dolly

International Centre for Neurotherapeutics, Dublin City University, Dublin, Ireland (G.L., J.W., J.O.D); Hematology Department, Imperial College, London, United Kingdom (C.K.N.K.C); and Biological Sciences, Allergan LLC, Irvine, California (K.R.A)

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ABSTRACT

The unique ability of a family of botulinum neurotoxins to block neuroexocytosis specifically—by selective interaction with peripheral cholinergic nerve endings, endocytotic uptake, translocation to the cytosol, and enzymic cleavage of essential proteins—underlies their increasing therapeutic applications. Although clinical use of type A is most widespread due to its prolonged inactivation of the synaptosomal-associated protein of 25 kDa, botulinum neurotoxin E cleaves this same target but at a different bond and exhibits faster onset of neuromuscular paralysis. Herein, insights were gained into the different dynamics of action of types A and E toxins, which could help in designing variants with new pharmacological profiles. Natural and recombinant type E dichain forms showed similar proteolytic and neuromuscular paralytic activities. The neuroparalysis induced by type E toxin was accelerated between 21 and 35°C and attenuated by bafilomycin A1. Temperature elevation also revealed an unanticipated bipartite dose response indicative of two distinct internalization processes, one being independent of temperature and the other dependent. Although elevating the temperature also hastened intoxication by type A, a second uptake mechanism was not evident. Increasing the frequency of nerve stimulation raised the uptake of type E via both processes, but the enhanced trafficking through the temperature-dependent pathway was only seen at 35°C. These novel observations reveal that two membrane retrieval mechanisms are operative at motor nerve terminals which type E toxin exploits to gain entry via an acidification-dependent step, whereas A uses only one.

Seven serotypes (termed A–G) of botulinum neurotoxin (BoNT), produced by the bacteria Clostridium botulinum, cause the disorder botulism that is characterized by flaccid neuromuscular paralysis (Dolly et al., 2002). BoNTs are proteins (mol. wt. 150 × 10^3) with a heavy chain (≈100 × 10^3) and light chain (50 × 10^3) linked by a disulfide bond and noncovalent interactions. They are synthesized as single-chain (SC) precursors that require proteolytic nicking to yield the potent dichain (DC) forms. Each acts by inhibiting acetylcholine release via a complex multiphasic mechanism (Simpson, 1980, 2004): binding to high-affinity ectoacceptors on cholinergic nerve endings (Dolly et al., 1984), endocytotic uptake (Black and Dolly, 1986a,b), translocation of a toxic moiety, and specific cleavage of proteins essential for the release of transmitters from vesicles (Schiavo et al., 2000). Their heavy chain contributes to binding (Poulain et al., 1989) plus internalization (Dolly et al., 1994). Synaptic vesicle proteins have been identified as acceptors for some serotypes: SV2 for BoNT/A (Dong et al., 2006; Mahrold et al., 2006) and synaptotagmins I and II for BoNT/B and BoNT/G (Nishiki et al., 1994, 1996; Dong et al., 2003; Rummel et al., 2004a). An intact DC is needed for translocation (de Paiva et al., 1993) of the light chain to the presynaptic cytosol where, in the cases of BoNT/A or BoNT/E, it cleaves and disables SNAP-25 (Schiavo et al., 2000; Meunier et al., 2003). Due to these unique properties and, especially, an extremely long duration of neuroparalysis induced by BoNT/A, it is being used very successfully for the treatment of a wide and continually expanding range of neuromuscular disorders and other conditions associated with overactive peripheral cholinergic nerves (Jankovic, 2004). Although BoNT/E produces a more transient muscle weakness (Eleopra et al., 1998), this toxin exhibits the therapeutically desirable properties of higher potency and faster onset of neuromuscular paralysis, compared with BoNT/A (Simpson, 1980; Simpson and Das-Gupta, 1983). Hence, investigation of the basis for these advantageous properties should yield useful information for...
the design and protein engineering of improved therapeutic versions of BoNT.
For this study, a recombinant form of BoNT/E expressed in Escherichia coli was largely used because of displaying similar biological activities to its counterpart from C. botulinum, as well as being cheaper to prepare and easier to purify. BoNT/E uptake was found to occur via two processes displaying different temperature dependencies. Both were accelerated by an increased frequency of nerve stimulation and involved endocytotic vesicle acidification by a bafilomycin-sensitive proton pump. BoNT/A, in contrast, seems to use only one such uptake pathway. In view of the recent discovery of SV2 as an acceptor for BoNT/A, these timely new findings on the distinctive kinetics of neuromuscular paralysis by BoNT/A and BoNT/E may help identify differences in the trafficking of SV2 and the protein acceptor(s) for BoNT/E.

Materials and Methods

Materials. Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA). Substrates for the chemiluminescent detection of horseradish peroxidase and photographic film were bought from Millipore Corporation (Billerica, MA). Precast 4 to 12% polyacrylamide gels and electrophoresis running buffer were purchased from Invitrogen (Carlsbad, CA). L-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin, soybean trypsin inhibitor, a horse-

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ardish peroxidase-conjugated antibody to goat immunoglobulin G, and all other chemicals were obtained from Sigma Chemical (Poole, Dorset, UK). An antibody to the 18 C-terminal residues of SNAP-25 was purchased from Autogen-Bioclear UK (Devizes, Wiltshire, UK). Purified natural BoNT/E was obtained from Metabiologies, Inc. (Madison, WI) and Prof. E. Johnson; the expression and purification of BoNT/E or its light chain from E. coli will be described elsewhere. BoNT/A was purified as described previously (Shone and Tranter, 1995). BoNT/E SCs were nicked with 2 μg/ml trypsin for 1 h at 37°C before adding 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml soybean trypsin inhibitor; conversion to DC was confirmed by SDS-PAGE and protein staining as described previously (Maisey et al., 1988). The protein concentration of natural and recombinant BoNT samples was determined using Bradford’s dye-binding protein assay using a kit from Bio-Rad (Hemel Hempstead, UK).

Animals. Female TO mice (20–25 g) were purchased from Harlan UK Limited (Bicester, Oxon, UK) and housed in the animal care facility at Dublin City University with unrestricted access to food and water. The mice were killed by cervical dislocation immediately before experiments. All procedures involving animals were reviewed and approved by the Institutional Ethics Committee and licensed by the Irish Government, Department of Health and Children.

Measurement of the Toxins’ Proteolytic Activities. A model substrate [GST-SNAP-25 C65 (residues 142–206)] was expressed in E. coli and affinity-purified (Chen et al., 1999); the amount of protein was determined using Bradford’s Assay (as above). For studying the effect of temperature, it was more convenient to use recombinant light chain (to be described elsewhere) rather than BoNT/E, which would require nicking and reduction to reveal its full proteolytic activity. BoNT/A, E, or light chain samples were diluted to 250 or 500 nM in HEPES-buffered saline (HBS) containing bovine serum albumin (HBS/BSA; 20 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM CaCl2, and 10 μg/ml BSA) and incubated for 30 min at 37°C in the absence or presence of dithiothreitol before the addition of 10 mM iodoacetate to alkylate the free thiols. GST-SNAP-25 C65 was added to serial dilutions of the toxin and incubated for 30 min at 37°C (unless stated otherwise in figure legend). For reverse-phase high-performance liquid chromatography, the reactions were stopped by an equal volume of HBS/BSA containing 2% (v/v) trifluoroacetic acid and the 28-amino acid product (from cleavage at Arg180–Ile181) separated on a C18 column, as detailed previously (Foran et al., 1994). A synthetic peptide, residues 181 to 206 of SNAP-25, was used to verify the elution volume and to construct a standard curve. Initial reaction rates were calculated from experiments in which the amount of product represented less than 5 to 15% of the substrate. For SDS-PAGE and Western blotting assays, reactions were terminated by adding a one-third volume of SDS-PAGE sample buffer [100 mM Tris, pH 8.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.01% (w/v) bromphenol blue] and heating to 80°C for 2 min. Substrates and protease fragments were separated by 4 to 12% poly-

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am ide precast gels and visualized by Coomassie staining. Alternati-

dely, after transfer to polyvinylidene difluoride membrane, Western blotting with goat anti-SNAP25 (0.5 μg/ml) followed by horseradish peroxidase-conjugated rabbit anti-goat secondary antibo-
dies (diluted 1:10,000, following manufacturer’s instructions) was used to reveal intact substrate. Bound antibodies were detected by enhanced chemiluminescence with exposure to photographic film. Digital images of protein stained gels and photographs developed from Western blots were transferred to a computer using a flat-bed scanner; signal intensities were assigned nominal values using NIH Image. In protein-stained gels, uncleaved substrate and one of the cleavage products were resolved; the fraction of substrate converted to product was calculated by dividing the product intensity value by the sum of the those for product and uncleaved substrate. Fractional cleavage was plotted against BoNT concentrations (light chain); curve fitting and EC50 calculations were performed using Sigmaplot software (Systat Software, Inc., San Jose, CA). A single batch of substrate was used for all gel-based protease assays, and another was used for the reverse-phase high-performance liquid chromatography experiments; n values represent the number of separate experiments, and probability values were obtained from paired sample Student’s t tests.

Assay of Neuromuscular Paralytic Activity and Lethality of BoNTs. Neuromuscular transmission was recorded, as detailed previously (Li et al., 2001), in mouse phrenic nerve hemidiaphragms incubated in static baths with Krebs-Ringer (KR) buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 23.8 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM CaCl2, and 11.7 mM glucose) containing 0.1% (w/v) BSA and gassed with 5% CO2/95% O2. In all experiments, except those involving bafilomycin A1, the preparations were exposed for 30 min to BoNT/E under conditions designed to permit binding but attenuate internalization (4°C, without nerve stimulation in a mod-

dified KR buffer equivalent to KR except for the addition of 3.8 mM MgCl2 and the reduction of CaCl2 to 0.5 mM (Simpson, 1980; Maisey et al., 1988) continuously aerated with 95% O2/5% CO2). After wash-
ing off unbound toxin, the modified buffer was replaced with normal KR (this was pre-equilibrated with 95% O2/5% CO2 and aeration was continued throughout the recordings). The temperature was raised (see figure legends for details), and muscle tension was recorded in response to nerve stimulation (square pulses of 10–15 V, 0.2-ms duration, and 0.2-Hz frequency, except where stated other-

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wise). The force of muscle contractions was measured using Fort 10 force transducers (World Precision Instruments, Inc., Sarasota, FL), amplified, and transferred to a computer by a Quad bridge amplifier linked to a MacLab4e (ADInstruments Pty Ltd., Castle Hill, Australia) and analyzed using Chart software (ADInstruments Pty Ltd.). In experiments involving bafilomycin A1, the diaphragms were pre-
exposed to the latter at 1.5 μM in modified KR for 30 min at 24°C before lowering the temperature to 4°C and adding BoNT/E; after 30 min, the tissues were transferred to normal KR, which also included 1.5 μM bafilomycin, and nerve-induced muscle contractions were recorded. In accord with previous findings (Simpson et al., 1994), 1.5 μM bafilomycin itself had minimal effect on neuromuscular trans-

mission. Curve fitting was performed using Sigmplot software. n Values represent the number of diaphragms used to generate the data shown. The toxins’ lethalities were measured in mice (body weight, 20 g; four per group) after i.p. injection, and the LD50 values were calculated, as described previously (Maisey et al., 1988).
TABLE 1
Rates of cleavage of a SNAP-25 substrate by BoNT/E preparations: nicking together with reduction potententiates the protease activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dithiothreitol*</th>
<th>Initial Rate of Substrate Proteolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural nicked BoNT/E</td>
<td>None</td>
<td>53.3 ± 10.9 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>4 mM</td>
<td>409 ± 125 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>73.3 ± 9.4 (n = 3)</td>
</tr>
<tr>
<td>DC BoNT/E</td>
<td>None</td>
<td>82.1 ± 19.1 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>4 mM</td>
<td>427 ± 81 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>139 ± 9 (n = 3)</td>
</tr>
<tr>
<td>SC BoNT/E</td>
<td>None</td>
<td>93.9 ± 14 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>4 mM</td>
<td>115 ± 11 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>71.1 ± 8.1 (n = 3)</td>
</tr>
</tbody>
</table>

*Toxins were prereduced as described under Materials and Methods.

Results

Purified rBoNT/E Exhibits All the Characteristics of Natural E Toxin

The suitability of rBoNT/E as a convenient alternative to its native counterpart was demonstrated by their biological activities being similar. After expression and purification of the SC form and proteolytic nicking, the resultant DC showed slightly higher protease activity compared with that of its natural counterpart (Table 1) toward a fusion of glutathione S-transferase with residues 142 to 206 of SNAP-25. Proteolytic activity was detected with each in the absence of reducing agent; however, prior reduction with 4 mM dithiothreitol (optimal concentration; Fig. 1, B and C) increased the activities of natural or recombinant DC more than 5-fold (Table 1) but had minimal effect on the SC. Higher concentrations of dithiothreitol proved less able to increase protease activity (Fig. 1C; Table 1). The ability of nicked BoNT/E to induce neuroparalysis of the phrenic nerve diaphragm matched that of the natural DC (Fig. 1A), including the different inhibitory profiles seen at two temperatures (detailed below); in the absence of toxin, the force of muscle contractions remained undiminished for more than 500 min (data not shown). Note that to simplify examination of the multistep intoxication process, the binding of BoNT/E to neuronal acceptors was carried out in advance under conditions (Simpson, 1980) that minimize internalization [i.e., 4°C, without nerve stimulation and in the presence of an elevated [Mg^{2+}] (3.8 mM) and reduced [Ca^{2+}] (0.5 mM)]. Finally, the median lethal doses of recombinant and natural DC BoNT/E in mice were found to be 400 and 100 pg, respectively. Thus, the recombinant protein faithfully reproduces the multiple properties (binding, intracellular uptake, membrane translocation, and protease activity; see Introduction) required for potent neuromuscular paralysis and neurotoxicity in vivo.

Investigations on Paralysis of Motor Nerve Terminals by BoNT/E Unveil Two Uptake Processes with Distinct Dynamic Properties

Parallel Power-Function Relationships Exist between BoNT/E Concentrations and Neuromuscular Paralysis Times That Reach a Plateau at Different Concentrations of DC and SC. The dose-response plot obtained for inhibition by BoNT/E DC of nerve-evoked muscle tension at 24°C approximates to a straight line on double-log axes, indicating a power-function relationship up to ~100 pM (Fig. 1A). Similar profiles have been reported for natural BoNT/E and other serotypes (Simpson, 1980; Simpson and DasGupta, 1983; Rummel et al., 2004b) and can be described by eq. 1:

\[
PT = a/[BoNT/E]^b
\]

where PT represents the time taken to reduce muscle tension by 90%, and a and b are constants. Constant a (minutes per
picomolar) equals PT when \([\text{BoNT/E}] = 1 \mu\text{M}\), whereas the power constant \(b\) represents the slope and is a measure of the concentration dependence for toxin-induced paralysis. For rDC, the values observed were: \(a = 1300 \pm 250\) and \(b = 0.78 \pm 0.07\). Using the experimentally determined value for \(b\), it can be calculated that reduction of PT by half requires the toxin concentration to be increased by a factor of \(\sqrt{2} \approx 2.4\)-fold. This highlights a tight inverse relationship between applied concentration and PT. A similar dose-response pattern was observed for BoNT/E SC, albeit with a shift to \(\sim 63\)-fold higher concentrations (Fig. 1A); the data fit a power function up to 2 nM (\(a = 30300 \pm 9500\) and \(b = 0.77 \pm 0.06\)). Note that the power value corresponds to that measured for DC, in accord with the lines being parallel. From the ratio of the different \(a\) values extrapolated for SC and DC using eq. 1, it can be determined that similar concentrations of the former would require \(\sim 23\)-fold longer paralysis times than DC. This ratio can also be calculated from the relative toxin potency raised to the power \(b\) (i.e., 63\(^{0.77} = 24.3\); a slight discrepancy arises because the data for SC and DC are not perfectly parallel). It is noteworthy that the optimal protease specific activity (i.e., in the presence of 4 mM dithiothreitol) observed for the SC was only \(\sim 3.7\)-fold lower than the DC (Table 1), which is insufficient to explain the disparity in their neuroparalytic potencies.

The above-noted power-function concentration-time relationships broke down at higher toxin concentrations, \(>100\) pM and \(>2\) nM DC and SC, respectively (Fig. 1A). This disappearance of proportionality between toxin concentration and PT indicates the presence of a rate-limiting step in the intoxication process. Because the SC retains proportionality up to a higher concentration than DC, it appeared that acceptor occupancy is not a limiting factor (see Discussion).

The Dose Responses for BoNT/E Can Be Resolved into Two Parts with Differing Temperature Sensitivities: Evidence for Kinetic Cooperativity. Dose-response relationships, recorded at four temperatures, showed complex patterns for BoNT/E-induced neuromuscular paralysis. As expected, the paralysis times for low concentrations (e.g., 20 pM) of BoNT/E DC became shorter as the temperature was raised from 21 to 35°C (Fig. 2A). Most notable, however, was the continuous decrease in paralysis time for higher toxin concentrations at increased temperatures, in contrast to the leveling off observed at 24°C or below (Fig. 2A).

Fig. 2. Raising the temperature causes a greater shortening of the time taken for high than low concentrations of BoNT/E to cause neuromuscular paralysis; bafilomycin A1 attenuates the toxin’s action. A, diaphragms were pre-exposed to various concentrations of rBoNT/E DC in modified KR for 30 min at 4°C before removal of unbound toxin, extensive washing, and transfer to KR buffer. Electrical stimulation was performed at four different temperatures 21 (○), 24 (□), 30 (△), and 35°C (○), and the paralysis times are plotted in A against [rBoNT/E DC]. Inset, paralysis times for different concentrations of rSC (●) and natural DC BoNT/E (▲) at 35°C. In B, the data from A for rBoNT/E DC is replotted versus temperature, with best-fit power functions (---), calculated using eq. 2 (see Results), fitted for data sets obtained with concentrations: 10 pM (▲), 20 pM (○), 33 pM (□), 50 pM (○), 100 pM (▼), 450 pM (▲), 1 nM (○), and 5 nM (●). A plot of the power value against [rBoNT/E DC] between 450 pM and 5 nM can be fitted by a hyperbolic function (C). D, diaphragms were exposed to modified KR containing 1.5 μM bafilomycin A1 for 30 min at 24°C, then the temperature was lowered to 4°C and 1 nM rBoNT/E DC added. After a further 30 min, the tissues were transferred to KR, also containing 1.5 μM bafilomycin A1, and the time to paralyze nerve-evoked muscle contractions was recorded at 24 or 35°C. Control samples were treated identically, except for the omission of bafilomycin A1.
The phenomenon was also observed with SC and natural DC (Fig. 2A, inset). When paralysis times were plotted against temperature (Fig. 2B), the patterns became somewhat clearer and resolved into two parts. The experimental data observed at most concentrations of BoNT/E for PT and temperature could be fitted by power functions using eq. 2:

$$PT = \frac{a}{Temp}^b$$

(2)

At low concentrations of BoNT/E (10–33 pM), the temperature sensitivity observed was constant ($b = 2.0 \pm 0.4$ at 10 pM and $2.2 \pm 0.2$ at 20 pM), hence, the near-parallel lines in the upper half of Fig. 2B; a $-1.41$- and $-1.38$-fold increase in temperature ($^\circ$C) is needed to halve the PT for diaphragms pre-exposed to 10 or 20 pM BoNT/E, respectively. On the other hand, this linearity disappeared at 50 pM, with increases in the temperature from 24 to 35$^\circ$C having very little effect on PT (Fig. 2B). Moreover, a new pattern emerged at yet higher concentrations of toxin, with the slopes of the temperature dependencies plots now increasing with toxin concentration, as manifested by a fanning out of the lines (Fig. 2B). A plot of the power function $b$ in eq. 2 against only the higher concentrations (0.1–5 nM) of BoNT/E fitted a hyperbolic function (Fig. 2C) that predicts a maximum power value of 4.8 ± 0.2. These collective findings are consistent with the existence of two components in the intoxication process. It is noteworthy that both mechanisms of BoNT/E uptake involve acidification of a subcellular compartment, presumably endocytotic vesicles, because the PTs for 1 nM rBoNT/E are significantly prolonged at both 24 and 35$^\circ$C by bafilomycin A1 (Fig. 2D), which inhibits vacuolar-type H⁻-ATPases (Bowman et al., 1988).

The change from parallel shifts in the responses for low [BoNT/E] to increasing slopes of the plots seen with higher concentrations indicates that in the former case, elevated temperatures reduce PT uniformly irrespective of concentration (~2.5-fold for a shift from 24 to 35$^\circ$C), whereas increasing slopes reflect larger reductions in PT at higher toxin concentrations. The latter behavior translates into sigmoidal dose-response relationships (Fig. 2A); the higher the temperature, the greater the inflection in the curve. Such sigmoidal dose responses are reminiscent of cooperative ligand-receptor binding relationships, in that small increases in concentration produce larger than expected decreases in PT over a narrow concentration range (50–1000 pM; Fig. 2A).

**Acceleration of Neuroparalysis at Raised Temperatures Is Not Due Solely to Enhancement of the Protease Activity.** The concentration dependence of proteolysis of GST-SNAP-25 C65 by BoNT/E light chain (Fig. 3, A and B) gave the amount required for 50% substrate cleavage (EC$_{50}$) at each temperature (Fig. 3C). Although the EC$_{50}$ was reduced slightly upon raising the temperature (Fig. 3D), the $1.2$-fold increase in protease activity observed between 25 and 35$^\circ$C was not statistically significant ($p < 0.05$) and does not seem adequate to explain the 2.5-fold reduction in PT at low [BoNT/E] (Fig. 2, A and B; calculated by solving eq. 1 at 25 and 35$^\circ$C).

**Reducing the Temperature Only Blocks One of the Two Intoxication Processes, But Both Are Enhanced by Nerve Stimulation.** An alternative means of accelerating toxin uptake and paralysis of nerve terminals is to increase the frequency of electrical stimulation (Hughes and Whaler, 1962; Simpson, 1980; Black and Dolly, 1986b).
like increments in temperature, raising the stimulation frequency would not alter the BoNT protease activity; thus, any reduction in PT can be attributed to faster internalization of toxin. At 24°C, increasing the stimulation frequency gave parallel downward shifts in the dose-response curves, reflecting consistent reductions in PT, but only up to 33 pM BoNT/E (Fig. 4A); likewise, the power-function slopes between PT and stimulation frequency showed similar parallel downward shifts for these same limited concentrations of toxin (Fig. 4A, inset). Contrasting, raising the stimulation frequency failed to exert any effect on PT for higher concentrations at 24°C (Fig. 4A, inset). Thus, elevating the stimulation frequency enhances the responses to low [BoNT/E], but, unlike increases in temperature (c.f. Fig. 2A), is unable to shorten PT for higher concentrations (c.f. Fig. 4A). Nevertheless, at 35°C, the PT observed for 1 nM toxin could be further reduced by increasing the stimulation frequency (Fig. 4B). Hence, elevating the stimulation frequency accelerates uptake of BoNT/E via both temperature-insensitive and -sensitive pathways.

**BoNT/A Exhibits a Shallower Dose-Response Relationship Than BoNT/E and a Steeper Temperature Dependence, But a Second Uptake Mechanism Is Not Discernible**

BoNT/A and BoNT/E seemingly bind to different extracellular acceptors because a specific and potent competitor of binding and neuromuscular paralysis by BoNT/A was almost ineffective toward BoNT/E (Dolly et al., 1994); also, the SV2 acceptor for type A toxin does not bind BoNT/E (Dong et al., 2006). Because acceptor binding is likely to influence endocytotic trafficking, comparison of the effects of temperature and concentration on neuromuscular paralysis by each of these toxins should reveal insights into the dynamics of their actions. Diaphragms were exposed to different concentrations of BoNT/A DC, in the same manner as described for BoNT/E, before raising the temperature to 24°C (Fig. 5A) or 35°C (Fig. 5B) and recording PT. At 24°C, BoNT/A required far longer to induce paralysis than equivalent concentrations of BoNT/E. Moreover, a lower power relationship was evident for BoNT/A DC, in the same manner as described for BoNT/E, (Fig. 4A, inset). Contrasting, raising the stimulation frequency failed to exert any effect on PT for higher concentrations at 24°C (Fig. 4A, inset). Thus, elevating the stimulation frequency enhances the responses to low [BoNT/E], but, unlike increases in temperature (c.f. Fig. 2A), is unable to shorten PT for higher concentrations (c.f. Fig. 4A). Nevertheless, at 35°C, the PT observed for 1 nM toxin could be further reduced by increasing the stimulation frequency (Fig. 4B). Hence, elevating the stimulation frequency accelerates uptake of BoNT/E via both temperature-insensitive and -sensitive pathways.

**Discussion**

The successful expression and ease of isolation of rBoNT/E have provided a convenient alternative for examining this toxin’s functional properties because it displays all the biological activities of the natural protein. Examination of the concentration dependencies of neuromuscular paralysis at 24°C by BoNT/E and BoNT/A showed power-function relationships that leveled off at higher concentrations (Figs. 1 and 5). It is noteworthy that BoNT/E appears to reach a plateau at much lower concentration than BoNT/A, presum-
ably due to some limitation in one or more of the multiple intoxication steps (i.e., acceptor binding, endocytosis, translocation, and SNAP-25 proteolysis). The power relationship was shallower for BoNT/A than BoNT/E (b/H11005 0.25/H11006 0.03 and b/H11006 0.78/H11006 0.07, respectively, at 24°C), reflecting more efficient internalization of the type E protease. Indeed, it has been reported that internalization into cultured neurones was much faster for BoNT/E than BoNT/A (Keller et al., 2004).

Presumably, paralysis is achieved when a threshold amount of SNAP-25 is inactivated by cleavage, PT being the time taken for this to be realized. If BoNT uptake was 100% efficient (b = 1 in eq. 1), doubling extraneuronal [BoNT] would double its [protease] inside the neuron such that PT would be halved (because cleavage of the necessary amount of SNAP-25 would be accomplished in half the time, a reasonable assumption because it is likely that [SNAP-25] >> [BoNT] inside the nerve terminal). However, BoNT internalization is less than 100% efficient (b < 1); likely reasons include a delay in trafficking the protease to the cytosol, failure to internalize all the externally bound BoNT, and diffusion of toxin in the cytosol away from the transmitter release sites. Nevertheless, increases in [BoNT/A] are very effectively translated into reductions in PT; using eq. 1, it can be calculated that doubling external [BoNT/A] reduces PT by ~40%. In contrast, a 2-fold increase in [BoNT/A] shortens PT by only ~16%. Put another way, halving PT for BoNT/E only needs ~2.5-fold higher concentration, whereas [BoNT/A] must be raised 16-fold. These values apply for the linear power relationships observed at low concentrations. The notion that shallower dose-response relationships reflect less efficient toxin internalization is supported by the effects of bafilomycin A1, a vesicular H+/ATPase inhibitor, which blocks the transfer of the toxin protease from the vesicle lumen to the cytosol and, thereby, reduces the power value for BoNT/A, BoNT/B, and TeTx (Simpson et al., 1994).

Although significant structural rearrangements of BoNT/E are known to occur upon nicking (the prevalence of β-sheet increases from 37–47%; DasGupta, 1989), the SC exhibited substantial neuroparalytic activity. This could arise from conversion to DC by tissue proteases, although the consistent 63-fold lower potency of SC, irrespective of concentration and PT, mitigates such a deduction. The binding of SC and DC BoNT/E to neuronal membranes is similar (DasGupta, 1989). Interestingly, the protease site seems to be almost fully accessible in SC, which showed only ~4-fold lower activity (measured in the presence of dithiothreitol) than DC. Even without reduction, both SC and DC BoNT/E exhibited 19 to 22% of the optimal protease rate of 4 mM dithiothreitol-reduced DC, a surprising finding considering the low activi-
ties found for other serotypes, including BoNT/A (Fig. 5F), when reducing agents are omitted.

Upon raising the temperature from 24 to 35°C, the plateau in the dose-response curve for BoNT/E, but not that for BoNT/A, was largely overcome (Fig. 2). Such a large apparent increment in the amount of BoNT/E internalized implicates a high-capacity uptake process that becomes more active at the higher temperature. It is noteworthy that neuroparalysis was accelerated more at high than low [BoNT/E] (Fig. 2B), which suggests that BoNT/E is acting cooperatively at some step that is inactive at 24°C but induced at 35°C. Because the binding step, which is temperature-insensitive (Simpson, 1980), was performed at 4°C in advance of monitoring paralysis, the observed effects cannot be attributed to alteration in acceptor binding. Raising the temperature also accelerated the neuroparalysis induced by low, subsaturating concentrations of BoNT/E (Fig. 2; PT was reduced 2.5-fold at 35°C compared with 24°C), apparently by speeding up toxin internalization more than the protease rate, which was only 1.2 times faster at 35°C compared with 25°C (Fig. 3). For subsaturating concentrations of BoNT/E, increasing the temperature produced the same -fold reduction in PT regardless of the toxin concentration (hence, the parallel dose-response relationships in the upper parts of Fig. 2, A and B). BoNT/A protease was 1.8-fold enhanced at 35°C compared with 25°C, and this contributed to a greater acceleration of neuroparalysis by BoNT/A (Fig. 5; PT reduced 4.3-fold, in good agreement with prior studies; Simpson, 1980), with the remainder being due, presumably, to faster toxin internalization. Raising the temperature did not alter the slope (i.e., power) of the dose response for BoNT/A (b = 0.25 ± 0.03 and 0.24 ± 0.03 at 24 and 35°C, respectively).

It remains unclear whether the BoNT/E uptake processes occurring at low and high concentrations involve the same or different acceptors; in this regard, it is notable that these toxins have been proposed to bind to gangliosides, proteins, and combinations thereof (Nishiki et al., 1994, 1996; Montecucco et al., 2004; Rummel et al., 2004a; Dong et al., 2006; Mahrhold et al., 2006). Moreover, binding studies have demonstrated the existence at motor nerve endings of high- and low-affinity acceptors for BoNT/A that can be distinguished by its heavy chain; it competes for binding to the latter only and fails to antagonize high-affinity toxin uptake (Daniels-Holgate and Dolly, 1996) but does delay intoxication by very high concentrations of BoNT/A (Bandyopadhyay et al., 1987). However, no evidence was obtained herein for multiple entry mechanisms for type A toxin.

Due to the complex and bipartite dose-response relationships for BoNT/E, it is reasonable to deduce that this toxin enters motor nerve terminals via two functionally distinguishable transport pathways, exhibiting different temperature dependencies. This proposal was given further support by the observation that nerve stimulation accelerates only the uptake operative for low toxin concentrations at 24°C but enhances both pathways at 35°C (Fig. 4). Moreover, uptake of BoNT/E was inhibited at both temperatures by antagonism of endocytotic vesicle acidification with bafilomycin A1. Our observation of two BoNT/E trafficking pathways is important because of a widespread interest in multiple mechanisms of neuronal endocytosis (Murthy and De Camilli, 2003; Sudhof, 2004). This finding was made possible by prebinding of the toxin under conditions that minimized uptake and use of stimulation at various temperatures. Prebinding removed a delay due to the time taken for toxin diffusion to nerve terminals and interaction with acceptors (4.1 ± 14 min at 1 nM; Simpson and DasGupta, 1983) that would have obscured observation of the rapid onset of neuroparalysis at 35°C. Indeed, BoNT/E prebound on cultured neurons is fully endocytosed within 4 min after stimulation (Keller et al., 2004); moreover, within 1 further min, a large fraction of the protease activity was translocated to the cytosol. The dual uptake mechanism described here for BoNT/E parallels to some extent the more complex trafficking of tetanus toxin that also seems to enter peripheral nerves via both low- and high-affinity pathways. At low doses, tetanus toxin is mainly directed away from motor terminals (Lalli et al., 2003), but it is released locally if large quantities are presented; this phenomenon causes a switch from spastic to flaccid paralysis in severe cases of tetanus intoxication (Matsuda et al., 1982).

It is tempting to speculate on how BoNT/E could partake in different neuroexo-/endocytosis mechanisms that are differentially influenced by temperature. Exocytosed synaptic vesicle proteins are recycled by multiple endocytotic routes (Sudhof, 2004), and this could apply also to the BoNT/E acceptor, or acceptors if multiple binding partners exist (Montecucco et al., 2004). Another important outcome of this investigation on motor endplates is that both uptake mechanisms for type E are potentiated by overactivity; thus, internalization should occur more rapidly in nerves needing attenuation by therapeutic application of such toxins.

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Address correspondence to: Dr. J. Oliver Dolly, International Centre for Neurotherapeutics, Dublin City University, Dublin 9, Ireland. E-mail: oliver.dolly@dcu.ie

Dr. J. Oliver Dolly, International Centre for Neurotherapeutics, Dublin City University, Dublin 9, Ireland. E-mail: oliver.dolly@dcu.ie