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Synaptobrevin/vesicle-associated membrane protein (VAMP) of *Aplysia californica*: Structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F

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ABSTRACT **Synaptobrevin/vesicle-associated membrane protein (VAMP) and syntaxin are potential vesicle donor and target membrane receptors of a docking complex that requires *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment proteins as soluble factors for vesicle fusion with target membranes. Members of this docking complex are the target of clostridial neurotoxins that act as zinc-dependent proteases. Molecular cloning of the *Aplysia californica* synaptobrevin cDNA revealed a 180-residue polypeptide (M_r, 19,745) with a central transmembrane region and an atypically large C-terminal intravesicular domain. This polypeptide integrates into membranes at both the co- and posttranslational level, as shown by modification of an artificially introduced N-glycosylation site. The soluble and membrane-anchored forms of synaptobrevin are cleaved by the light chains of the botulinal toxins type D and F and by tetanus toxin involving the peptide bonds Lys^{49}-Ile^{50}, Gln^{48}-Lys^{49}, and Gln^{66}-Phe^{67}, respectively. The active center of the tetanus toxin light chain was identified by site-specific mutagenesis. His^{233}, His^{237}, Glu^{234}, and Glu$^{270/271}$ are essential to this proteolytic activity. Modification of histidine residues resulted in loss of zinc binding, whereas a replacement of Glu^{234} only slightly reduced the zinc content.**

Clostridial neurotoxins, tetanus toxin (TeTx), and the seven related but serologically distinct botulinal neurotoxin types (BoNT/A to G) are dichain toxins that potently inhibit neurotransmitter release. Both toxins enter the nervous system at peripheral motoneurons. Botulinal neurotoxins act directly at the primary site of entry, thus blocking release of acetylcholine, which results in a flaccid paralysis, the clinical manifestation of botulism. In contrast, TeTx is retrogradely transported into the central nervous system where it blocks the release of inhibitory neurotransmitters leading to the clinical manifestation of tetanus. It is generally accepted that the heavy chains (100 kDa) of the individual neurotoxins are responsible for neuroselective binding, internalization, different intraneuronal sorting, and translocation of the light (L) chains from an acidic compartment into the cytosole (1). The L chains (50 kDa) contain in their middle a putative zinc-binding motif, HEXXH (2, 3). Indeed, once released from the heavy chains, the L chains evoke their detrimental activity as metalloproteases by selectively cleaving synataxin (BoNT/C1) (4), SNAP-25 (25-kDa synaptosome-associated protein) (BoNT/A) (5), or synaptobrevin/vesicle-associated membrane protein (VAMP) (TeTx, BoNT/B, or BoNT/F) (6–8). These three target proteins were shown (9) to be members of a multiprotein complex that controls vesicular fusion with target membranes.

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Synaptobrevins are class II membrane proteins that expose most of their sequence at the cytoplasmic face of small synaptic vesicles (10, 11, 37). The proteins are structurally highly conserved in evolution from *Drosophila* through mammals (12, 13), suggesting a central role in the regulated release of neurotransmitters, perhaps as the donor or ligand peptide that binds to a receptor protein of the docking complex located on the cytoplasmic surface of the plasma membrane.

The identified pre- and postsynaptic neurons of the buccal ganglia of *Aplysia californica* (14) have been useful for determining the action of clostridial neurotoxins either applied as peptides (15) or microinjected in form of encoding mRNA (3, 16). As a first step to identifying target proteins of clostridial neurotoxins in *Aplysia* neurons, we have cloned and sequenced the synaptobrevin gene.** *Aplysia* synaptobrevin integrates into membranes equally well at the co- and posttranslational level. We show that both membrane-associated and free synaptobrevin are proteolyzed at distinct sites by the recombinant L chains of BoNT/D, TeTx, and BoNT/F. Using an *in vitro* cleavage assay with *Aplysia* synaptobrevin as a substrate and a set of TeTx L chain mutants, we characterized the active center of this metalloprotease.

MATERIALS AND METHODS

Molecular Cloning and Expression of the Synaptobrevin Gene from *A. californica*. A cDNA library established from the total nervous system of *Aplysia* was screened with a random primer-labeled rat synaptobrevin 2-specific probe at low stringency [5× standard saline citrate/5× Denhardt's reagent in 10 mM sodium phosphate (pH 6.8) containing denatured salmon sperm DNA at 100 μg/ml and 0.1% SDS at 42°C]. For expression of a soluble synaptobrevin fragment, a His_6-tag, after Leu^{87}, was added by PCR. Expression in *Escherichia coli* and isolation of the peptide on Ni-nitrilotriacetic acid-agarose were performed according to protocols of the manufacturer (Diagen, Düsseldorf, F.R.G.). The peptide was further purified by HPLC using a Nucleosil 5μ C_8 column (250 × 4 mm) (Macherey & Nagel).

***In Vitro* Transcription/Translation.** The following transcription vectors derived from pSP72/73 (Promega) were used: pASB-1 contained the *Aplysia* synaptobrevin-specific *Hin*cII–*Eco*RI fragment. pLC-2 contained a resynthesized

Abbreviations: TeTx, tetanus toxin; BoNT/A through G, botulinal neurotoxin type A through G; L, light.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. U00997).

TeTx L chain gene (17). pBN2 contained the authentic BoNT/D L chain gene, as generated by PCR using BoNT/D-encoding bacteriophage DNA (18) and the two primers 5′-GGTCTAGATCTAGGAGATTAGTATTATGACATGGCCAGTAAAAG-3′ and 5′-GGTCTAGATCTTTAACTATTTTTTGTTAATCTTAAACATAC-3′. pE1Δ45-132 encodes a truncated form of the coronaviral E1 glycoprotein used to control membrane integration (19). Transcription/translation was performed in the absence or presence of canine microsomal membranes (Promega) as described (19). Samples were analyzed by SDS/PAGE (20) and autoradiography.

Neurotoxins. BoNT/D and BoNT/F holotoxin were isolated by published procedures (21, 22). L chains were obtained from the individual holotoxins by reduction (23). The TeTx L chain containing a C-terminal His_6-tag was expressed from a resynthesized gene (17) in *E. coli* and isolated by binding to Ni-nitrilotriacetic acid-resin. To generate TeTx L chain mutants with substitutions within the $H^{233}ELIH^{237}$ zinc-binding motif or to change Tyr^{243} into Phe^{243}, we replaced a *Ppu*MI–*Sph* I fragment of pLC-2 (17) by specific synthetic oligonucleotides. Similarly, for mutation of Glu^{258} or Glu^{270} and Glu^{271}, the *Sph* I–*Cla* I fragment of pLC-2 was replaced with suitable oligonucleotides. All mutations were verified by DNA sequencing. Protein concentrations were determined according to Bradford (24).

Toxin-Induced Proteolysis of *Aplysia* Synaptobrevin. Radiolabeled membrane-associated synaptobrevin and unlabeled L chains were generated by *in vitro* translation and incubated for 1 h at 37°C. Material was separated by SDS/PAGE and the amount of radioactivity present in the cleavage products was quantified by laser densitometry. For determination of the cleavage sites, the recombinant N-terminal fragment (residues 2–87) was incubated in 20 mM Hepes (pH 7.0) containing 100 mM NaCl with purified L chains.

Other Methods. Amino acid sequences were determined on a model 473A protein sequencer from Applied Biosystems. Zinc content was measured with a Zeaman atomic absorption spectrometer 4100ZL from Perkin–Elmer.

RESULTS AND DISCUSSION

Molecular Cloning and Sequencing of the *A. californica* Synaptobrevin Gene. To search for the synaptobrevin homologue in *Aplysia* as a potential target of clostridial neurotoxins, we isolated *Aplysia* synaptobrevin cDNA clones using low-stringency hybridization conditions and a rat synaptobrevin 2-specific probe. The *Aplysia* synaptobrevin open reading frame encodes a polypeptide of 180 residues with a calculated molecular mass of 19.7 kDa (Fig. 1). The distal N-terminal domain (residues 1–21) shows little homology to synaptobrevin isoforms from other species. This domain is thought to play a role in intracellular targeting. The central core portion (residues 22–84) is highly conserved among synaptobrevins from heterologous species showing between 33 or 87% identity with synaptobrevins from yeast (25) to rat (26), respectively. The hydrophobic transmembrane anchor (residues 85–105) is followed by an atypically large intravesicular domain (residues 106–180) (see below). The biological function of this portion of the molecule is unclear. It contains a surplus of 6 basic amino acid residues including 5 His residues that could interact with other vesicular proteins.

***Aplysia* Synaptobrevin Inserts into Microsomal Membranes Both at the Co- and Posttranslational Levels.** To study membrane integration and topology of *Aplysia* synaptobrevin, we introduced an artificial N-glycosylation site into the presumed intravesicular domain and translated the corresponding mRNA in the presence or absence of microsomal membranes added either co- or posttranslationally (Fig. 2). The translation mixtures were then separated by sedimentation

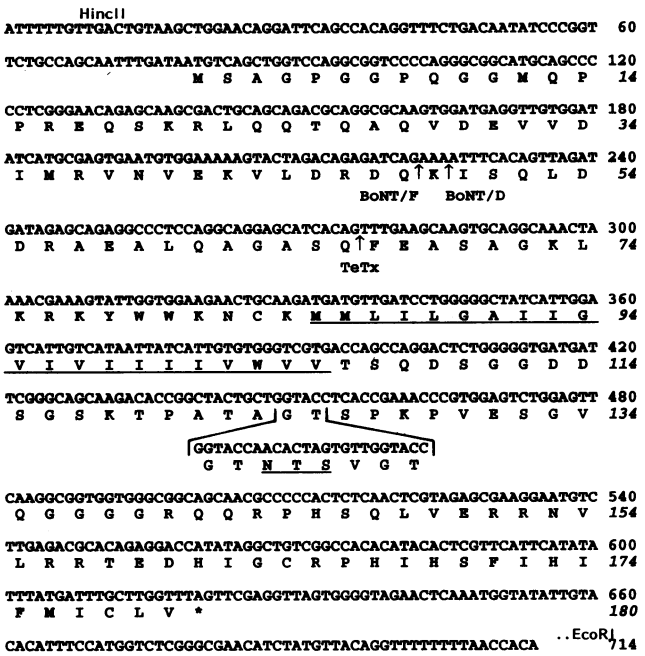

FIG. 1. Nucleotide and deduced amino acid sequence of the *A. californica* synaptobrevin. The putative transmembrane region is underlined. A self-complementary oligonucleotide encoding a target site for N-glycosylation was introduced into the *Kpn* I site to ascertain membrane translocation of the C-terminal tail. Restriction sites used for subcloning are indicated. Cleavage sites of TeTx, BoNT/D, and BoNT/F determined in this report are indicated by arrows.

through sucrose cushions into supernatant fractions and membrane pellet fractions both of which were analyzed

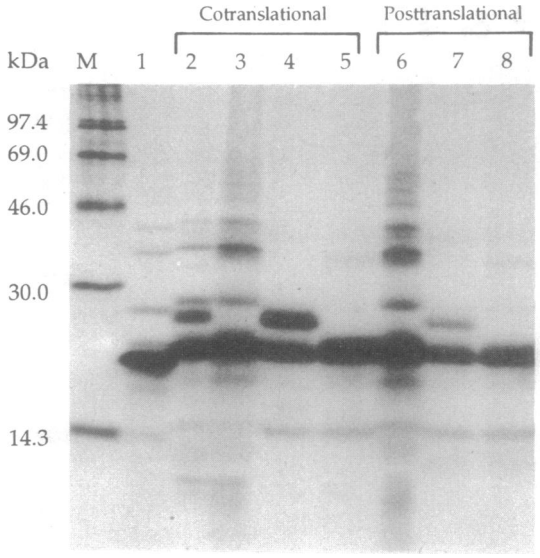

FIG. 2. Posttranslational glycosylation of a synaptobrevin mutant carrying a C-terminal N-glycosylation site. mRNA encoding wild-type (lane 1) or mutant (lanes 2–8) *Aplysia* synaptobrevin was translated in the presence of canine microsomal membranes added either cotranslationally (lanes 2–5) or after translation was blocked with cycloheximide (250 μM, final concentration; lanes 6–8). Membranes were recovered by centrifugation through a neutral sucrose cushion (5 min at 50,000 rpm, Beckman TLA 100 rotor). Protein in the supernatants was precipitated with trichloroacetic acid (lanes 3 and 6). The pellet fractions were resuspended in 100 mM sodium citrate (pH 5.5) and analyzed either directly (lanes 4 and 7) or after addition of 1% Triton X-100 and incubation for 16 h at 22°C with 100 microunits of endo-glucosaminidase H (lanes 5 and 8). Lane M, molecular mass markers.

separately for the presence of synaptobrevin. In comparison to the wild type (22.4 kDa) (lane 1), the mutant synaptobrevin produced, in addition, a larger membrane-associated species migrating with an apparent molecular mass of 25.5 kDa, no matter whether the membranes were added co- or posttranslationally. This electrophoretic shift was due to N-glycosylation of synaptobrevin, because it was exclusively observed with the membrane pellet fractions (lanes 4 and 7) whereas it was not detected with the supernatant fractions (lanes 3 and 6). In addition, treatment with endo-β-*N*-acetylglucosaminidase H reverted this shift (lanes 5 and 8). In control experiments we applied the same microsomal membrane preparation to demonstrate that a previously well-characterized coronaviral membrane glycoprotein carrying an internal membrane anchor (19) associated with the membranes only when these were added cotranslationally (data not shown). Thus our findings indicate that *Aplysia* synaptobrevin is capable of integrating into membranes and of translocating the C-terminal domain to the lumenal side at the co- and posttranslational level.

***Aplysia* Synaptobrevin Is Proteolyzed by BoNT/D, TeTx, and BoNT/F.** We next determined the susceptibility of membrane-associated *Aplysia* synaptobrevin to the L chains of BoNT/D, TeTx, and BoNT/F, employing L-chain derivatives that were either by themselves generated by *in vitro* translation (BoNT/D and TeTx) or highly purified by ion-exchange chromatography (BoNT/F). After incubation with the toxin, the reaction mixtures were again sedimented through sucrose cushions, thus allowing a direct comparison of the released fragments in the supernatant and of the membrane-anchored fragments in the pellet fractions.

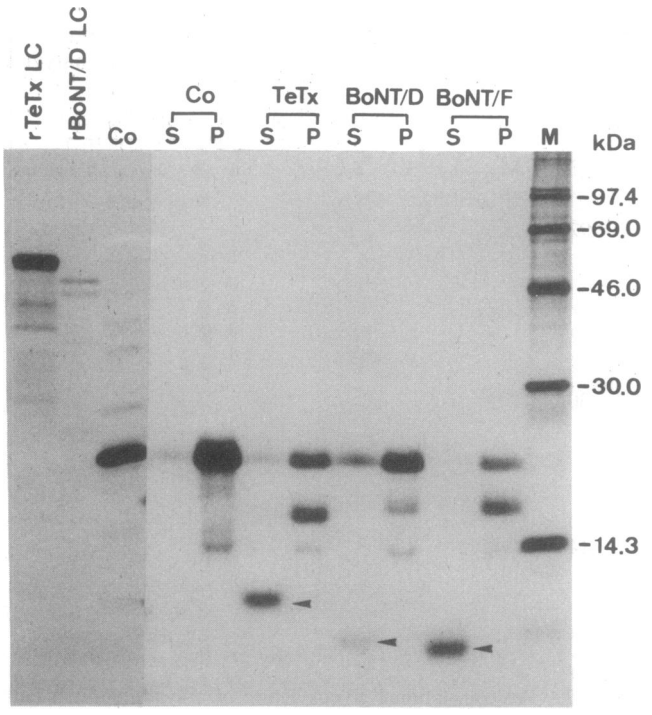

FIG. 3. *Aplysia* synaptobrevin is proteolyzed by BoNT/D, TeTx, and BoNT/F at different sites. Membrane-associated radiolabeled *Aplysia* synaptobrevin and the unlabeled L chains of TeTx (lane rTeTx LC) and BoNT/D (lane rBoNT/D LC) were produced by *in vitro* translation, whereas the type BoNT/F L chain (50 nM, final concentration) was derived from reduced holotoxin. The left two lanes show the products of control translations of TeTx and BoNT/D L chains in the presence of [^{35}S]methionine. After incubation (1 h, 37°C), the material was centrifuged through a neutral pH sucrose cushion to analyze the released fragments in the supernatant (S) and the membrane-retained fragments in the pellet (P) fractions. Lane M, molecular mass markers.

As shown in Fig. 3, BoNT/D, TeTx, and BoNT/F cleaved *Aplysia* synaptobrevin at different sites resulting in fragments of 7.5 kDa and 16.5 kDa (TeTx), 4.8 kDa and 17.0 kDa (BoNT/D), and 4.6 kDa and 17.0 kDa (BoNT/F), respectively. Dose-dependency studies indicated that *Aplysia* synaptobrevin was proteolyzed by the three toxins at different rates in the order BoNT/F > TeTx > BoNT/D, requiring 8.5 nM, 30 nM, or 130 nM, respectively, for semiquantitative cleavage (data not shown). By employing these concentrations, we then tested whether proteolysis was inhibited by preincubation with nonspecific inhibitors of metalloproteinases including EDTA (10 mM), *o*-phenanthroline (20 mM), dipicolinic acid (0.2 mM), and captopril (2 mM). In this proteolysis assay system, only *o*-phenanthroline was capable of inhibiting all three toxins, whereas captopril had no effect.

To determine the peptide bonds cleaved by the individual toxins, we used a recombinant fragment representing the entire N-terminal domain of *Aplysia* synaptobrevin up to Leu^{87} as a substrate. This fragment migrated as a single peak in reverse-phase HPLC (Fig. 4*A*). Incubation with the L chain of BoNT/D generated two smaller-sized fragments (Fig. 4*B*). Microsequencing yielded the sequences ISQLDDRAEA and (M)SAGPGGPQPP, indicating that BoNT/D cleaved the Lys^{49}–Ile^{50} bond. In a similar manner, we showed that TeTx proteolyzed the Gln^{66}–Phe^{67} bond, whereas BoNT/F affected the Gln^{48}–Lys^{49} bond. Thus these data suggest that each of the three toxins cleaves *Aplysia* synaptobrevin at different sites, whereby the latter two sites correspond to those mapped recently for rat synaptobrevin 2 (6, 8).

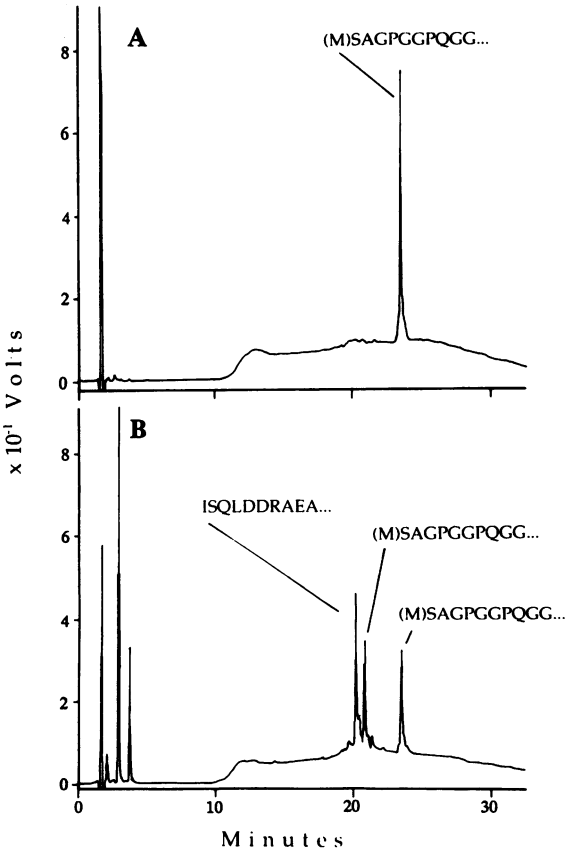

FIG. 4. HPLC profiles of a soluble *Aplysia* synaptobrevin fragment before and after treatment with BoNT/D. An HPLC-purified recombinant *Aplysia* synaptobrevin fragment (residues 2–87) was incubated (1 h, 37°C) alone (*A*) or with 500 nM (final concentration) BoNT/D L chain (*B*). Samples were separated by reverse-phase HPLC; peak fractions were subjected to microsequencing. Amino acid sequences specify the N-terminal sequences of the fragments.

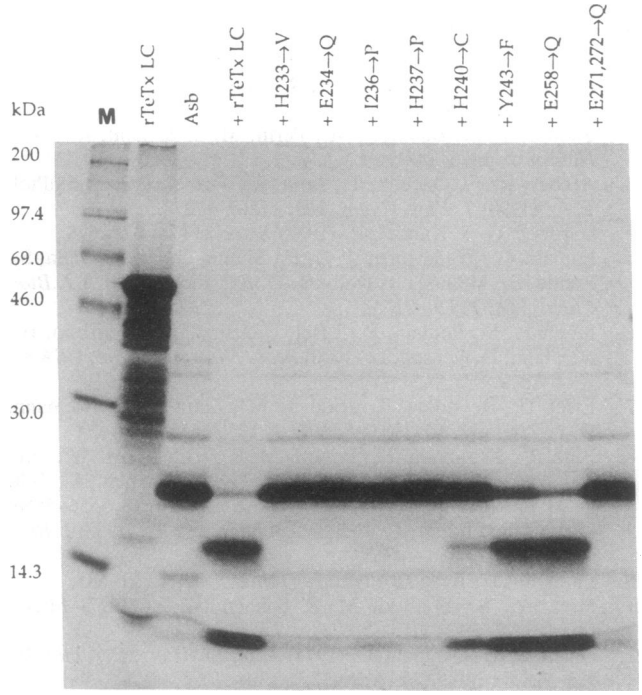

FIG. 5. Characterization of TeTx L chain mutants defective in zinc coordination or proteolysis. Unlabeled wild-type or mutant L chains of TeTx were generated by *in vitro* translation. rTeTxLC specifies control translation products in the presence of [^{35}S]methionine. *In vitro* synthesis of radiolabeled *Aplysia* synaptobrevin (lane Asb) was blocked by cycloheximide prior to addition of preformed mutant L chains and incubation for 3 h at 37°C.

Mutational Analyses of Residues in the Putative Zinc-Binding Motif of TeTx. The *in vitro* cleavage assay was applied to investigate which of the residues within the putative zinc-binding motif, H^{233}ELIHVLH^{240}, of TeTx was essential for proteolysis of synaptobrevin. First, we replaced the individual His residues, each of which could play a role in coordinating the catalytic zinc ion (2, 3, 27). As shown in Fig. 5, the His^{233} → Val mutation completely abolished protease activity. Mutants carrying Leu, Cys, Phe, Gly, or Pro in this position were also inactive (data not shown). Furthermore, a mutation in this position caused a significant reduction in zinc binding, as determined by atomic absorption spectroscopy of the Val^{233} mutant (Table 1). His^{237} was replaced by Pro (Fig. 5), Asp, Gly, or Val residues. In all these instances, protease activity was lost. The same result was obtained with the double mutant Val^{233,237}, which, as expected, lacked detectable amounts of zinc.

In contrast, His^{240} could be replaced by Arg and Ala without diminishing protease activity (data not shown). A replacement of this residue by Cys, however, reduced the activity to ≈15% of the wild-type L chain (Fig. 5), and mutants carrying either Asp or Pro in this position were inactive (data not shown).

We then replaced Glu^{234} by a Gln residue. This caused a complete loss of proteolytic activity, whereas zinc binding was only slightly reduced (Fig. 5 and Table 1). This finding is in agreement with mutational analyses of other zinc-dependent metalloproteases in which the δ-carboxyl group of the equivalent glutamate serves to coordinate a hydrogen-bonded water molecule in one of the four tetrahedral positions around the catalytic zinc ion (27).

Thus, our data suggest that the side groups of His^{233}, His^{237}, and Glu^{234} and the hydrogen-bonded water reside in three of the four positions of the tetrahedron around the catalytic zinc ion. Apparently, this configuration is facilitated, because the HEXXH motif of TeTx resides in an α-helix, as also determined for thermolysin, a zinc-dependent protease for which the crystal structure has been determined (28). The Pro^{236} mutant was generated because we wanted to distort the presumed α-helical configuration in the HELIH-motif. As shown in Fig. 5, this mutation indeed reduced protease activity to ≈3% of the wild-type L chain.

In thermolysin, the residue occupying the fourth position of the tetrahedron is a Glu, Glu^{166}, separated from the H^{142}EXXH^{146} motif by 19 amino acid residues. To determine the corresponding residue of TeTx, we first mutated Glu^{258} because this residue is conserved in TeTx and BoNT/D, and a Glu is present in a similar position in BoNT/B (1). However, the Gln^{258} mutant cleaved synaptobrevin as efficiently as the wild-type L chain (Fig. 5). According to computer-assisted structure predictions (SYBYL molecular modeling software; Tripos Associates, St. Louis), the δ-carboxyl groups of both Glu^{270} and Glu^{271} of TeTx could be positioned into a 2- to 3-Å range of the prospected zinc ion. These residues are highly conserved in the L chains of botulinal neurotoxins (1). Therefore, we simultaneously substituted both Glu^{270} and Glu^{271} with Gln residues. As shown in Fig. 5, the Gln^{270,271} double mutant was completely inactive suggesting that either Glu^{270} or Glu^{271} occupies the fourth position of the tetrahedron.

Our mutant data regarding the zinc-binding motif are in complete agreement with results obtained with other metalloproteases sharing the same motif (2, 27, 29, 30). They are, however, in conflict with previous observations (1) made with microinjected mRNAs. In particular, the previously noticed complete activity of mutants involving His^{233} (with the exception of His^{233} → Pro) appears difficult to interpret in view of the data presented here, as none of these mutants exhibited protease activity in this study. At this moment, we have no explanation for these discrepancies. Whereas it is conceivable that the mutant L chains could inhibit neurotransmitter release *in vivo* merely by physically binding to synaptobrevin, they could also possess more than just one activity. An activation of vesicular transglutaminase has been reported (31, 32); however, we do not know as yet whether our mutants would indeed retain this activity.

Table 1. Zinc content of the recombinant TeTx L chain and some inactive mutants

Designation	Sequence	% residual activity	Zinc content
rTeTx L chain	**His^{233}-Glu^{234}**-Leu-Ile-**His^{237}**-Val-Leu-**His^{240}**	100	1.16 ± 0.18
His^{233} → Val	**Val^{233}-Glu^{234}**-Leu-Ile-**His^{237}**-Val-Leu-**His^{240}**	0	0.21 ± 0.22
His^{233,237} → Val	**Val^{233}-Glu^{234}**-Leu-Ile-**Val^{237}**-Val-Leu-**His^{240}**	0	<0.06*
Glu^{234} → Gln	**His^{233}-Gln^{234}**-Leu-Ile-**His^{237}**-Val-Leu-**His^{240}**	0	1.27 ± 0.13

Wild-type and mutant TeTx L chains were expressed in *E. coli* M15 and chains were added at 100 nM. Percent residual activity was determined with radiolabeled *Aplysia* synaptobrevin as substrate. For zero value samples, no cleavage products were detectable after 16 h incubation at 37°C. Zinc content was determined by atomic absorption spectrophotometry of three samples. A zinc standard curve was established with a zinc standard solution. Boldface type indicates residues in the zinc-binding motif. *Below the parental cell background.

The precise function of synaptobrevin in the regulated exocytosis of neurotransmitters remains to be established. A TeTx-sensitive synaptobrevin homologue, termed cellubrevin, has recently been identified in vesicles of the constitutive pathway in all nonneuronal cells (33). Furthermore, Rothman and coworkers (9) identified synaptobrevin as a member of a protein complex consisting, in addition, of syntaxin and the synaptosomal-associated protein SNAP-25. This complex binds several cytoplasmic proteins, *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment proteins (SNAPs) required in a series of intracellular membrane fusion events. These latter proteins are highly conserved in evolution, with NSF and α-SNAP being the mammalian homologues of the yeast proteins SEC18p and SEC17p, respectively (34). Thus, members of the synaptobrevin family in association with SNAP-25 and syntaxin are likely to form the core of a vesicular docking/fusion complex that is structurally and functionally conserved in regulated and constitutive exocytosis. This complex may be further controlled by other proteins including synaptotagmin and neurexins (35, 36). By taking into account that SNAP-25 is selectively cleaved by BoNT/A (5), syntaxin is cleaved by BoNT/C1 (4), and synaptobrevin isoforms are hydrolyzed by TeTx, BoNT/B, BoNT/F, and BoNT/D (refs. 6–8 and 33 and this study), we may conclude that, during evolution, each of the clostridial neurotoxins has developed specificity for a subcomponent of a conserved protein complex controlling regulated and constitutive exocytosis in neuronal and nonneuronal cells.

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