Tetanus neurotoxin (TeNT) causes neuroparalytic disease by entering the neuronal soma to block the release of neurotransmitters. However, the mechanism by which TeNT translocates its enzymatic domain (light chain) across endosomal membranes remains unclear. We found that TeNT and a truncated protein devoid of the receptor binding domain (TeNT-LHN) enters the neuronal soma to block the release of neurotransmitters. However, the mechanism by which TeNT translocates into the cytosol of mammalian cells and disrupt normal cellular function. Numerous bacterial pathogens produce toxins that enter the cytosol of mammalian cells and disrupt normal cellular function. Many of these toxins are referred to as A-B toxins because of their structural and functional organization (1). The B (binding) moiety, composed of one or more subunits, binds to a cell surface receptor and facilitates delivery of the A (active) moiety into the cytosol, where it enzymatically modifies a cellular target. In a subset of A-B toxins, the B domain has the ability to undergo a series of structural changes that allows integration into lipid membranes and formation of a protein conducting channel through which the A domain may be delivered. What drives the structural changes within the B moiety and how polypeptides are translocated across membranes are fundamental questions yet to be fully resolved for any A-B toxin.

The clostridial neurotoxins (CNTs) are a family of bacterial A-B toxins that are among the most lethal natural agents known to humans (2, 3). Nine CNTs have been described to date: tetanus neurotoxin (TeNT) produced by Clostridium tetani and eight botulinum neurotoxins (BoNTs, serotypes A–H) produced by strains of Clostridium botulinum, Clostridium butyricum, and Clostridium baratii (4–7). CNTs are synthesized as single chain polypeptides with a molecular mass of ~150 kDa. The precursor is subsequently proteolytically cleaved into an ~50-kDa light chain (LC, A subunit) and an ~100-kDa heavy chain (HC, B subunit) linked by an essential interchain disulfide bond (8). HC contains an ~50-kDa N-terminal translocation domain (HCT) and an ~50-kDa C-terminal receptor binding domain (HCR) (9). The HCT facilitates translocation of the LC into the neuronal cytosol, whereas the HCR binds neuronal co-receptors (10–19).

How CNTs are able to convert from fully folded water-soluble proteins into membrane-integrated protein-translocating channels remains unclear. Traditionally low pH was proposed to trigger the translocation process, presumably by promoting structural changes facilitating the insertion of the HCT into the membrane bilayer. However, the recent demonstration that the isolated HCT of BoNT/A can form ion-conducting channels in the absence of a transmembrane pH gradient brings this model into question (20, 21). Rather, it appears that low pH serves to (i) relieve the inhibition of the translocation process mediated by the HCR and (ii) facilitate the partial unfolding of the LC into a conformation necessary for passage through the translocation channel (22, 23). The presence of reductant and neutral pH in the cytosol promotes release of the LC from the HC after completion of translocation. Although our understanding of the

**Significance:** This represents a new paradigm for how A-B toxins translocate enzymatic domains across cellular membranes.
translocation process has grown in recent years, the precise molecular mechanisms driving the conversion of the water-soluble form into the membrane-integrated form of TeNT remain to be determined.

In the present study, we investigated mechanisms leading to the formation of membrane channels using a combination of full-length TeNT and variants defective in the ability to bind ganglioside co-receptors. Here we demonstrate that ganglioside binding enhances the rate of channel formation, presumably by tethering TeNT close to the target membrane. Furthermore, we demonstrate that membrane association is moderated by the presence of acidic phospholipids, suggesting that the transition from a water-soluble protein into a translocase channel occurs close to the membrane interface. Based on our observations, we propose a sequential two-step model for TeNT channel formation that differs from the mechanisms employed by diphtheria and anthrax toxins, the current paradigms for cell entry of bacterial toxins.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Molecular biology grade reagents were purchased from either Fisher or Sigma-Aldrich. *Escherichia coli*-optimized DNA encoding TeNT residues 1–1315 was synthesized by EZBiolab (Carmel, IN). Gangliosides, cholesterol, and the following phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-α-phosphatidylserine (porcine brain), and 1-α-phosphatidylcholine soy 20% (asolecithin).

**Tetanus Neurotoxin Expression and Purification**—DNA encoding TeNT was cloned into the pET28a expression vector (Merck) using appropriate restriction endonuclease sites resulting in an N-terminal His tag fusion protein. To generate a catalytically inactive form of TeNT, two point mutations within the light chain (R372A and Y375F) were generated using the QuikChange II site-directed mutagenesis kit (Agilent). Proteins were expressed in *E. coli* BL-21 AI cells and purified as described previously (24, 25). Peak fractions from the Sephacryl S-200 column were concentrated using an Amicon filtration device (YM-100 type filter), dialyzed into 10 mM HEPES-phosphate buffer, and lyophilized in potassium buffer (10 mM HEPES-KOH, 150 mM KCl, 1 mM EDTA, pH 7.4) to a final concentration of 40 mM by lyophilization.

**Trypsinization of TeNT Proteins**—Trypsin-agarose (500 μl) was washed three times in phosphate-buffered saline (PBS) prior to incubation with 2 mg of TeNT or TeNT variants for 60 min at 4 °C. Proteins were separated from agarose beads by gentle centrifugation, and a sample was resolved by SDS-PAGE. SDS-PAGE analysis of trypsinized proteins in the presence or absence of reducing agent confirmed that the proteins were converted to dichain molecules of the anticipated sizes (data not shown).

**Triton X-114 Partitioning**—Triton X-114 partitioning assays were performed as described previously (27). For pH 7.4 samples, buffer containing 10 mM HEPES-NaOH, 150 mM NaCl, and 1 mM EDTA was used (hereafter referred to as neutral buffer). For samples at pH 6.5, 6.0, and 5.5, HEPES was replaced with 10 mM MES-NaOH, and for pH 5.0 and 4.0, HEPES was replaced with 10 mM sodium acetate-acetic acid. Final concentrations were 600 nM for TeNT or TeNT variants and 113 μM ganglioside. After partitioning, the aqueous and detergent phases of each sample were collected and resolved on 8% (w/v) SDS-PAGE

**Association and Proteoliposome Isolation**—Liposomes (100 μl) were combined with 10 μg of TeNT or TeNT variants in 500
μl of neutral buffer or low pH buffer (10 mM sodium acetate-acetic acid, 150 mM NaCl, 1 mM EDTA, pH 4.0). Liposomes were isolated by centrifugation at 100,000 × g for 30 min at 4 °C. Supernatants containing unbound proteins were recovered and held on ice for further analysis. Liposomes were suspended in 500 μl of fresh neutral or low pH buffer and recovered by centrifugation as above. Supernatants (wash fractions) were collected and, along with those from the first centrifugation step, concentrated to 50 μl using Microcon centrifugal filter devices. Liposomes were suspended in 50 μl of neutral buffer, and all fractions were mixed with an equal volume of SDS-PAGE buffer. Volume equivalents of each fraction were resolved on SDS-polyacrylamide gels and visualized by staining with Coomassie Blue dye.

K⁺ Release Assay—Liposomes (100 μl) were diluted into 5 ml of neutral or low pH buffer with constant stirring and allowed to equilibrate for 1–5 min. TeNT or TeNT variants (2.5 nmol) were then added to the solution, and potassium ion release was monitored using a potassium-selective electrode (Orion, ThermoFisher Scientific). After 5 min, 0.01 M KCl was added to the solution to ensure that the electrode was functioning as expected. Specific K⁺ release was determined by subtraction of basal release values obtained from liposomes incubated in buffer alone.

Statistical Analysis—Densitometric analysis was performed using Protein Simple AlphaView version 3.0 software (Santa Clara, CA). Data were analyzed using GraphPad Prism, version 6.0 (La Jolla, CA). One-way analysis of variance with a Student-Newman-Keuls post-test was performed to determine the difference between means after treatment. Two-way analysis of variance with a Bonferroni post-test was used to determine the difference between pH and treatments and the possible interactions of each. Differences were considered significant at p < 0.05.

RESULTS

Functional Entry of Recombinant Tetanus Toxin and Protein Variants into Cortical Neurons—The mechanism by which the LC protease of TeNT is translocated across the endosomal membrane is currently unresolved. To investigate this mechanism further, a series of TeNT variants was constructed (Fig. 1A) and validated by monitoring their ability to enter primary cortical neurons.

FIGURE 1. TeNT cleavage of VAMP2 in rat cortical neurons. A, schematic representation of TeNT, ciTeNT, and TeNT-LHN, showing domain organization. The disulfide bond between the LC and the HC in the mature toxin is indicated by S-S. B, rat cortical neurons (10–14 days in vitro) were incubated for 24 h with the indicated concentrations of TeNT (white bar), ciTeNT (gray bar), or TeNT-LHN (black bar). VAMP2 cleavage was visualized by Western blotting and quantified as described above.
ing domain of TeNT is not essential for toxicity. Finally, a recombinant, full-length tetanus toxin was engineered with two point mutations within the light chain (R372A and Y375F). Arg-372 and Tyr-375 are conserved across all CNT family members and are known to contribute to catalysis by facilitating correct alignment of conserved histidine and glutamate residues for the zinc coordination sphere (29–31). Treatment of cells with a 10,000-fold higher concentration of the catalytic inactive form of toxin (ciTeNT) also failed to cleave VAMP2 (Fig. 1B). This is consistent with the recent study of Blum et al. (32), which showed that mice injected with 5 mg of a protein containing the same two mutations (R372A and Y375F, equivalent to 110,125 lethal doses) did not develop any signs of disease. Thus, ciTeNT was substituted for wild type protein in the remaining assays to maximize safety. To further validate the use of TeNT-LHN as a tool to study translocation, the requirement for passage through an acidified compartment was investigated. In agreement with previous studies, the activity of recombinant TeNT was inhibited by the vacuolar proton pump inhibitor bafilomycin A1 (33). TeNT-LHN was also inhibited by bafilomycin A1, indicating that translocation of the light chain by TeNT-LHN was still dependent on exposure to an acidified environment (Fig. 1C).

**FIGURE 2. Polysialogangliosides induce changes in TeNT secondary structure at low pH.** A, far-UV CD spectra of TeNT, TeNTyw, and TeNT-LHN at pH 7.4 and 4.0 in the absence (solid lines) and presence (dashed lines) of 30 μM GT1b. B, far-UV CD spectra of TeNT at pH 4.0 in the absence (solid lines) or presence (dashed lines) of 1 μM GT1b, 30 μM GA1, or both.

**TeNT Undergoes Secondary Structural Changes in the Presence of Polysialogangliosides at Low pH**—Recent studies demonstrated that interaction of ganglioside GT1b with BoNT/B and BoNT/E triggers conformational changes within the two proteins that facilitates transformation into hydrophobic proteins at low pH (34, 35). To test whether gangliosides trigger a similar conformational change in TeNT, CD spectroscopy was performed at neutral and acidic pH in the absence and presence of GT1b. At neutral pH, the far-UV CD spectra at 196–265 nm overlapped extensively, and the helical content was estimated at 18.4 and 19.6%, respectively (Fig. 2A and Table 1). Reducing the pH to 4.0 had little effect on the CD spectrum of TeNT, indicating that the protein secondary structure remains largely unchanged. By comparison, the addition of GT1b at low pH caused a dramatic shift in the CD spectrum, with a reduction in helical content to 7.8% and a marked increase in β-strand content to 40.8%.

The binding of several CNTs to gangliosides is mediated by a conserved binding pocket located within the HCR (25). It was therefore anticipated that TeNT-LHN, which lacks the HCR, would not undergo secondary structural changes at low pH in the presence of ganglioside. However, similar to full-length TeNT, TeNT-LHN shifted to a largely β-strand dominated
structure at low pH in the presence of ganglioside (Fig. 2A and Table 1). It is therefore assumed that the conformational changes occurring in the LC and HCT domains of both proteins are largely the same. Finally, the CD spectra of a mutated TeNT protein deficient in the ability to bind gangliosides (previously termed TeNT RW) (10, 24) were acquired. The CD spectra of TeNT RW at both neutral and acidic pH are similar to those obtained with the wild-type protein (Fig. 2 and Table 1). This is consistent with data indicating that mutations at Arg-1226 and Trp-1289 have little effect on the tertiary structure of the HCR (36). Only by the addition of GT1b at low pH could a change in the secondary structure of TeNT RW be observed (Fig. 2A). Furthermore, pH-triggered conformational changes in TeNT could also be observed by the addition of alternative polysialo-gangliosides (GD3, GM1a, GD1a, and GD1b) previously demonstrated to bind TeNT in an HCR-dependent manner (data not shown) (24). These data strongly argue against a role for the HCR in detecting the presence of gangliosides at low pH.

Next, the assay was performed using GT1b at concentrations below the reported critical micelle concentration (~1 × 10^{-5} M (37)). Under these conditions, the addition of GT1b did not promote a shift in the CD spectrum of TeNT (Fig. 2B). This implied that structural changes in TeNT might simply be a function of ganglioside micelle formation. If this assertion was correct, then it follows that micelles formed from asialo-GM1a should also stimulate a change in TeNT secondary structure. However, as shown in Fig. 2B, the addition of GA1 alone or as mixed micelles composed of GA1 and GT1b (30:1 molar ratio) did not result in secondary structure changes (Fig. 2B). These observations suggest that both micelle formation and the presence of sialic acid(s) are necessary to drive conformational changes in TeNT at low pH.

### TABLE 1

| Toxin       | Buffer only | Buffer + GT1b | Buffer only | Buffer + GT1b |
|-------------|-------------|---------------|-------------|---------------|
| TeNT        | %           | %             | %           | %             |
| TeNT RW     | α = 19.6    | α = 18.4      | α = 17.9    | α = 7.8       |
| β = 27.8    | β = 27.9    | β = 28.1      | β = 40.8    |
| TeNT-LH3a   | α = 21.6    | α = 21.1      | α = 19.5    | α = 6.5       |
| β = 25.6    | β = 26.6    | β = 27.9      | β = 42.6    |
| TeNT-LH3b   | α = 36.9    | α = 34.3      | α = 32      | α = 5         |
| β = 13.8    | β = 14.5    | β = 16.3      | β = 45.7    |

### FIGURE 3.

**A**

Gangliosides facilitate transformation of TeNT into a hydrophobic protein at low pH. Triton X-114 partitioning assays were performed at various pH values in the absence or presence of ganglioside. TeNT (A) and TeNT-LH3a (B) were detected by silver staining, and the percentage distribution in the detergent phase was determined by densitometry. Mean values ± S.E. (error bars) for at least three independent experiments are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001, two-way analysis of variance with Bonferroni post-test. n.s., not significant.

**B**

Protein associated with detergent fraction (%) at pH values ranging from 4.0 to 7.4 was investigated. In the absence of GT1b, TeNT was largely recovered in the aqueous phase. However, the addition of ganglioside caused TeNT to transition from the aqueous to detergent phase only as the pH decreased below ~6.5 (Fig. 3A). In comparison, the partitioning of TeNT-LH3b into the detergent phase was dependent on the addition of GT1b but unaffected by the pH of the system (Fig. 3B). This observation suggests that the HCR may function in part to mask hydrophobic membrane binding regions until the toxin is exposed to a low pH environment.

### TelNT Associates with Liposomal Membranes Enriched in Acidic Lipids

The ability of TeNT to bind to liposomal membranes as a function of pH was determined. Initial experiments employing liposomes composed of zwitterionic lipids (base liposomes, phosphatidylcholine/phosphatidylethanolamine/cholesterol, 45:45:10, mol %) did not result in detectable binding at either neutral or low pH (data not shown). However, in agreement with previous studies (38, 39), pH-dependent binding of both TeNT and TeNT-LH3b to asolectin liposomes was observed (Fig. 4A). Based on these opposing observations, it was postulated that the increased anionic charge present in asolectin liposomes might facilitate toxin binding. To directly examine this possibility, base liposomes containing increasing amounts of the acidic phospholipid, phosphatidylserine (PS), were prepared. As shown in Fig. 4B, binding of TeNT to liposomes (base liposomes + PS) at low pH showed a clear depen-
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![Diagram](image-url)

**FIGURE 4.** TeNT associates with liposomal membranes. A. top, ~70 pmol of TeNT or TeNT-LHN was mixed with 4 μmol of asolectin liposomes in buffer at either pH 7.4 or pH 4.0. Proteoliposomes (Lipo.) were isolated by centrifugation, washed with buffer, and recovered by a second centrifugation step. Supernatant (Supt.) from the initial centrifugation step and wash fractions (Wash) were analyzed along with the proteoliposomes by SDS-PAGE. A representative example of TeNT and TeNT-LHN association with asolectin liposomes at pH 7.4 and pH 4.0 is shown. Bottom, the relative distribution of TeNT (white bars) and TeNT-LHN (black bars) ± S.E. (error bars) from three independent experiments was determined by densitometry. B. ~70 pmol of TeNT or TeNT-LHN was mixed with 4 μmol of base liposomes (45% phosphatidylcholine, 45% phosphatidylethanolamine, 10% cholesterol, mol %) containing increasing amounts of acidic lipids (0–40 mol%) in buffer at pH 4.0. Proteoliposomes were isolated as above and resolved by SDS-PAGE, followed by staining with Coomassie Blue. The image is representative of at least three independent determinations. The relative distribution of TeNT (white bars) and TeNT-LHN (black bars) ± S.E. from the indicated number of independent experiments was determined by densitometry.

Acidic Lipids Enhance TeNT-mediated Release of K⁺ from Liposomes—To further characterize the role of acidic lipids in triggering membrane association of TeNT. Although binding of TeNT-LHN to liposomes also showed a clear dependence on PS, association with base liposomes (no PS) was also increased relative to TeNT (Fig. 4B). The reason for this difference is not yet clear, but it may reflect exposure of hydrophobic surfaces by removal of the HCR.

Binding of TeNT to Liposomes through Ganglioside Receptors Mediates Efficient Channel Formation—To determine what effect cellular receptors may play in the translocation process, base liposomes + PS were doped with gangliosides to facilitate direct binding of TeNT to the membrane. Initial experiments were conducted using liposomes containing 2 mol % GT1b and 10 mol % PS. Under these conditions, the rate of K⁺ release was too rapid to allow for the contribution of GT1b to be assessed (data not shown). Therefore, the concentrations of PS and TeNT were reduced to the minimum levels necessary to detect K⁺ release as compared with base liposomes. Under these conditions (base liposomes + 2 mol % GT1b and 5 mol % PS), binding of TeNT⁻ ww or TeNT-LHN, was observed at both neutral and low pH (Fig. 6A). This demonstrates that binding of TeNT to liposomes containing gangliosides is mediated through HCR-ganglioside interactions and not through electrostatic interactions between the toxin and the charged membrane environment. Coupling of the toxin to the liposomal membrane resulted in significantly enhanced release of K⁺ content when compared with base liposomes containing PS only. However, no such effect was observed when
DISCUSSION

CNTs cause neuroparalytic diseases by preventing the release of neurotransmitters at nerve terminals. In recent years, our understanding of their structure-function relationships (e.g. catalytic LC, HCT, and HCR domains), enzymatic mode of action, and identification of neuronal cell surface receptors have all rapidly evolved (9). By comparison, the mechanism by which low pH drives the conversion of CNTs from water-soluble molecules into protein translocase channels remains elusive. Here we investigated the mechanism of channel formation by TeNT to gain new insights into this enigmatic step in the intoxication process. Our data are summarized in Fig. 7, where we propose a novel two-step model for TeNT channel formation.

Initially, the HCR binds to ganglioside co-receptors present in the neuronal plasma membrane (Step 1) (10, 24). This interaction presumably promotes partitioning into the bilayer by tethering the HCT close to the membrane, such that its orientation relative to the bilayer is optimal for subsequent channel formation. The interaction of HCR with ganglioside is independent of pH ranging from 4.0 to 7.4 (data not shown), suggesting that the toxin remains bound to the membrane within the acidified endosome. Intriguingly, the interaction of BoNT/B with synaptotagmin II was also reported to be independent of pH, potentially signifying a shared mechanism among the CNTs (40). The need to orient the HCT relative to the membrane may be related to the large -helices of the domain, which probably insert into the bilayer. By comparison, the interaction of diphtheria toxin with its cellular receptors is sensitive to pH, and consequently, formation of membrane channels occurs independently of receptor (41). Furthermore, CNTs do not undergo changes in secondary structure in response to low pH alone (Fig. 2), as has been reported for diphtheria toxin (42). Thus, the initial interaction of TeNT with the membrane does not appear to require the formation of a

base liposomes + PS and gangliosides were incubated with either TeNT or TeNT-LHN (Fig. 6B).

FIGURE 6. Ganglioside incorporation enhances K+ release from liposomes at low pH. A, 70 pmol of TeNT was mixed with 4 µmol of base liposomes + PS and gangliosides (5% PS, 2% mixed gangliosides, mol %) in buffer at either pH 7.4 or pH 4.0. Proteoliposomes were isolated by centrifugation, washed with buffer, and analyzed by SDS-PAGE followed by staining with Coomassie Blue. A representative example of TeNT association with liposomes at pH 7.4 and 4.0 is shown. B, base liposomes + PS (5% PS, mol %; white bars) or base liposomes + PS and gangliosides were diluted into buffer at pH 4.0 and incubated with the indicated proteins (1 nmol) for 5 min at 24 °C, and K+ release was recorded using an ion-specific electrode. K+ release values (mV) are the mean ± S.E. of six independent experiments. Base liposomes + PS and gangliosides were p < 0.001 (***) or p < 0.0001 (****) (one-way ANOVA with Student-Newman-Keuls post-test) compared with base liposomes + PS only. PC, phosphatidylcholine; PE, phosphatidylethanolamine; n.s., not significant.
membrane-competent state in solution. In step 2, low pH triggers the formation of an interfacial intermediate state that is regulated by the presence of acidic lipids within the membrane. The molecular basis of this regulation is currently unknown and will be the subject of future investigations. The requirement for acidic lipids further distinguishes TeNT from the interaction of diphtheria toxin with the bilayer, which is largely unaltered by the physicochemical nature of the membrane. Once the toxin has formed the interfacial intermediate state, we posit that a rapid and possibly irreversible transformation into the channel state occurs (Fig. 7).

Recent studies have demonstrated that BoNT/B and BoNT/E undergo pH-triggered conformational changes and transformation into oligomeric membrane proteins in the presence of ganglioside GT1b. We observed similar conformational changes in TeNT, a ganglioside-binding deficient form of TeNT (TeNT\textsuperscript{RGW}), and a truncated protein lacking the entire receptor binding domain (TeNT-LH\textsubscript{rc}) at low pH in the presence of GT1b (Fig. 2A). These data indicate that the observed structural changes in response to GT1b are not dependent on the HCR. Future studies are planned to determine whether the HCRs of BoNT/B and BoNT/E are necessary for the reported effects of ganglioside on secondary structure and oligomerization. How, therefore, is GT1b able to stimulate the observed changes in TeNT secondary structure? Based on the data presented in Fig. 2, we hypothesize that the observed changes in secondary structure result from insertion of the HCT into the hydrophobic core of the ganglioside micelle. Moreover, given that the addition of GA1 alone or GA1 in combination with a low amount of GT1b did not stimulate a change in secondary structure, the negative charge of the sialic acids also appears to play an important role in the interaction. Thus, it is posited that polysialoganglioside micelles mimic an acidic membrane environment necessary to drive the formation of the interfacial intermediate state as proposed in Fig. 7.

Low pH within the lumen of endosomal compartments has long been postulated as the trigger for translocase channel formation. This is in agreement with previous studies demonstrating single-channel activity of BoNTs in planar bilayers and membrane patches excised from neuroendocrine cell lines. Therefore, what role, if any, does lipid composition play in channel formation by BoNTs? Interestingly, channel formation in planar bilayer systems employed either a asolectin or defined lipid mixtures containing both phosphatidylserine and ganglioside GT1b (21, 43). Thus, the requirement for acidic lipids in regulating the formation of the TeNT interfacial intermediate state may be a shared property among the CNTs. Indeed, Fischer et al. (21) previously noted that translocation activity of BoNT/A devoid of the receptor binding domain could not be observed in non-neuronal cell lines and speculated that membrane lipid composition might contribute to this effect.

In summary, the data presented provide new insights into the mechanism by which the HCT is able to form a channel capable of translocating the LC moiety across the endosomal membrane. Moreover, they suggest a new mechanism for A-B toxin translocation, which differs significantly from the current paradigms of diphtheria and anthrax toxins.

Acknowledgments—We thank Michael Henzl, Ph.D., for assistance with CD data collection and Joseph Barbieri, Ph.D., David Lee, Ph.D., Donald Burke, Ph.D., and Marc Benson, Ph.D., for comments that greatly improved the manuscript.

REFERENCES
1. Lemichez, E., and Barbieri, J. T. (2013) General aspects and recent advances on bacterial protein toxins. Cold Spring Harb. Perspect. Med. 3, a013573
2. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) Neurotoxins affecting neuroexocytosis. Physiol. Rev. 80, 717–766
3. Johnson, E. A. (1999) Clostridial toxins as therapeutic agents: benefits of nature’s most toxic proteins. Annu. Rev. Microbiol. 53, 551–575
4. Barash, J. R., and Arnon, S. S. (2014) A novel strain of Clostridium botulinum that produces type B and type H botulinum toxins. J. Infect. Dis. 209, 183–191
5. Smith, T. J., Lou, J., Geren, I. N., Forsyth, C. M., Tsai, R., Laporte, S. L.,
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Tepp, W. H., Bradshaw, M., Johnson, E. A., Smith, L. A., and Marks, J. D. (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect. Immun. 73, 5450–5457

6. McCroskey, I. M., Hatthew, C. L., Woodruff, B. A., Greenberg, J. A., and Jurgenson, P. (1991) Type F botulism due to neurotoxicogenic Clostridium baratti from an unknown source in an adult. J. Clin. Microbiol. 29, 2618–2620

7. Poulet, S., Hauser, D., Quanz, M., Niemann, H., and Popoff, M. R. (1992) Sequences of the botulinal neurotoxin E derived from Clostridium botulinum type E (strain Beluga) and Clostridium butyricum (strains ATCC 43181 and ATCC 43755). Biochim. Biophys. Acta. 1083, 107–113

8. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clastridial neurotoxins. J. Mol. Biol. 291, 1091–1104

9. Binz, T., and Rummel, A. (2009) Cell entry strategy of clostridial neurotoxins. J. Neurochem. 109, 1584–1595

10. Chen, C., Fu, Z., Kim, J. J., Barbieri, J. T., and Baldwin, M. R. (2009) Gangliosides as high affinity receptors for tetanus neurotoxin. J. Biol. Chem. 284, 26569–26577

11. Pirazzini, M., Rossetto, O., Bolognese, P., Shone, C. C., and Montecucco, C. (2011) Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. Cell. Microbiol. 13, 1731–1743

12. Dong, M., Liu, H., Tepp, W. H., Johnson, E. A., Janz, R., and Chapman, E. R. (2008) Glycosylated SV2A and SV2B mediate the entry of botulinum neurotoxin E into neurons. Mol. Biol. Cell 19, 5226–5237

13. Dong, M., Yeh, F., Tepp, W. H., Dean, C., Johnson, E. A., Janz, R., and Chapman, E. R. (2006) SV2 is the protein receptor for botulinum neurotoxin A. Science 312, 592–596

14. Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A., and Chapman, E. R. (2003) Synaptogamins I and II mediate entry of botulinum neurotoxin B into cells. J. Cell Biol. 162, 1293–1303

15. Peng, L., Bertntsson, R. P., Tepp, W. H., Pitkin, R. M., Johnson, E. A., Stemmark, P., and Dong, M. (2012) Botulinum neurotoxin D-C uses synaptogamin I and II as receptors, and human synaptogamin II is not an effective receptor for type D, D-C and G toxins. J. Cell Sci. 125, 3233–3242

16. Rummel, A., Hafner, K., Mayhord, S., Darashchonak, N., Holt, M., Jahn, R., Beermann, S., Karoth, T., Bigalke, H., and Binz, T. (2009) Botulinum neurotoxins C, E and F bind gangliosides via a conserved binding site prior to stimulus-induced uptake with botulinum neurotoxin F utilizing the three isoforms of SV2 as second receptor. J. Neurochem. 110, 1942–1954

17. Rummel, A., Karoth, T., Henke, T., Bigalke, H., and Binz, T. (2004) Synaptogamins I and II act as nerve cell receptors for botulinum neurotoxin G. J. Biol. Chem. 279, 30865–30870

18. Fu, Z., Chen, C., Barbieri, J. T., Kim, J. J., and Baldwin, M. R. (2009) Glycosylated SV2 and gangliosides as dual receptors for botulinum neurotoxin serotype A. Biochemistry 48, 5631–5641

19. Karalewitz, A. P., Fu, Z., Baldwin, M. R., Kim, J. J., and Barbieri, J. T. (2012) Botulinum neurotoxin serotype C associates with dual ganglioside receptors to facilitate cell entry. J. Biol. Chem. 287, 40806–40816

20. Fischer, A., Sambashivan, S., Brunger, A. T., and Montal, M. (2012) Beltless translocation domain of botulinum neurotoxin A embodies a minimum ion-conductive channel. J. Biol. Chem. 287, 1657–1661

21. Fischer, A., Mushrush, D. J., Lacy, D. B., and Montal, M. (2008) Botulinum neurotoxin devoid of receptor binding domain translocates active protease. PLoS Pathog. 4, e1000245

22. Cai, S., Kukreja, R., Shoesmith, C., Chang, T. W., and Singh, B. R. (2006) Botulinum neurotoxin light chain refolds at endosomal pH for its translocation. Protein J. 25, 455–462

23. Fu, F. N., Busath, D. D., and Singh, B. R. (2002) Spectroscopic analysis of low pH and lipid-induced structural changes in type A botulinum neurotoxin relevant to membrane channel formation and translocation. Biochemistry 41, 8903–8911

24. Binz, T., Bade, S., Rummel, A., Kollewe, A., and Alves, J. (2002) Arg362 and Tyr365 of the botulinum neurotoxin type A light chain are involved in translocation state stabilization. Biochemistry 41, 1717–1723

25. Blum, F. C., Przepelski, A., Tepp, W. H., Johnson, E. A., and Barbieri, J. T. (2013) Entry of a recombinant full-length, atoxic tetanus neurotoxin into Neuro-2a cells. Infect. Immun. 81(1128):1:1539–13

26. Simpson, L. L., Coffield, J. A., and Bakx, N. (1994) Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. J. Pharmacol. Exp. Ther. 269, 256–262

27. Sun, S., Suresh, S., Liu, H., Tepp, W. H., Johnson, E. A., Edwardson, J. M., and Chapman, E. R. (2011) Receptor binding enables botulinum neurotoxin B to sense low pH for translocation channel assembly. Cell Host Microbe 10, 237–247

28. Sun, S., Tepp, W. H., Johnson, E. A., and Chapman, E. R. (2012) Botulinum neurotoxins B and E translocate at different rates and exhibit divergent responses to GT1b and low pH. Biochemistry 51, 5655–5662

29. Rummel, A., Bade, S., Alves, I., Bigalke, H., and Binz, T. (2003) Two carbohydrate binding sites in the H(CC)-domain of tetanus neurotoxin are required for toxicity. J. Mol. Biol. 326, 835–847

30. Ulrich-Bott, B., and Wiegandt, H. (1984) Micellar properties of glycosphingolipids in aqueous media. J. Lipid Res. 25, 1233–1245

31. Sreerama, N., and Woody, R. W. (2004) On the analysis of membrane sphingolipids in aqueous media. J. Mol. Biol. 326, 8291–8302

32. Schiavo, G., Boquet, P., Dasgupta, B. R., and Montecucco, C. (1990) Membrane interactions of tetanus and botulinum neurotoxins: a photolabelling study with photoactivatable phospholipids. J. Physiol. 84, 180–187

33. Boquet, P., and Duflot, E. (1982) Tetanus toxin fragment forms channels in lipid vesicles at low pH. Proc. Natl. Acad. Sci. U.S.A. 79, 7614–7618

34. Fisch, R., Rummel, A., Binz, T., and Brunger, A. T. (2006) Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. Nature 444, 1092–1095

35. Petosa, C., Collier, R. J., Klimpel, K. R., Leppla, S. H., and Liddington, R. C. (1997) Crystal structure of the anthrax toxin protective antigen. Nature 385, 833–838

36. Kyrchenko, A., Posokhov, Y. O., Rodnin, M. V., and Ladokhin, A. S. (2009) Kinetic intermediate reveals staggered pH-dependent transitions along the membrane insertion pathway of the diphtheria toxin T-domain. Biochemistry 48, 7584–7594

37. Hoch, D. H., Romero-Mira, M., Ehrlich, B. E., Finkelstein, A., DasGupta, B. R., and Simpson, L. L. (1985) Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. Proc. Natl. Acad. Sci. U.S.A. 82, 1692–1696