Differences in the Protease Activities of Tetanus and Botulinum B Toxins Revealed by the Cleavage of Vesicle-Associated Membrane Protein and Various Sized Fragments†

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ABSTRACT: Botulinum neurotoxin serotype B (BoNT/B) and tetanus toxin (TeTx) block neuroexocytosis through selective endoproteolysis of vesicle-associated membrane protein (VAMP). The enzymological properties of both toxins were compared for the first time in their cleavage of VAMP and various sized fragments using a sensitive chromatographic assay. The optimal substrate sizes for the zinc-dependent protease activities of the light chains of TeTx and BoNT/B were established using synthetic peptides corresponding to the hydrophilic core of VAMP (30−62 amino acids in length). TeTx was found to selectively cleave the largest peptide at a single site, Gln76−Phe77. It exhibited the most demanding specificity, requiring the entire hydrophilic domain (a 62-mer) for notable hydrolysis, whereas BoNT/B efficiently cleaved the much smaller 40-mer. Thus, an unusually long N-terminal sequence of 44 amino acids upstream of the scissile bond is required for the selective hydrolysis of VAMP by TeTx. Using the largest peptide, BoNT/B and TeTx exhibited ~50% and 35%, respectively, of the activities shown toward intact VAMP, detergent solubilized from synaptic vesicles. Given the large size of the smallest substrates, it is possible that these neurotoxins recognize and require a three-dimensional structure. Although both toxins were inactivated by divalent metal chelators, neither was antagonized by phosphoramidon or ASQFETS (a substrate-related peptide that spans the cleavage site), and TeTx was only feebly inhibited by captopril; also, they were distinguishable in their relative activities at different pHs, temperatures, and ionic strengths. These collective findings are important in the design of effective inhibitors for both toxins, as well as in raising the possibility that TeTx and BoNT/B interact somewhat differently with VAMP.

Botulinum neurotoxin serotype B (BoNT/B)[1] and tetanus toxin (TeTx), dichain proteins from *Clostridium botulinum* and *Clostridium tetani*, respectively, are specific inhibitors of neurotransmitter release. Their heavy chains mediate binding to distinct neuronal ectoacceptors, enabling penetration of the light chain (LC) into the cytosol where it blocks exocytosis [reviewed in Dolly (1992)]. The observation that the TeTx-LC sequence contains the zinc-binding consensus motif (HExxH) typical of neutral endoproteases (Jogeneel *et al.*, 1989) was the first evidence that these toxins possess protease activity, a feature later shown to be conserved in all BoNT serotypes (Kurazono *et al.*, 1992). Subsequently, the LCs of TeTx and BoNT/B were shown to contain an essential Zn^{2+} and the two histidines in the consensus sequence proposed to contribute to chelating the metal ion (Schiavo *et al.*, 1992a,b). Within the latter motif of thermolysin (TLN), a well-studied metalloprotease, Zn^{2+} binding is mediated via both histidines, together with a distant glutamate; the fourth ligand is water. The glutamate residues present in this motif of TLN and neutral protease E-24.11 are essential for catalysis (Matthews *et al.*, 1972; Devault *et al.*, 1988). An equivalent glutamate in the LCs of TeTx and BoNT/B is also essential for both proteolytic activity toward VAMP and the subsequent neurotoxicity (Link *et al.*, 1992; McMahon *et al.*, 1993; Li *et al.*, 1994), suggesting active-site similarities between these enzymes. Activation of the proteolytic activities of BoNTs and TeTx requires nicking to form the dichain, as well as reduction of the interchain disulfide (Schiavo *et al.*, 1992c).

VAMP (also called synaptobrevin), the target of TeTx, BoNT/B, -D, and F- (Schiavo *et al.*, 1992a−c), is a neuronal protein that is inserted into SCVs by a single hydrophobic C-terminal region, with the remainder of the protein being exposed cytoplasmically (Trimble *et al.*, 1988; Baumert *et al.*, 1989). In vertebrates, two isoforms of VAMP (1 and 2) are differentially expressed within the central and peripheral

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[1] Abbreviations: DTT, dithiothreitol; SCVs, small synaptic clear vesicles; TeTx, tetanus toxin; BoNT/B, botulinum neurotoxin serotype B; LC, light chain; EDTA, ethylenediaminetetraacetic acid; PTL, 1, 10-phenanthroline; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine anhydride; VAMP-2, vesicle-associated membrane protein isoform 2; Fmoc, (9-fluorenylmethoxy)carbonyl; HV30−HV62, synthetic peptides corresponding to human VAMP-2 30−62-mers; HVF2, the carboxyl terminal peptide product (residues 77−94) resulting from the proteolysis by neurotoxin of HV30 to HV62; FA-Gly-Leu-NH_2, *N*-[3-(2-furyl)-acryloyl]-Gly-Leu amide; DMSO, dimethyl sulfoxide; RP-HPLC, reverse-phase high-performance liquid chromatography; BSA, bovine serum albumin; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; FAB-MS, fast atom bombardment mass spectrometry; ESMS, electrospray mass spectrometry; TFA, trifluoroacetic acid; TLN, thermolysin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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nervous systems (Elferink *et al.*, 1989). Each isoform can be hypothetically subdivided into three domains, consisting of a highly variable N-terminal head of 40−44 residues, followed by a highly conserved 60 residue hydrophilic core and, finally, a hydrophobic transmembrane anchor. Interestingly, isoform 1 of VAMP from rat (unlike other species) is not efficiently cleaved by TeTx or BoNT/B because there is a substitution of V for Q76 at the P1 residue (nomenclature for protease−substrate subsites; Schecter & Berger, 1967). In addition, TeTx-LC has been shown to cleave cellubrevin (McMahon *et al.*, 1993), a ubiquitous non-neuronal vesicle protein with a strong homology to the hydrophilic domain of VAMP but containing a dissimilar N-terminal head region.

Further studies have identified the substrates for some of the other BoNT serotypes; BoNT/A and -E selectively cleave SNAP-25 (Blasi *et al.*, 1993a), and BoNT/C1 splits syntaxin (Blasi *et al.*, 1993b). All of the toxins' targets are components of the proposed docking−fusion protein complex, operating in the neuroexocytotic process (Sollner *et al.*, 1993). It is likely that cleavage of VAMP or syntaxin by the above-noted toxins (which remove the majority of their cytosolic portions) results in a blockade of neurotransmitter release, by preventing the docking of synaptic vesicles (SCVs) with the presynaptic release sites or active zones. However, the blockade of exocytosis through the selective proteolysis of SNAP-25 by BoNT/A and -E may not act in this way, because in its complete absence VAMP isoforms have been shown to specifically bind syntaxins 1A and 4 (Calakos *et al.*, 1994). Instead, SNAP-25 probably performs some equally essential function in regulated exocytosis and, accordingly, has been shown to associate directly with syntaxin (Hata *et al.*, 1993).

Since the amino acid sequence homology (52%) shared by the LCs of BoNT/B and TeTx is greater than that (~35%) retained by all clostridial neurotoxin LCs (Kurazono *et al.*, 1992), it may be expected that BoNT/B and TeTx cleave the same peptide bond of VAMP (Schiavo *et al.*, 1992c); nevertheless, appreciable divergence in their sequences might be reflected in some differences in their enzymological properties. In this study, dissimilarities were observed between the proteolytic activities of TeTx and BoNT/B in their very different minimum sized VAMP substrates, pH and temperature profiles, and sensitivities to inhibitors such as captopril; such findings predict subtle differences in their intracellular actions.

EXPERIMENTAL PROCEDURES

Materials. Hepes and all other buffer components were purchased from BDH Chemical Co. Ltd. (Poole, UK). Captopril, ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline (PTL), tetrakis(2-pyridylmethyl)ethylenediamine anhydride (TPEN), dithiothreitol (DTT), zinc (Zn^{2+}) acetate, dimethyl sulfoxide (DMSO), *N*-[3-(2-furyl)acryloyl]-Gly-Leu amide (FA-Gly-Leu-NH_2), bovine serum albumin fraction 5 (BSA), trypsin (chymotrypsin free), phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), and gelatin were purchased from Sigma (St. Louis, MO). Immobilon P (PVDF) membranes were from Pharmacia (Milton Keynes, UK). Anti-rabbit immunoglobin conjugated to alkaline phosphatase was purchased from Bio-Rad (Hemel, Hempstead, UK). All high-performance liquid chromatography (HPLC) grade S solvents were obtained from Rathburn (Walkerburn, UK). Lubrol PX detergent (peroxide free) was obtained from Boehringer Mannheim (Lewes, UK). A neutral endopeptidase substrate (3-carboxypropanoyl)alanylalanylleucine-4-nitroanilide (Suc-Ala-Ala-Leu-NH-Np) was a gift from Prof. A. J. Turner. All chemicals and solvents purchased were of the highest purity commercially available.

Purification of Botulinum Neurotoxins. BoNT/B was purified by a modification of the process described for BoNT/F (Wadsworth *et al.*, 1990), as specified in Shone *et al.* (1993). The detection of background contaminating protease activities, monitored using endoprotease substrates FA-Gly-Leu-NH_2 (Feder & Schuck, 1970), Suc-Ala-Ala-Leu-NH-Np (Indig *et al.*, 1989), BSA, and gelatin, were removed by an additional purification step on a Pharmacia Mono S ion-exchange column in 0.05 M succinate−NaOH buffer (pH 5.5) with a gradient of 0−0.3 M NaCl. BoNT/B was dialyzed against 0.05 M Hepes−NaOH (pH 7.5) containing 0.15 M NaCl and stored in small aliquots at −20 °C; its repeated freeze−thawing and vortex mixing should be avoided. BoNT/B was fully nicked as outlined in Evans *et al.* (1986), with the slight modification that trypsin was inactivated with 0.5 mM PMSF as well as with SBTI. TeTx and its LC were a gift from Dr. U. Weller, Universität Mainz, Mainz, Germany. TeTx and BoNT/B concentrations were determined from extinction coefficients calculated from amino acid sequence data, using the methods of Gill and von Hippel (1989). The absorbance values predicted for TeTx and BoNT/B at 1 mg/mL in 6 M guanidine hydrochloride are 1.26 and 1.07, respectively. The specific neurotoxicities of BoNT/B and TeTX measured by mouse bioassay (Maisey *et al.*, 1988) were $\sim 1 \times 10^8$ and $\sim 2 \times 10^7$ mouse LD_{50} units/mg, respectively, and are equivalent to published values (Evans *et al.*, 1986; Weller *et al.*, 1989). Concentrations of TeTx-LC were quantified by a colorimetric assay (Bradford, 1976).

Synthesis and Purification of VAMP Peptides. Human VAMP-2 (HV) peptides, 30−62 residues in length (Figure 1), were synthesized as outlined in Shone *et al.* (1993), using solid-phase Fmoc methods (Applied Biosystems Inc.). Peptides were cleaved from the resin, deprotected, diethyl ether precipitated, and washed, in accordance with Applied Biosystems guidelines. The resultant crude peptides were stored dry at −20 °C. All peptides were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on Micropax C18 (5 or 10 mm × 300 mm), using repetitive 1 h, 0−60% (v/v) acetonitrile gradients in 0.05% (v/v) trifluoroacetic acid (TFA). All of the peptides were judged by HPLC analysis to be of high purity: 90−95% for ASQFETS, HV40 and -50 plus residues 53−68 of HV; 80−85% for HV50 and -62. Positive ion electrospray mass spectrometry (ESMS) allowed the direct molecular weight determination of the large VAMP peptides (kindly performed by Dr. Maria Panico). The values obtained for the major components were as follows: HV30, 3480.71 ± 0.14; HV40, 4695.26 ± 0.32; HV50, 5863.89 ± 0.26; HV62, 7204.89 ± 0.37. These values were in good agreement with predicted values. The identity of the peptide ASQFETS and residues 53−68 of VAMP-2 (with an added N-terminal cysteine) was confirmed by FAB-MS to be correct, having M_r values of 768 and 2005, respectively. The HPLC-purified peptides were desiccated to remove solvents, redissolved in double-distilled water, and stored at −20 °C. Peptide concentration was routinely

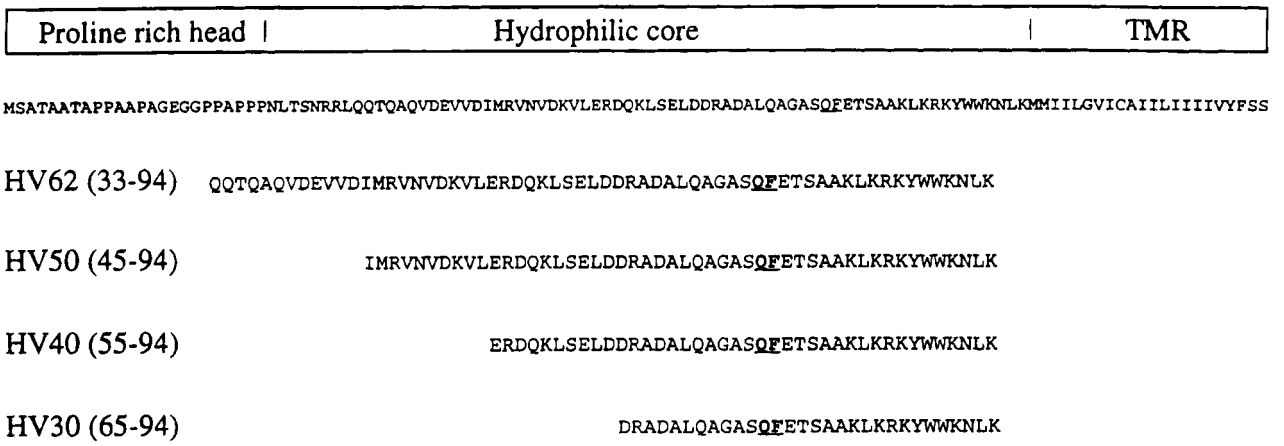

FIGURE 1: Structure of human VAMP-2 and synthetic substrates. The hydrophilic domain of VAMP isoforms is approximately 95% homologous, whereas the proline-rich head region exhibits great diversity; this is suggestive of the former alone being sufficient for cleavage by toxin. The HV prefix is an abbreviation for human VAMP-2, followed by the number of residues in each polypeptide; bracketed numbers locate their position in the sequence. The cleavage site QF shared by TeTx and BoNT/B is underlined. TMR indicates the transmembrane region.

determined by absorbance measurements at 210 nm (Scopes, 1974) and at 280 nm, on the basis of extinction coefficients calculated from sequence data (Gill & von Hippel, 1989). Amino acid analyses were also performed to accurately assess true peptide concentrations (i.e., for the HVF2 standard curve and VAMP peptide concentrations used for enzymatic kinetic measurements).

Quantitation by RP-HPLC of Toxin-Mediated Endoproteolysis of VAMP Peptides. Toxins were activated by prereduction with 20 mM DTT in 100 mM Hepes (pH 7.0) for 30 min at 37 °C and subsequently diluted to either 1 or 2 mM DTT using 100 mM Hepes−NaOH (pH 7.0) containing 0.02% (w/v) Lubrol PX to eliminate protein losses when small amounts were used. This nonionic detergent had no apparent effect on the toxins' activities when tested at up to 0.2% (w/v). Typical incubations with toxins contained, in addition, 10 μM VAMP substrate and 1−10 μM Zn^{2+} in a final volume of 40 or 80 μL. The reactions were terminated by the addition of an equal volume of 2% (v/v) TFA and 5 mM EDTA, conditions known to cause immediate toxin inactivation. Preincubations with potential inhibitors and chelators were routinely performed for 30 min at 37 °C, following dichain toxin reduction, using a 2-fold higher concentration of reagent than that present in the final incubation. Captopril stocks were prepared before use and dissolved in 0.1 M Hepes (pH 7.0). Purified ASQFETS and phosphoramidon were stored frozen in the latter buffer.

The amounts of VAMP peptides hydrolyzed were measured by monitoring the peak height of the cleavage product, HVF2, at either 220 or 280 nm, following RP-HPLC separation on a Micropax C18 column (5 × 300 mm) equilibrated in 0.05% (v/v) TFA using a 0−60% (v/v) acetonitrile gradient, at either 1.0 or 1.5 mL/min. A standard curve was constructed by plotting absorbance peak height against known quantities of injected HVF2 subjected to similar acetonitrile gradients. The peptide standards were dissolved in 100 mM Hepes (pH 7.0) containing 0.02% (w/v) Lubrol PX (necessary to reduce the loss of standard) and an equal volume of termination buffer. When assays were performed at a flow rate of 1 mL/min, the recovery of peptide increased, necessitating a different standard curve. None of the reagents used appeared to perturb the recovery of HVF2; however, longer gradient times were necesssary to completely resolve HVF2 from other constituents with similar retention times, e.g., captopril (when present) and HV40 or -30. Toxin activity was measured as an initial rate, up to 20% hydrolysis of the total substrate.

Immunological Measurement of Cleavage by Toxins of VAMP and HV62. Bovine cerebrocortical small clear vesicles (SCVs) were prepared by the method of Hell *et al.* (1988), using the modifications of Matsuoka *et al.* (1990). Toxin activity toward native VAMP and HV62 was measured by Western blotting. Purified SCVs (1 mg of protein/mL) were detergent solubilized using 1% (w/v) Lubrol PX for 30 min at 4 °C with occasional mixing in 100 mM Hepes−NaOH (pH 7.0), and the unsoluble material was removed by centrifugation at 15000*g* (maximum) for 30 min. Prereduced toxins were incubated for 20 min at 37 °C with either detergent-solubilized SCVs (0.5 mg of protein/mL; 0.5% (w/v) Lubrol PX) or 0.4 μM HV62 in buffer B (100 mM Hepes−NaOH (pH 7.0) containing 1 mM DTT and 100 μM Zn^{2+}). Incubations were terminated by the addition of SDS−PAGE sample buffer, followed by immediate boiling. Samples were subjected to SDS−PAGE (on 15% acrylamide gels), electrophoretically transferred by Immobilon P (PVDF) membranes, and blotted with antibodies raised against HV62 (de Paiva *et al.*, 1993). Anti-VAMP immunoglobins bound to blots were visualized by an antispecies antibody linked to alkaline phosphatase and were quantified using a densitometer. The amounts of VAMP remaining were calculated after subtraction of the background; in addition, standard curves of amounts of HV62 or SCV proteins plotted against band intensity were found to be linear.

RESULTS

Chromatographic Quantitation of Toxin-Mediated Endoproteolysis of VAMP Substrates. Hydrolysis of VAMP peptides (listed in Figure 1) was measured by monitoring the peak height at 220 or 280 nm of the common C-terminal cleavage product, HVF2, following RP-HPLC (Figure 2A). The quantity of HVF2 was extrapolated from a standard curve (Figure 2B) relating the peak absorbance to the known quantities injected; this was found to be linear between 25 and 1000 pmol of HVF2. Confirmation that TeTx cleaves VAMP at a single peptide bond (Q76−F77) was achieved

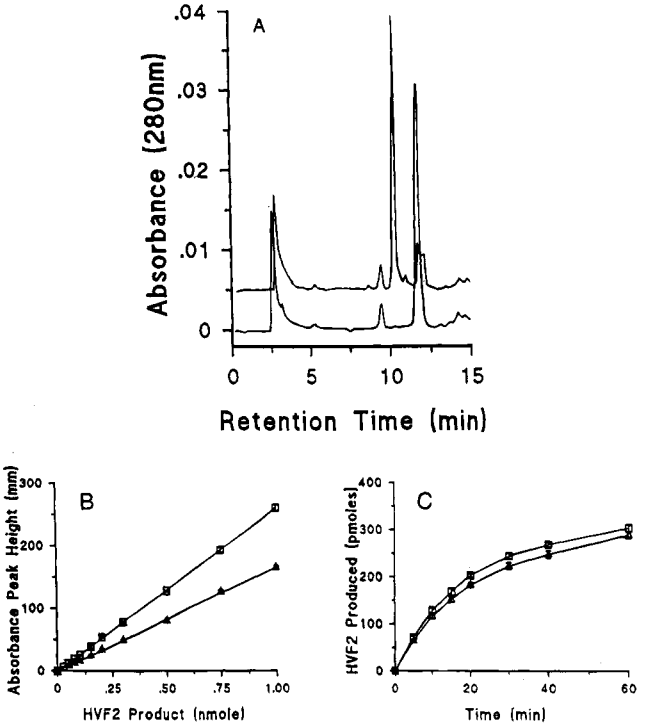

FIGURE 2: Quantitation of toxin-mediated selective endoproteolysis of VAMP peptides using RP-HPLC. (A) Incubations were performed in buffer A (100 mM Hepes (pH 7.0), containing 0.5 mM DTT, 0.02% (w/v) Lubrol PX, and 10 μM Zn^{2+}) for 45 min at 37 °C with 10 μM purified HV62, in the absence or presence (displaced trace) of 100 nM TeTx. Reactions were terminated by the addition of an equal volume of 2% (v/v) TFA/5 mM EDTA; the mixtures were then subjected to RP-HPLC on a Micropax C18 column (5 mm × 300 mm) in 0.05% TFA, using a 0–60% acetonitrile gradient (10 mL) at 1.0 mL/min. The retention time (minutes) of gradient components were as follows: HV62, 12.0; HVF2 (cleavage product), 10.5; DTT, 9.5; all other minor peaks were system background. (B) Standard curves were constructed to relate absorbance or chart peak height (millimeters) at either 220 nm (△) or 280 nm (□) to known amounts of injected HVF2 produced. HVF2 standards (n = 2 or 3) were subjected to RP-HPLC (over 7 min at 1.5 mL/min), after which their peak heights were measured. (C) The time courses of HV62 cleavage by prereduced toxins; 25 nM BoNT/B (□) or 100 nM TeTx (△) was incubated with 10 μM HV62 at 37 °C in the specified buffer for various times. The results shown are means (±SEM) from two independent experiments, each performed in duplicate.

by ESMS mass determination of the RP-HPLC-isolated proteolytic product (residues 77–94), giving the expected value, 2296.03 ± 0.09. In addition, no other major cleavage peaks were visible upon RP-HPLC, suggesting that TeTx is selective for this bond (Figure 2A). Both TeTx and BoNT/B exhibit a typical time course for an enzyme-catalyzed reaction, an initial linear rate that eventually becomes nonlinear because of the depletion of substrate or accumulation of product (Figure 2C); note that a 4-fold higher concentration of TeTx was required to give a similar rate.

TeTx and BoNT/B Require Different Minimal Sizes of VAMP Peptides for Optimal Cleavage. Recently, Shone *et al.* (1993) demonstrated that BoNT/B could selectively proteolyse large peptides corresponding to the hydrophilic domain of human VAMP-2 at a single peptide bond (Gln76–Phe77). Thus, in this study, we examined the minimum length of VAMP peptide necessary for efficient hydrolysis by TeTx. Figure 1 lists the sequences of human VAMP peptides tested. TeTx required the longest peptide, HV62 (a 62-mer, residues 33–94), before it exhibited notable

Table 1: Proteolytic Activities of TeTx and BoNT/B toward a Series of Synthetic Peptides Homologous to the Hydrophilic Domain of Human VAMP-2

		activity (nmol/min/mg)[a]		% relative rate	
substrate	sequence	BoNT/B	TeTx	BoNT/B	TeTx
HV62	33–94	104 ± 5	21.3 ± 0.8	100	100
HV50	45–94	126 ± 4	0.23 ± 0.07	121	1.1
HV40	55–94	109 ± 7	0.08 ± 0.03	105	<0.4
HV30	65–94	7.3 ± 1.7	0.07 ± 0.03	7	<0.3

[a] Prereduced toxins (4 nM) were incubated with 10 μM peptide, and initial rates were calculated as specified in the Experimental Procedures. Toxin activity is expressed as nanomoles/minute/milligram; mean values (±SEM) shown are from three independent experiments, each performed in duplicate. The initial rate values displayed represent an underestimate of the true figure, because the LC accounts for only one-third of total toxin protein.

activity (Table 1), whereas BoNT/B is significantly less demanding, as it was able to cleave HV40 (a 40-mer, residues 55–94) with rates similar to that of the 62-mer (Table 1). Truncation of HV62 to HV50 (residues 45–94) resulted in a dramatic loss of TeTx cleavage activity to approximately 1% of that seen with the larger peptide, suggesting that amino acids 33–44 are essential for maximal activity. Losses of activity with BoNT/B were not incurred (Table 1) until the peptide was shortened to HV30 (65–94) or HV25 (60–94) (Shone *et al.*, 1993), establishing the importance of residues 55–65 for BoNT/B activity. These toxins additionally failed to detectably proteolyse the much smaller ASQFETS peptide that encompasses the scissile bond, even when 400 nM BoNT/B or 1 μM TeTx was incubated with 0.1 mM peptide for 2 h; this finding highlights both toxins' lack of endoprotease activity toward relatively small peptides. TeTx exhibited a 5-fold lower activity toward HV62 compared to BoNT/B, yet they have been demonstrated to display roughly equivalent activities toward VAMP in SCVs (Schiavo *et al.*, 1992c). Such a discrepancy could be explained by the inclusion of 0.3 M NaCl in the assays for the latter study, which would have preferentially reduced the activity of BoNT/B (~5-fold) as it is more sensitive to high ionic strength, whereas TeTx displays an ~1.4-fold decrease (see the following).

Comparison of the Proteolytic Activities of BoNT/B and TeTx toward VAMP and HV62. The relative hydrolytic activities of VAMP and HV62 were compared in order to assess the importance of the N-terminal head region. To make this comparison, it was necessary to determine the amount of VAMP on SCVs. This was accomplished by Western blotting and densitometry of known amounts of SCV protein and HV62. This yielded a value of 8–10 μg of VAMP/mg total protein, representing an initial molarity (VAMP M_r = 13 600) of ~0.4 μM in the incubation mixture (0.5 mg of SCV protein/mL). This same concentration of HV62 was also used. The relative toxin activities toward the two substrates were compared by determining the toxin concentrations required to cleave ~50% of the initial substrate in a specified time. BoNT/B and TeTx displayed activities toward HV62 that were ~50% and 35%, respectively, of that observed toward detergent-solubilized VAMP (Figure 6A,B); this shows that the N-terminal head domain does influence cleavage by the toxins. Thus, while toxins cleave the detergent-solubilized native VAMP more efficiently, their activities toward soluble peptides are similar,

Table 2: Cleavage of HV62 by BoNT/B and TeTx LC Requires the Reduction of Their Interchain Disulfide

	% relative rate[a]	
	BoNT/B	TeTx
reduced dichain	100 ± 5	100 ± 4
unreduced dichain	5 ± 1	10 ± 3
isolated LC		94 ± 17
partially nicked (~40%), reduced toxin	42 ± 8	

[a] Hydrolysis of 10 μM HV62 was performed using standard conditions (±DTT) and was quantified as described in the Experimental Procedures; the initial rates determined are relative to the maximum value obtained for each reduced dichain toxin. Average values (±SEM) shown are from two or three independent experiments, each performed in duplicate.

and the latter represents a more quantitative procedure for assessing toxin activity.

Prereduction of the Toxins' Interchain Disulfide Is Necessary for Maximal Proteolytic Activity. Low levels of proteolytic activity were detected in the unreduced dichain form of TeTx or BoNT/B (Table 2). Although this may be due in part to the small amounts of free chains seen in silver-stained SDS−PAGE gels (results not shown) of the preparations, the proportion of the latter was too low to account for the amount of activity observed. After reduction, the chains of TeTx and BoNT/B are known to remain associated (denaturing agents are required for the separation and purification of the individual chains; Weller *et al.*, 1989; Sathyamoorthy *et al.*, 1985). Despite this, the reduced TeTx dichain exhibited levels of activity similar to that of the isolated LC, suggesting that noncovalent associations do not interfere with the endoproteolytic activities of LC from TeTx and, possibly, BoNT/B. Although separation of the chains might occur *in vivo*, it is not a prerequisite for the enzymic activity of LC. Limited trypsinolysis of the initial single-chain toxin forms is also necessary for full proteolytic activities, as conversion of BoNT/B 60% single chain to completely nicked was accompanied by a 50−65% increase in enzyme activity (Table 2). On the basis of this limited data, it cannot be excluded that the un-nicked and nonreduced toxins possess minimal levels of activity (Shone *et al.*, 1993).

Metalloprotease Inhibitors Distinguish the Activities of TeTx and BoNT/B Although These and Substrate-Related Peptides Are Poor Antagonists. Proteolysis of the HV62 by TeTx, but not by BoNT/B, was antagonized by captopril (Figure 3A); however, the concentration found to be effective (IC_{50} ~ 5 mM) is far higher than that for the carboxypeptidase, an angiotensin-converting enzyme whose IC_{50} is 20 nM (Cushman *et al.*, 1977). A previous study (Schiavo *et al.*, 1992c) has demonstrated significant blockade of the activities of both toxins with 2 mM captopril, but the preincubations were performed with 20 mM of this agent in a buffer (5 mM Hepes/0.3 M glycine (pH 7.4)) that is unable to prevent the resultant pH drop to 3.4 (de Paiva *et al.*, 1993), a condition that was found herein to inactivate TeTx and BoNT/B to 90% and 20%, respectively. Phosphoramidon, an inhibitor of only a small proportion of metalloproteases, failed to perturb either toxin's activity toward VAMP substrates when used at a final concentration of up to 0.5 mM. Phosphoramidon, like captopril, has been shown to significantly delay both TeTx- and BoNT/B-mediated neuroparalysis in both *Aplysia* (BoNT/B not examined) and the mouse hemidiaphragm, without an apparent blockade of

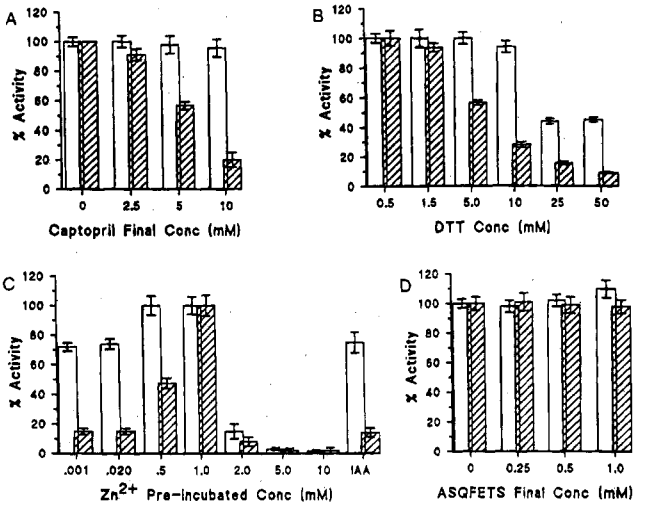

FIGURE 3: Effects of metalloprotease inhibitors and substrate-related compounds on toxin-mediated endoproteolysis. Prereduced toxins were preincubated for 30 min at 37 °C with captopril (A), DTT (B), Zn^{2+} (C), or ASQFETS (D). Subsequent cleavage was performed with 10 μM HV62 in buffer A for 15 min at 37 °C containing either 10 nM BoNT/B (open bars) or 50 nM TeTx (hatched bars), with or without the test reagent. The concentrations of the latter in the preincubations were 2-fold higher than those in the final reaction mixture, except in the case of DTT. Toxin activity was measured as initial rates (as outlined in Experimental Procedures), which were converted to relative activity by division by the rates calculated for the control toxin samples. The results are averages (±SEM) from three independent experiments, each performed in duplicate. In C, reduced toxins in buffer A but containing 2 mM DTT were preincubated with zinc(II) acetate (pH adjusted); iodoacetamide (6 mM) was used similarly except that the final incubation was supplemented with 10 μM Zn^{2+}.

VAMP cleavage on isolated SCVs *in vitro* (Schiavo *et al.*, 1992b; de Paiva *et al.*, 1993). Clearly, this anomaly requires further study, but it is likely to reflect feeble inhibition by phosphoramidon that is, apparently, only detectable in highly sensitive electrophysiological analysis in intact neuronal systems.

DTT can associate with Zn^{2+} in the active sites of metalloproteases through its weakly chelating sulfhydryl groups, resulting in inactivation (e.g., matrix metalloproteases; Woessner & Taplin, 1988); however, it is not a particularly effective inhibitor of these toxins (Figure 3B). TeTx exhibits a significantly higher sensitivity to DTT than BoNT/B, resulting in an IC_{50} ~ 5 mM, which equals the value for captopril; thus, this suggests that the weakly inhibitory effect of the latter is solely due to its thiol.

Zn^{2+} at concentrations higher than that (10−100 μM) employed in the standard assay, when used in the presence of a 2-fold higher concentration of DTT (i.e., 0.5 mM Zn^{2+}/1 mM DTT, shown in Figure 3C), can dramatically enhance the activity of TeTx by ~6-fold; interestingly, only a minimal increase (30%) in the activity of BoNT/B was noted. Although the mechanism of this enhancement is unclear, it certainly has no physiological relevance because only a micromolar amount of Zn^{2+} exists *in vivo*; as much lower Zn^{2+} concentrations (up to 100 μM) or the use of different buffers failed to elevate TeTx activity, it is unlikely that the reduced activity seen in lower Zn^{2+} concentrations arose from contaminating chelators. Similarly, it is unlikely that Zn^{2+} improves substrate folding, as neither reduced BoNT/B nor the residual activities of nonreduced toxins showed such a substantial enhancement. Iodoacetamide alkylation of the

thiols of DTT and TeTx following the latters' prereduction failed to enhance TeTx activity (Figure 3C), suggesting that the reducing agent is not directly involved in this phenomenon. Iodoacetamide alkylation of reduced toxins did not alter their proteolytic activities (not shown). None of the other divalent cations tested at 1 mM (Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+}, Cu^{2+}, or Hg^{2+}), in the presence of 2 mM DTT, enhanced or antagonized either toxin. Conversely, both toxins are similarly inactivated by higher Zn^{2+} concentrations (>2 mM), provided only 1 mM DTT is present (Figure 3C); if a 2-fold molar excess of the latter is employed, inhibition is completely prevented (not shown). The activities of other metalloproteases, e.g., TLN (Pangburn & Walsh, 1975), are reduced when sub-millimolar concentrations of Zn^{2+} are used, an effect believed to result from the inactivation of catalytic histidine residues.

Several substrate-related peptides were examined for the ability to antagonize toxin-mediated hydrolysis of VAMP substrates, presumably via association with the toxins' substrate binding sites. As none of the peptides used in this study perturbed either toxin's activity, it was concluded that they probably possess significantly lower affinities for these toxins compared to the cleavable substrate, HV62. The peptide ASQFETS that spans the cleavage site in VAMP failed to block either toxin, even when tested at concentrations of up to 1 mM (Figure 3D). ASQFETS previously has been shown to delay the neuroparalysis time of TeTx in *Aplysia* (Schiavo *et al.*, 1992c), but not in mouse hemidiaphragm with BoNT/B or TeTx, when 50 μM was applied liposomally (de Paiva *et al.*, 1993). The latter study did report that ASQFETS at 2 mM was able to inhibit (55%) VAMP cleavage by TeTx, but not by BoNT/B, a condition where the peptide was probably in >10 000-fold molar excess over the maximum possible VAMP concentration (in the 0.25 mg/mL SCV incubation used). The importance of residues 55–64 in VAMP to the minimal N-terminal sequence requirement of BoNT/B (discussed previously) led us to examine whether a peptide corresponding to this region might perturb proteolysis. However, residues 53–68 of human VAMP-2 (including the latter region and completely homologous in all mammalian VAMPs) failed to antagonize either toxin when employed at a concentration of 0.1 mM, which was a 10-fold molar excess over HV62. In a similar study employing only TeTx, the abilities of the poorly cleaved peptides (HV30, -40, and -50) were examined for the possibility that they may behave as competitive inhibitors and block the cleavage of HV62. As these smaller peptides failed to perturb TeTx activity toward HV62 (using 5-fold molar excesses of HV30, -40, and -50), lower affinities for toxin are suspected.

Inhibitory Effects of Chelators. EDTA, TPEN, and PTL (chelators of divalent cations, the latter being highly selective for Zn^{2+}) were tested for their relative abilities to inactivate BoNT/B and TeTx (Figure 4A,B). TPEN was the most effective antagonist, despite implications that its large branched aliphatic structure only allows binding of free Zn^{2+} (Schiavo *et al.*, 1992b). A 30 min preincubation with 10 μM at pH 7 caused an 80% reduction in the cleavage activities of both toxins. At the lower pH of 5.5, 10 μM TPEN was able to completely block activity; this is expected for metalloproteases because histidine residues (the Zn^{2+} ligands) become protonated at acid pH and subsequently release their metal. Both toxins exhibited similar sensitivities

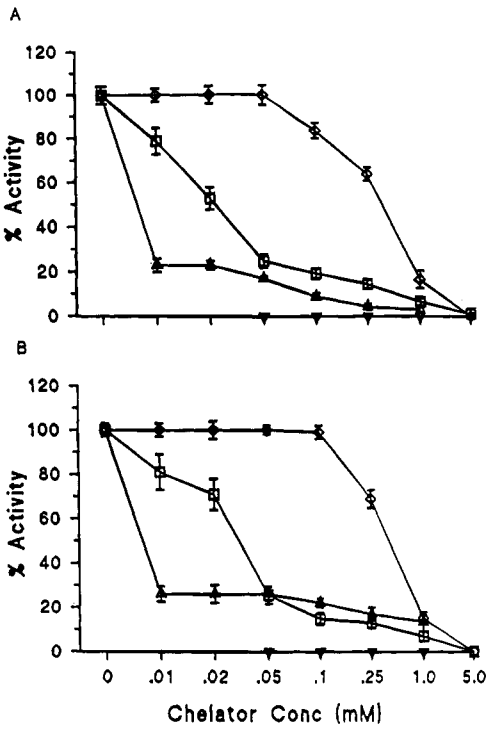

FIGURE 4: Comparison of the potencies of several chelators in inactivating TeTx and BoNT/B. Prereduced toxins, either 20 nM BoNT/B (A) or 100 nM TeTx (B), were preincubated with the chelator concentrations shown for 30 min at 37 °C [EDTA (□), PTL (◇), TPEN (△)] in buffer A (pH 7) without Zn^{2+}. An equal volume of 20 μM HV62 in the specified buffer was added to each. Other procedural details are the same as for Figure 2. TPEN was additionally preincubated with the toxins at pH 5.5 using 25 mM Mes buffer (▽); in this case, a similarly treated chelator-free toxin control was used. These results are means (±SEM) from two independent experiments, each performed in duplicate.

to each chelator, PTL being the least potent. The inhibitory actions of these chelators appear to be specific for Zn^{2+} chelation because the metal-treated chelators (at up to 0.25 mM) did not alter the toxins' activities. The inactivation of these proteases by >80% using 50 μM TPEN or EDTA could be largely reversed (to recoveries of ~80% of the original activity) if the chelator was diluted 5-fold and the apotoxins were incubated with 50 μM Zn^{2+} at pH 7 for 30 min. Also, the inactivation by chelators is markedly time dependent, i.e., comparisons of 5 and 30 min preincubations with 50 μM TPEN or EDTA resulted in the retentions of 80% and 20% of the control activities, respectively, in the subsequent 10 min incubation with substrate.

Differential Effects of pH, Temperature, and Ionic Strength on the Enzymatic Activities of BoNT/B and TeTx. With the aim of defining the optimum conditions for the toxins' endoproteolytic activities, several parameters were examined (Figure 5). The pH optimum was ascertained using two different buffers, Mes–Hepes pH 5.5–8.3 (Figure 5A) or sodium phosphate–Tris, both giving equivalent optimum profiles, albeit at slightly different levels of activity. BoNT/B and TeTx exhibit considerably different pH profiles; the former had a characteristic bell-shaped profile with a sharp pH optimum between 6.5 and 7.0, whereas TeTx displayed an unusually broad pH optimum of 6.5 to 7.5. This dissimilarity may result from variations in the ionizable groups (with pK values close to neutrality, i.e., histidine) at the catalytic or substrate binding site. pH variations can also cause changes in the substrates so as to diminish their

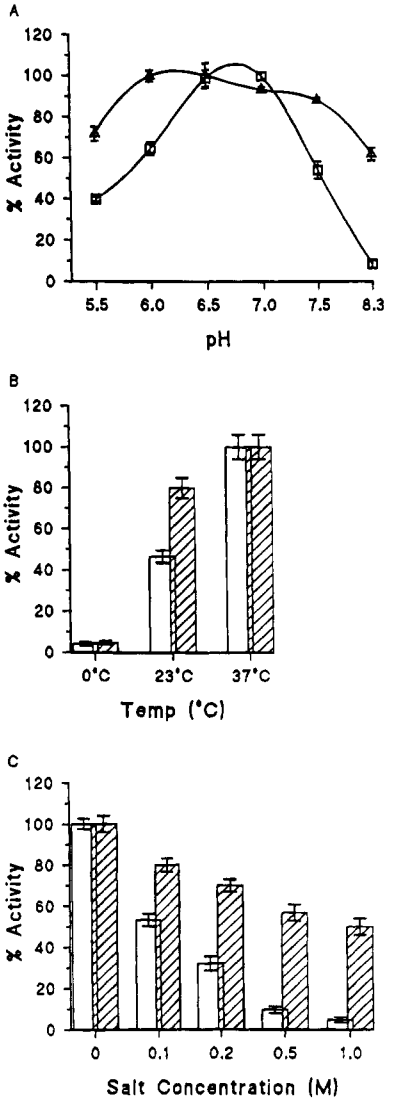

FIGURE 5: Differential effects of pH, temperature, and ionic strength on the proteolytic activities of BoNT/B and TeTx. (A) The pH values were obtained by mixing appropriate volumes of 100 mM Mes (pH 5.5) with 100 mM Hepes (pH 8.3). Reduced toxins, 10 nM BoNT/B (□) or 50 nM TeTx (△), were diluted 20-fold into the desired pH buffer that also contained 10 μM HV62, 1 mM DTT, 0.02% (w/v) Lubrol PX, and 10 μM Zn^{2+}. Incubations were performed at 37 °C for 15 min. (B) Reduced toxins, 10 nM BoNT/B (open bar) or 50 nM TeTx (hatched bar), were equilibrated at the appropriate temperatures in buffer A, before the addition of HV62 to a final concentration of 10 μM. (C) Reduced toxins, 5 nM BoNT/B (open bar) or 25 nM TeTx (hatched bar), were incubated with 10 μM HV62 in 50 mM Hepes−NaOH (pH 7.0) containing the appropriate NaCl concentrations, as well as 0.5 mM DTT, 0.02% (w/v) Lubrol PX, and 10 μM Zn^{2+}. Other details are given in the legend to Figure 2. Mean values shown (±SEM) are from two independent experiments, each performed in duplicate.

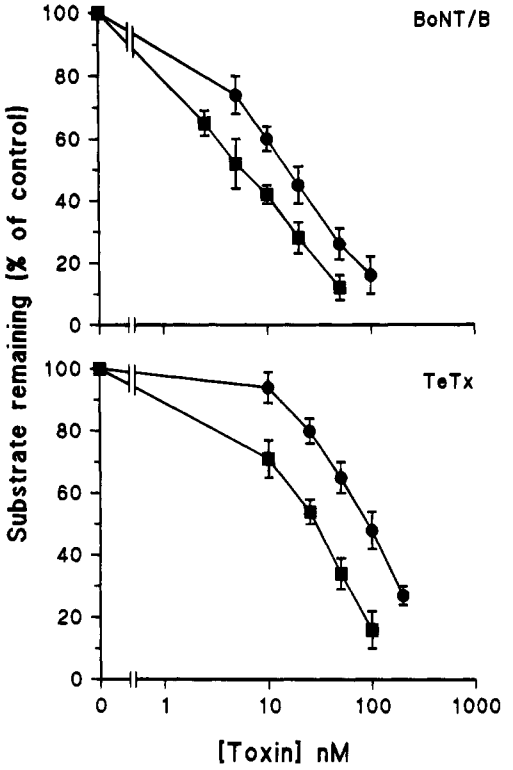

FIGURE 6: Relative activities of BoNT/B and TeTx toward VAMP and HV62. BoNT/B or TeTx, at the specified concentrations, were incubated in buffer B for 20 min at 37 °C with either (■) detergent-solubilized SCVs (0.5 mg of protein/mL) or (●) 0.4 μM HV62. The initial VAMP concentration in SCV incubations was determined immunologically to be ~0.4 μM (see Results). The reactions were terminated by the addition of SDS−PAGE sample buffer and immediate boiling, prior to SDS−PAGE, electrophoretic transfer to PVDF membranes, and immunoblotting with an anti-HV62 peptide-derived antibody. The relative amounts of primary antibody bound were visualized using antispecies immunoglobin conjugated with alkaline phosphatase and quantified using a densitometer. The amounts of substrates (either VAMP or HV62) remaining after toxin treatment are expressed (±SEM, $n = 2$ or 3) relative to toxin-free control incubations.

Table 3: Comparison of the Kinetic Parameters of TeTx and BoNT/B[a]

toxin	K_m (mM)	V_{max} (M s^{-1})	K_{cat} (s^{-1})
TeTx	2.4	5.5×10^{-8}	2.7
BoNT/B	0.65	6.2×10^{-8}	12.3

[a] Pre-reduced BoNT/B (5 nM) or TeTx (20 nM) was incubated at 37 °C with five concentrations (0.16−0.93 mM) of HV62 in buffer A. Determination of initial rates was performed using RP-HPLC. K_m and V_{max} were obtained from Lineweaver−Burk plots. Data represent the means of two determinations.

cleavability in a pH-dependent fashion. The small level of activity remaining at pH 8.3 in the case of BoNT/B, but not TeTx, may aid in the identification of an ionizable group involved in the catalytic mechanism of this particular toxin.

TeTx also differed from BoNT/B in its temperature dependence, being more active than BoNT/B at 23 °C but not 37 °C. It is uncertain whether this phenomenon also occurs *in vivo* or whether it is a reflection of the thermal conformational stability of toxins or HV62. The effects of increasing ionic strength were also studied; maximal activities of both toxins were observed at low values, with BoNT/B activity being more drastically reduced. Yet, again, interpretation of these results is difficult because increasing ionic strength may perturb either the substrate's folded structure (as well as those of the toxins) or the toxin−substrate interactions.

Kinetic Analysis of the Selective Endoproteolysis of HV62 by TeTx and BoNT/B. As shown in Table 3, the K_m value of HV62 for TeTx (2.4 mM) is ~4 times greater than that exhibited by BoNT/B (0.65 mM), while the respective K_{cat} values were 2.7 and 12.3 s^{-1}. Therefore, BoNT/B has both a higher affinity for this substrate and is catalytically more efficient. The K_m values calculated for this substrate and these toxins were uncharacteristically large (millimolar) compared to the substrates of other highly selective proteases,

e.g., endothelin-converting enzyme (ECE) (Okada *et al.*, 1993) and isoprenylated peptide protease (Ma & Rando, 1993), which are 41 and 8 μM, respectively.

DISCUSSION

The present study has demonstrated that optimal protease activities for TeTx and BoNT/B are obtained by using unusually large polypeptides requiring sections of the VAMP sequence, 44 and 22 residues, respectively, on the N-terminal side of the peptide bond cleaved. In the case of BoNT/B, Shone *et al.* (1993) established the importance of the 18 residues located after the cleavage site; therefore, this has not been evaluated herein for TeTx. In general, it is the residues spanning the cleavage site in a substrate that define whether it is efficiently cleaved by a protease. As these toxins require the largest minimal substrates of any proteases previously reported, they must recognize their substrates in a unique fashion. Perhaps the closest relative, also requiring fairly large minimal substrates (16−20 residues in total), is the selective metalloprotease ECE (Okada *et al.*, 1993). However, it is exceptional among proteases, many of which simply require one or two strong interactions with the P1 or P1′ moieties of the substrates; eight residues are adequate for more selective proteases such as HIV protease 1 or renin (Miller *et al.*, 1989; Blundell *et al.*, 1987). Generally, all of these clostridial neurotoxins comprise a new group of metalloproteases and do not appear to favor a particular type of P1 or P1′ residue at the point of cleavage (i.e., QR, BoNT/A; QF, BoNT/B and TeTx; KL, BoNT/D; RI, BoNT/E; QK, BoNT/F; AA, BoNT/G; Schiavo *et al.*, 1992c, 1993a−c; Yamasaki *et al.*, 1994), a property very commonly shared by members of less selective protease groups; for the latter, P1 sites are the primary selectivity determinants.

The mechanism of substrate recognition by the toxins that limits proteolysis to such large polypeptides may involve the following. Firstly, these toxins are likely to have extended substrate binding regions in which a large number of amino acids interact with moieties in the active-site/substrate binding cleft. Each amino acid of the substrate could vary in overall importance to binding and proteolysis. The observation that a Q to V substitution at the P1 site makes rat VAMP-1 uncleavable (Schiavo *et al.*, 1992c) suggests that this residue is important. Clearly, analysis of amino acid substitution in the minimal sized substrate will be necessary to uncover important residues in the vicinity of the cleavage site. However, the inability of these toxins to cleave peptides of less than 25 residues, a length that must logically exceed the maximum permissible extended substrate binding cleft, implicates other factors in target recognition. Another factor that might limit toxin activity to large substrates includes the structural conformation of the scissile bond, i.e., a folded structure not obtainable in smaller peptides. Unexpectedly, Shone *et al.* (1993) stated that there seems to be a lack of regular secondary structures (i.e., α-helix or β-structure) in the largest substrate HV62, as deduced from circular dichroism spectroscopy. Thus, this 62-mer peptide may assume random flexible conformations in aqueous solution; however, interaction with the toxins' active and substrate binding sites may introduce the correct form necessary for proteolysis. This hypothesis has been suggested for the small hormonal peptides, such as glucagon (29 amino acids in length), which also lacks a rigid conformation in solution but assumes the correct structure upon binding to its receptor (Gunning & Blundell, 1981). Consistent with this notion, computer-simulated annealing−molecular dynamics calculations [performed by M. Olsen and F. Lebeda (unpublished observation)] suggest that HV40 is in a coiled conformation in aqueous solution and that the cleavage site may be part of a short β-turn; however, if restraints are placed on residues predicted to be α-helices (which may exist in the toxin−substrate complex), then an α−α-hairpin loop was predicted to occur adjacent to the toxin cleavage site. Thus, these toxins may recognize and bind to discontinuous sections of sequence in their substrate, possibly far removed from the scissile bond but brought together in a three-dimensional complex by toxin-induced folding of the substrates' polypeptide backbones. The corollary to this is that, as uncleavable short peptides displayed very low affinities for the toxin's active site, absolute specificity may involve several distinct but low-affinity discontinuous interactions that are mediated by the larger peptides and sum to a very much higher affinity. The dissimilar minimal substrate requirements of TeTx and BoNT/B suggest that they recognize VAMP in distinct fashions (at least in part), as would be expected for proteins that retain only 52% sequence homology, despite sharing an identical processing site.

The activities of TeTx and BoNT/B were further distinguishable by different sensitivities to pH, temperature, and ionic strength, as well as sensitivities to weak metalloprotease inhibitors such as captopril, which only perturb the activity of TeTx. The apparent necessity of having to use very large concentrations of these agents to achieve antagonism eliminates their potential for future clinical and biochemical applications. Perhaps through rational design methods based on the three-dimensional structures of VAMP and the toxins' LC catalytic sites, it may be possible to improve the affinities of small peptides and, eventually, acquire a useful peptide-based toxin antagonist.

The high sensitivity of the assay described here allows quantitative measurement of single-site proteolysis by toxin, a technique that is essential for the biochemical evaluation of antagonists as well as residues of importance in the toxins' LCs, i.e., site-directed mutants. A catalytic glutamate-deficient genetically engineered mutant of TeTx-LC has already been characterized in this way (work performed in collaboration with Dr. Yan Li of this laboratory; Li *et al.*, 1994). An important consideration when using a new proteolytic assay, which might be used for the evaluation of potential antagonists, is how it reflects the *in vivo* physiological intoxication. Although *in vitro* methods may never be equivalent, the demonstrated shared weak sensitivities of antagonists toward BoNT/B- and TeTx-mediated cleavage of intact VAMP and of substrate-derived peptides, as well as their similar proteolytic activities toward both, support its validity. Unexpectedly, the intracellular toxin concentrations may also be similar to the 4 and 25 nM used herein, i.e., a single toxin molecule in the minute internal volume (5×10^{-16} dm^{-3}), calculated for a cerebrocortical synaptosome (average diameter = 1 μm; McMahon & Nicholls, 1991) could yield a final molarity of ~3 nM. In reality, probably more than one toxin molecule is internalized (governed by the unknown number of specific acceptors per synaptosome, as well as incubation time), so that toxins could achieve even larger cytosolic concentrations. By comparison, the internal volume of an average motor nerve ending

[physiological site of action of BoNT; reviewed in Dolly (1992)] is vastly larger, but because an appreciable number of toxin acceptors reside there (Black & Dolly, 1986), BoNT may still reach nanomolar cytosolic concentrations. Nevertheless, the ability to mimic *in vivo* conditions is complicated by the intracellular environment. Firstly, the conditions inside a cell are not like the dilute solutions of an *in vitro* assay; in fact, the total cytosolic protein concentration may be 300 mg/mL and the fluid phase diffusion rates 4 times slower (Fulton, 1982; Hou *et al.*, 1990). Additionally, toxin cleavage *in vivo* may be slower if VAMP is complexed with other proteins (e.g., syntaxin) and not readily accessible. It can, thus, be speculated that the remarkable potencies of these neurotoxins *in vivo* result from their abilities to become concentrated in the cytosol of the susceptible nerve terminals. This is achieved by binding to specific high-affinity acceptors, followed by internalization through coated pits and translocation into the cytosol [reviewed in Dolly (1992)], where the reducing environment generated by thioredoxin breaks the interchain disulfide linking the dichain, thus producing maximal LC activity (Schiavo *et al.*, 1990). Upon arrival there, these toxins may not cleave their targets particularly rapidly (relative to other proteases), but as there may be no greater than a thousand SCVs (containing VAMP) per synaptosome [calculated from serial sectioning studies performed by Verhage *et al.* (1991)], blockade of neurotransmitter release could, nevertheless, be rapid.

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