Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus Toxin and Botulinum Neurotoxin Type A

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Hisao Kurazono‡, Sumiko Mochida§§, Thomas Binz**, Ulrich Eisel, Martin Quanz, Oliver Grebenstein, Karel Wernars‡‡, Bernard Poulain§§, Ladislav Tauč, and Heiner Niemann§§

From the Institute for Microbiology, Federal Research Center for Virus Diseases of Animals, Paul-Ehrlich-Strasse 28, Post Office Box 1149, D7400 Tübingen, Federal Republic of Germany, the 1Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, F31188 Gif-sur-Yvette, France, the Department of Physiology, Tokyo Medical College, 1-1 Shinjuku-6-chome, Shinjuku-ku, Tokyo 160, Japan, and the 2Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven 3720 BA, The Netherlands

To define conserved domains within the light (L) chains of clostridial neurotoxins, we determined the sequence of botulinum neurotoxin type B (BoNT/B) and aligned it with those of tetanus toxin (TeTx) and BoNT/A, BoNT/C1, BoNT/D, and BoNT/E. The L chains of BoNT/B and TeTx share 51.6% identical amino acid residues whereas the degree of identity to other clostridial neurotoxins does not exceed 36.5%. Each of the L chains contains a conserved motif, HExxHxxH, characteristic for metalloproteases. We then generated specific 5′- and 3′-deletion mutants of the L chain genes of TeTx and BoNT/A and tested the biological properties of the gene products by microinjection of the corresponding mRNAs into identified presynaptic cholinergic neurons of the buccal ganglia of Aplysia californica. Toxicity was determined by measurement of neurotransmitter release, as detected by depression of postsynaptic responses to presynaptic stimuli (Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., and Tauč, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7844–7848). Our studies allow the following conclusions. 1) Residues Cys140 of TeTx and Cys140 of BoNT/A, both of which participate in the interchain disulfide bond, play no role in the toxification reaction. 2) Derivatives of TeTx that lacked either 8 amino- or 65 carboxyl-terminal residues are still toxic, whereas those lacking 10 amino- or 68 carboxyl-terminal residues are nontoxic. 3) For BoNT/A, toxicity could be demonstrated only in the presence of added nontoxic heavy (H) chain. A deletion of 8 amino-terminal or 32 carboxyl-terminal residues from the L chain had no effect on toxicity, whereas a removal of 10 amino-terminal or 57 carboxyl-terminal amino acids abolished toxicity. 4) The synergistic effect mediated by the H chain is linked to the carboxyl-terminal portion of the H chain, as demonstrated by injection of Hc-specific mRNA into neurons containing the L chain. This finding suggests that the Hc domain of the H chain becomes exposed to the cytosol during or after the putative translocation step of the L chain.

Tetanus toxin (TeTx) and the seven serologically distinct botulinum neurotoxins, designated BoNT/A to BoNT/G, are synthesized as single-chain polypeptides (M, 150,000). Proteolytic activation generates di-chain species in which the L chains (Mr = 50,000) are disulfide linked to the corresponding H chains (Mr, 100,000) which provide the machinery for neuroselective binding, internalization, and translocation of the toxic L chains into the cytosol.

Although TeTx resembles BoNTs in regard to biosynthesis and molecular architecture (Fig. 1), the primary site of action differs for the two classes of neurotoxins, and therefore different clinical manifestations arise: the spastic disorder caused by TeTx is caused by a blockade of central synapses, but the flaccid paralysis observed in botulism involves inhibition of neuromuscular junctions. The toxic action has been proposed to develop in three consecutive steps (Simpson, 1980; Schmitt et al., 1981): binding of the toxins to neuronal membrane acceptors, internalization and intraneuronal targeting, and blockade of neurotransmitter release. The H chains bind to cellular receptors (Simpson, 1986) and mediate translocation of the corresponding L chains into the cytosol (Morris and Saelinger, 1989; Poulain et al., 1991). Although the molecular mechanisms(s) underlying toxification through the L chains remain obscure, it has been demonstrated convincingly that the L chains of TeTx and BoNT/A alone are sufficient to abolish catecholamine release from permeabilized bovine adrenal chromaffin cells used as a model for neuronal secretion (Ahnert-Hilger et al., 1989; Bittner et al., 1989). Central synapses in Aplysia have been found to be highly sensitive to clostridial neurotoxins (Poulain et al., 1988; 1989b; Mochida et al., 1989, 1990). As in vertebrate cells, a blockade of transmitter release was observed in the cholinergic synapses of buccal or in the non-cholinergic synapses of cerebral ganglia when the isolated L chain of TeTx was injected into presynaptic neurons (Mochida et al., 1989; Poulain et al., 1990). However, in this system toxicity of the L chains of BoNT/A or BoNT/B could be demonstrated only.

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§§ To whom correspondence and reprint requests should be addressed. Tel.: 7071-603-357; Fax: 7071-603-201.

1 The abbreviations used are: TeTx, tetanus toxin; BoNT/A--G, botulinum neurotoxin types A--G, respectively; L chain, light chain; H chain, heavy chain; Hc(BoNT/A), carboxyl-terminal fragment of the H chain comprising residues Asn152 to Leu199 of BoNT/A; ACh, acetylcholine; PCR, polymerase chain reaction.
FIG. 1. Common structure, biosynthesis, and nomenclature for clostridial neurotoxins and their subfragments. Single-chain toxins (sc-TeTx or sc-BoNT), as synthesized by Clostridium tetani or C. botulinum, are activated by bacterial or host proteases into their di-chain neurotoxic isoforms. Proteolytic digestion with specific proteases in vitro may generate L Hc, and Hc. Nomenclature according to Aguilar et al. (1992).

in the presence of the H chains which by themselves, however, are completely nontoxic (Poulain et al., 1988).

We have previously cloned and sequenced the structural genes of TeTx (Eisel et al., 1986), BoNT/A (Binz et al., 1990a), BoNT/C1 (Hauser et al., 1990), BoNT/D (Binz et al., 1990b), and BoNT/E (Poulet et al., 1992). In this study we present the sequence of the L chain of BoNT/B. An alignment of the individual L chain sequences reveals at least three highly conserved domains, suggesting that they should serve a conserved structural or biological function.

Each of the L chains contains, close to its COOH-terminal end, a single cysteine residue which mediates binding to the corresponding H chain. In this respect the clostridial neurotoxins resemble several postsynaptic snake venom neurotoxins all of which contain cysteine residues in this position (Endo and Tamiya, 1987). The L chain acts on permeabilized chromaffin cells only upon reductive separation from the H chain (Ahnert-Hilger et al., 1989; Stecher et al., 1989a), and reconstitution experiments between the two subchains indicated that the sulfhydryl group of this particular cysteine was indeed highly reactive (Weller et al., 1988). Furthermore, Schiaovo et al. (1990) have demonstrated that the interchain disulfide bond of TeTx is required for nerve cell penetration and that permeabilized cells are capable of reducing this bond. Therefore, it could be speculated that the newly generated free sulhydryl group within the carboxyl-terminal domain of the L chain could play a direct role in the intoxication process.

In this study we have applied amino- and carboxyl-terminal deletion mutants of the L chains of TeTx and BoNT/A to define the minimal essential domains required for toxicity. Furthermore, we demonstrate that the unexplained synergistic role of the botulinum H chain is mediated by its Hc portion.

MATERIALS AND METHODS

Cloning and Sequencing of Gene Fragments Encoding the L chains of BoNT/E and BoNT/B—Cloning and sequencing of the entire BoNT/E gene from Clostridium butyricum (strain Beluga) (DDBJ, EMBL, and GenBank nucleotide sequence data base accession number X62089) and from two strains of Clostridium butyricum (strains ATCC 43181 and 43755) (accession number X62088) have been published recently (Poulet et al., 1992). Cloning of chromosomal fragments from C. botulinum type B (strain Okra) encoding BoNT/B will be published elsewhere. To establish the sequence of the L chain, a 600-base pair Dral fragment was identified with a synthetic 23-mer oligonucleotide, 5'-AATATTATATGATATATGATCC-3', the sequence of which corresponded to a sequence published previously for the amino-terminal region of the L chain of BoNT/B (Schmidt et al., 1984). Hybridization conditions were as described (Binz et al., 1990a). Subsequent clones were obtained by PCR and characterized by direct sequencing of both strands using the chain termination method (Sanger et al., 1977).

Transcription Vectors Encoding L Chain Variants of TeTx and BoNT/A—To introduce a translational termination codon into the position exactly corresponding to the proteolytic cleavage site between the L and the H chains of TeTx, we applied site-directed mutagenesis (Taylor et al., 1985). A 1.9-kilobase EcoRI/HindIII fragment from pE97 (Eisel et al., 1986) was subcloned into M13mp19 (Yanisch-Perron et al., 1985) and the codons GCA (for Ala297) and TCA (for Ser356) were changed into GCC (no amino acid exchange) or GGC (to yield Gly356), respectively. The newly generated singular Ncol site was then used to insert the synthetic oligonucleotide 5'-CTGGATCACAT-3' providing an in-frame termination codon for translation, a singular BamHI site and an ATG codon for initiation of translation of the H chain. The orientation of the oligonucleotide was verified by sequencing. To generate an optimal Kozak's consensus sequence (Kozak, 1987) the SnaBI/BamHI fragment was treated with Klenow polymerase and cloned into the filled-in Accl site of the pSP64 vector (Promega, Madison, WI) to yield pEN14. Through this operation a G residue was brought into the -3 position upstream from the ATG codon, as desired for optimal initiation of translation, and the BamHI site was regenerated. The HindIII/BamHI fragment from pEN14 was inserted, ends were filled in with Klenow polymerase, and the fragment was then cloned into the EcoRV one of the pSP73 vector (Promega; Krieg and Melton, 1988), to yield pSA14 (Fig. 2A). This vector contained an additional terminator sequence providing termination codons in all three reading frames, and a poly(dA-dT) segment derived from an influenza A virus hemagglutinin gene (Min Jou et al., 1982) to allow for the synthesis of polyadenylated mRNA during in vitro transcription.

A similar vector, pBN1, was constructed for the synthesis of BoNT/A/L chain-specific mRNA. A chromosomal 1,480-base pair Sapl fragment (Binz et al., 1990a) was cloned into the EcoRV site of pSP73 (Fig. 2B).

Construction of Deletion Mutants—Synthetic oligonucleotides and PCR methodology were applied to generate specific 5'deletions in the coding regions for the L chains of TeTx and BoNT/A. For TeTx upstream primers were: 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', and 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', respectively. The newly generated singular Ncol site was then used to insert the synthetic oligonucleotide 5'-CTGGTATAATATCAGCTGAGGGTCC-3'. Translations derived from individual clones are characterized in greater detail below. The PCR products were digested with Hind111 and NcoI and ligated with the large fragment of pSA14 obtained by cleavage with the same enzymes.

For BoNT/A mutants the following synthetic oligonucleotides were used as upstream primers: 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', and 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', respectively. The newly generated singular Ncol site was then used to insert the synthetic oligonucleotide 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3', respectively. The newly generated singular Ncol site was then used to insert the synthetic oligonucleotide 5'-CAGGATCGACTCTAG-3'. Translations derived from individual clones are characterized in greater detail below. The PCR products were digested with Hind111 and Ncol and ligated with the large fragment of pSA14 obtained by cleavage with the same enzymes.

Polymerase Chain Reactions—PCR reactions were performed in a 50-μl reaction mixture containing 100 ng chromosomal DNA, 50 pmol each of the upstream and downstream primers, 250 μm each of the four deoxynucleotides, 5 units of Taq polymerase (Boehringer, Mannheim, Germany), and 1× reaction buffer (Boehringer, Mannheim, Germany). The following cycling conditions were used: an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. The PCR products were separated by electrophoresis on 1.4% agarose gel and visualized with ethidium bromide.

Recombinant Hc Fragment from BoNT/A—The synthetic oligonucleotide 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3' and 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3' were used to amplify a gene fragment encoding the entire Hc region from BoNT/A. Chromosomal DNA (4 μg) from C. botulinum (strain 62A) served as a template in the protocol detailed below. The PCR product was then treated with BamHI and cloned into the digested pPD109 vector. The correct sequence and orientation were confirmed by direct sequencing as well as by Northern blot hybridization using a 1.4-kilobase EcoRI fragment containing a synthetic oligonucleotide 5'-GAGGAGGCGTGCCTGGCTTTATAAATAATGATACA-3' as probe.
total volume of 50 µl containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 4 mM MgCl₂, 0.1% bovine serum albumin, a 100 µM concentration of each dNTP, 10 mM β-mercaptoethanol, 25 pmol of each primer, 2.5 units of Taq-polymerase (Boehringer, Mannheim, FRG), and 10 ng of pSA14 or pBN1 DNA previously linearized with XhoI. The reaction mixtures were denatured for 2 min at 95 °C and then subjected to 25 consecutive cycles consisting of denaturation (1 min at 93 °C), annealing (2 min at 55 °C), and polymerization (3 min at 73 °C). Amplified products were digested with the corresponding restriction endonucleases, purified by chromatography on QIAGEN columns (Diagen Dusseldorf, FRG), and characterized by DNA sequencing.

Preparation of L Chain-specific mRNAs—Protocols for in vitro synthesis of 5'-capped and 3'-polyadenylated RNA from recombinant pSP73 vectors have been published previously (Mayer et al., 1988). For the synthesis of radiolabeled TeTx LC-specific mRNA, pSA14 was linearized with XhoI or BamHI and transcribed with SP6 RNA polymerase (Bethesda Research Laboratories). For stability analyses of individual mRNA species, transcriptions were performed in the presence of 50 µCi of [α-32P]ATP (3,000 Ci/mmol; American Cyanamid).

Electrophysiological Analyses of Alterations Induced by Injection of L Chain-specific mRNA into Aplysia californica Presynaptic Neurons—Experiments were performed with buccal ganglia dissected from A. californica (Marinus Inc., Long Beach, CA) as described previously (Mochida et al., 1990). Briefly, preparations were pinned in a 1-ml experimental chamber and superfused continuously (10 ml/h and 3 ml/min during washing steps) with artificial seawater (460 mM NaCl, 10 mM KCl, 25 mM MgCl₂, 28 mM MgSO₄, 10 mM Tris/HCl, pH 7.8), except when the H chain of BoNT/A was added to the bath. Recordings were made from the couples of cholinergic neurons of the buccal ganglia which make well defined CI-dependent synapses (Tauc et al., 1974). Both pre- and postsynaptic neurons were impaled with two microelectrodes (3 M KCl, 1.5-4 meq/ml). The presynaptic cells were current clamped to ~50 mV and stimulated once/min. In this experimental setup, the amplitude of post-synaptic responses, as measured by conventional voltage-clamp technique and expressed as membrane conductances, is proportional to the amount of ACh released per impulse. mRNA (0.5 µg/µl) was injected into the presynaptic neuron by air pressure using an additional micropipette under visual and electrophysiological monitoring. To estimate the injected volume, the mRNA was mixed with a dye solution (1% (w/v) Fast Green FCF Sigma). The injected sample volume (<0.4 nl) was between 2 and 10% of the cell body volume leading to a maximal theoretical intracellular concentration of L chain mRNA of 100 nM. After injection, the injection micropipette was removed.

RESULTS AND DISCUSSION

Alignment of the L Chain Amino Acid Sequences of Various Clostridial Neurotoxins—We compared by visual inspection the newly established amino acid sequence of the L chain of BoNT/B with those of BoNT/D (Binz et al., 1990a), BoNT/C1 (Hauser et al., 1990), BoNT/A (Binz et al., 1990b), TeTx (Eisel et al., 1986), and with the recently determined sequences of BoNT/E, as derived from C. botulinum and C. butyricum (Poulet et al., 1992). The alignment in Fig. 3 shows at least four interesting features.

1. Three domains of increased homology can be coarsely defined based on the distribution of identical or similar amino acid residues. These domains comprise the 120 amino-termi- nally located residues, a central core region involving residues 186-241 of BoNT/B containing a highly conserved histidine-rich motif, HExxHxxH, characteristic for zinc-dependent metalloproteases, and a carboxyl-terminal domain consisting in the case of BoNT/B of residues 296-413. In metalloproteases, the first and the second histidine residue constitute zinc binding sites, whereas the glutamic acid residue provides the nuclease activity in the active center of the protease (Jongeneel et al., 1989). From studies designed to map the epitopes of monoclonal antibodies, we know that the conserved domains I and III fold by themselves into compact tertiary structures largely independent of the other two dom- ines. It remains to be shown whether carbonyl groups of residues Asp⁷⁶, Asp⁷⁷, Asn⁸⁵, Asp⁸⁶, or Glu³⁸⁶, Glu³⁸⁷, Asp³⁸⁸, Glu³⁸⁹, Asp³⁹⁰ of BoNT/B contribute to binding of Ca²⁺ ions, as observed in several thermolysin-related proteases (Stoeva et al., 1980). Furthermore, although it has been demonstrated

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**Fig. 2.** Transcription vectors used for in vitro synthesis of mRNA encoding the L chains of TeTx (A) or BoNT/A (B) or various deletion mutants. Sequences of the 5'- and 3'-noncoding regions are shown in greater detail. Polyadenylation was guaranteed by the presence of a poly(dA-dT) segment located between the XhoI and the HindIII sites. This fragment was isolated from a cDNA clone encoding the hemagglutinin of an influenza virus (Min Jou et al., 1980).
2. The L chain of BoNT/B is most closely related to that of Te'Tx (51.6% identity, Table I). This is in keeping with previous reports documenting that in the mouse motor nerve terminal Te'Tx and BoNT/B display electrophysiologically very similar inhibitory effects on the spontaneous and nerve-evoked transmitter release. Furthermore, the two toxins show similar sensitivity to 4-aminoypyridine (Gansel et al., 1987). Based on these and similar data, it has been suggested that Te'Tx or BoNT/B on the one side and BoNT/A on the other act on different intracellular targets (Gansel et al., 1987; Dreyer et al., 1987; Simpson et al., 1988). This is further supported by the finding that the L chains of both Te'Tx and BoNT/B exhibit only about 32% identity in comparison with BoNT/A (Table I). It should be noted, however, that the high degree of relatedness between Te'Tx and BoNT/B only applies to the L chains and does not extend into the H chains where the degree of identity amounts to only 37.4% (data not shown).

3. Only 7 identical residues are found within the 55 carboxyl-terminal amino acids of the individual L chains. This low degree of identity continues within the amino-terminal portion of the individual H chains (not shown). It is, therefore, tempting to speculate that the nonessential carboxyl-terminal portion of the L chains merely serves a spacer function to provide flexibility for the postulated translocation of the L chains through an H chain-specified pore (Boquet and Duflot, 1982; Boquet et al., 1984; Shone et al., 1987; Hoch et al., 1985; Donovan and Middlebrook, 1986). The hinge regions are exposed at the surface of the toxin molecules. This would explain why this region is accessible for a variety of proteases (Weller et al., 1988; Dekleva and DasGupta, 1990).

Stability of Microinjected mRNA—Previous studies (Mochida et al., 1990) have shown that the molecular genetic approach involving microinjection of in vitro generated mRNA into identified presynaptic neurons of A. californica could provide a safe experimental access to gain further insights into the mechanism(s) by which the neurotoxins block neurotransmitter release. We were initially concerned about the stability of microinjected mRNA derived from clostridial genes containing about 73% A+T in the coding and about 80% in the noncoding regions (Binz et al., 1990a, 1990b). Several studies on rapidly degraded mRNA have indicated that such degradation appears to be controlled by the presence of conserved A+U-rich sequences within the 3'-noncoding region (Brewer and Ross, 1988; Shaw and Kamen, 1986; Wilson and Treisman, 1988). The mRNA encoding the L chain of Te'Tx, for instance, contained within its 1,371 nucleotides eight copies of the motif 5'-AUUAUA-3' which has been proposed to constitute recognition sequences for an mRNA processing pathway that specifically degrades the mRNAs of

FIG. 3. Alignment of amino acid sequences of various clostridial neurotoxin L chains. The L chain sequences of BoNT/D (Binz et al., 1990b), BoNT/C1 (Hauser et al., 1990), BoNT/A (Binz et al., 1990a), Te'Tx (Eisel et al., 1988), BoNT/B (this study), BoNT/E from C. butyricum (strain Beluga) or C. botulinum (Butyr, ATCC strains 43755 and 43181, Poulet et al., 1992) were aligned by visual inspection. Identical or related residues are boxed. Cysteine residues involved in the disulfide linkage between the L and the H chains of the individual toxins can be aligned. This results in nicking regions of various length comprising 8 amino acid residues in BoNT/B and 27 residues in Te'Tx. Analyses of the unnicked single-chain toxins for surface probability suggested, however, that in all instances the hinge regions are exposed at the surface of the toxin molecules. This would explain why this region is accessible for a variety of proteases (Weller et al., 1988; Dekleva and DasGupta, 1990).
certain lymphokines, cytokines, and protooncogenes (Shaw and Kamen, 1986).

To see whether polyadenylation stabilized the mRNA encoding the L chain of TeTx, we linearized pSA14 either with \textit{BamHI} or with \textit{HindIII}, i.e. immediately upstream or downstream from the \textit{dA-dT} segment, respectively (Fig. 2A). Transcripts labeled with [\textit{\alpha}-\textit{\textsuperscript{32}P}]adenosine were separated from nonincorporated nucleotides by passage over a Sephadex G-50 spin column. They were then precipitated, dissolved in 10 mM TE buffer, pH 7.4, and microinjected into \textit{Xenopus laevis} oocytes. This system has been used previously by Drummond et al. (1985) to assay the stability of mRNAs exhibiting a balanced G+C content. Total RNA was reisolated at 1, 3, and 17 h after injection and analyzed by electrophoresis on a 2% formaldehyde gel and subsequent autoradiography (Fig. 4, \textit{lanes} 1–6). Polyadenylation clearly increased the stability of the microinjected RNA. This finding is in keeping with our observation that we were unable to detect any depression of postsynaptic responses in the \textit{Aplysia} system (see below) when mRNA was injected which lacked the poly(A) tail. Fig. 4 further demonstrates that mRNA encoding the deletion mutants TeTx'3'89 \textit{(lanes} 8 and 9) or TeTx'5'10 \textit{(lanes} 10 and 11) were not degraded within 3 h. None of the injected oocytes exhibited detectable morphologic alterations at 17 h after injection that could be assigned to toxic effects of newly synthesized gene products.

Characterization of the Individual Deletion Mutants by Combined In Vitro Transcription/Translation—To verify that the mRNAs obtained by \textit{in vitro} transcription of the individual 5'- and 3'-deletion mutants yielded polypeptides of the expected size, we performed translations in rabbit reticulocyte lysate in the presence of [\textit{\textsuperscript{35}S}]methionine and analyzed the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results obtained for the COOH-terminal L chain deletion mutants of TeTx and BoNT/A are summarized in Fig. 5. Unlabeled TeTx and BoNT/A were applied as controls. TeTx was prepared from single-chain TeTx by treatment with 1 \textit{µg/ml} \textit{Staphylococcus aureus} protease V8 as described by Weller et al. (1988). As shown in Fig. 5A the major translation products obtained from wild type TeTx L chain mRNA \textit{(lane} 3) had an electrophoretic mobility like authentic L chain of TeTx. The lower molecular weight material observed in translations of 3'-truncated mRNAs of TeTx and BoNT/A could either result from ribosomal slippage and internal initiation of translation or specific proteolytic degradation within the amino-terminal domain of the L chains. In principal, similar results were obtained with mRNA encoding various 5'-deletion mutants. Again, lower molecular weight species were observed (data not shown). In each case, however, the largest species was the most abundant product and the molecular weight of which corresponded to that listed in Table II.

\textit{Inhibition of Neurotransmitter Release from Presynaptic Neurons of \textit{A. californica} upon Injections of L Chain-specific mRNAs—Central synapses of \textit{A. californica} have been used extensively to study the action of clostridial neurotoxins (Mochida et al., 1989; Poulain et al., 1988, 1989a, 1989b). These studies have indicated that TeTx or BoNT/A, B, or E at nanomolar concentrations, when injected into presynaptic neurons, induced a depression of postsynaptic responses caused by inhibition of ACh release from the presynaptic neurons, beginning with a short delay of a few min. As shown in Fig. 6A, injection of mRNA derived from pSA14 (encoding the entire L chain of TeTx) also depressed ACh release, however, only after a delay of about 5 min. This delay has been ascribed to the time required for translation of the mRNA, diffusion and accumulation of the translation product within the anticipated site of action in the terminal (Mochida et al., 1990). Stimulation of the \textit{noninjected} presynaptic neuron afferent to the same postsynaptic neuron continued to cause about the same postsynaptic response amplitudes after 200 min (Fig. 6A, open circles) or even after overnight incubation (not shown) excluding the possibility that the postsyn-
carboxyl-terminal deletion mutants of the L chains of TeTx. Polyacrylamide gels were performed as described (Mayer et al., 1988). The translation products and analyses of the translation products on 12 sodium dodecyl sulfate-polyacrylamide gels were performed as described (Mayer et al., 1988).

The H chain (Krieglstein et al., 1989) is completely abolished toxicity (Table I). Similar depression curves were obtained with several carboxyl-terminal deletion mutants including TeTx3′392 (Table I; Fig. 6B, filled circles). This mutant lacked 65 carboxyl-terminal residues of the TeTx L chain including the cysteine residue that had been shown to be involved in the linkage to the H chain (Krieglstein et al., 1990). Apparently, toxicity of this derivative was only slightly reduced, as judged from the time required to reach 50% blockade of neurotransmission. However, the additional deletion of Thr3′63, Asp391, and Asp390 in TeTx3′389 (Fig. 6B, open circles) or of further residues, as is the case in the derivatives TeTx3′381 and TeTx3′356, completely abolished toxicity (Table II).

A similar set of experiments was performed with various carboxyl-terminal deletion mutants of the L chain of BoNT/A (Table II). In accordance with previous data (Mochida et al., 1990), postsynaptic responses remained unchanged after the injection of BoNT/A L chain-specific mRNA. Only when the H chain (50 nM) was added to the bath, an immediate onset of depression was observed (Fig. 7A). These findings support previous conclusions that in Aplysia both the L and the H chain of BoNT/A are required to generate toxicity (Poulain et al., 1988). However, it must be stressed that neither bath-applied nor internally applied H chain caused any change in the postsynaptic response even when applied at concentrations of up to 125 nM (Maisey et al., 1988; Poulain et al., 1990).

Theoretically, the pBN1-derived mRNA should yield a translation product that contains the entire L chain, 46 amino acid residues from the Hx region and 12 foreign residues specified by the vector (Table II). The conclusion that such additional carboxyl-terminal sequences do not interfere with toxicity, however, is not justified for two reasons. First, we cannot exclude proteolytic processing of the primary translation product at the nicking site. Second, because of the extreme neurotoxicity, undetectable amounts of truncated peptides arising from premature termination of translation could account for the toxic effects.

A deletion of the 65 carboxyl-terminal amino acids from the L chain of BoNT/A, as present in BoNT/A3′384, abolishes toxicity (Fig. 7C), whereas a derivative that is 22 residues longer retains full activity (Fig. 7B). From the deletion mutants summarized in Table II we can conclude that Cys439 of TeTx and Cys439 of BoNT/A are not required to evoke neurotoxicity. This finding argues against the possibility that these cysteines have to become posttranslationally modified, for instance by polyisoprenylation, as observed for a variety of biologically active molecules such as the ras and ras-related small GTP-binding proteins (Hancock et al., 1989) or the
nuclear lamins (Krohne et al., 1989). Furthermore, these data exclude any role for the conserved cysteine residues either in the toxification process itself, in a potential attachment to the interaction with an, as yet undefined, cellular cofactor.

We then analyzed various amino-terminal deletion mutants, as generated by PCR of the corresponding genes. The results are summarized in Table II and in Fig. 8. Whereas a deletion of 8 residues is tolerated in both the L chains of TeTx and BoNT/A without affecting their toxicity, a deletion of the conserved tyrosine residue in position 10, or of additional amino acid residues (Table II), completely abolished toxicity (Fig. 8). At present it is unclear whether Tyr<sup>10</sup> plays a role in merely stabilizing the tertiary structure of the individual L chains. Alternatively, this residue could be involved in binding to the putative subcellular target molecule or in the interaction with an, as yet undefined, cellular cofactor.

In general, the above conclusions are based on the assumption that the various translated protein products are equally resistant against proteolytic degradation in the microinjected Aplysia neuron. Since we were as yet unable to detect the corresponding polypeptides by Western blotting, processing of the gene products cannot be excluded.

**The HC Domain of the H Chain of BoNT/A Synergistically Activates the L Chain**—The well documented synergistic function of the botulinic H chains in the Aplysia system (Maisey et al., 1988; Poulain et al., 1988) is not understood, even more so since several reports have demonstrated that the same preparations of botulinic L chains alone are blocking transmitter release when introduced into the cytosol of bovine chromaffin cells (Bittner et al., 1989; Stecher et al., 1989b), PC12 cells (McInnes and Dolly, 1990), or into mammalian
nerve terminals (de Paiva and Dolly, 1990). The Aplysia-restricted helper function mediated by the botulinum H chain has been assigned to its Hc domain based on the following lines of indirect evidence: L-Hc of BoNT/A, a proteolytic cleavage product isolated from the di-chain toxin (Fig. 1), was found to be inactive in Aplysia neurons, no matter whether applied extra- or intracellularly (Poulain et al., 1989a). Neurotoxicity was restored, however, by bath application or microinjection of the entire H chain from BoNT/A, but not from TeTx (Poulain et al., 1990). Unfortunately, the contribution of Hc to the toxification process could not be assessed directly because of practical problems associated with the isolation of this peptide fragment in its native form (Shone et al., 1985). To overcome this problem, we generated a DNA fragment by PCR that encoded the entire Hc region (residues Asn872 to Leu1286) of the BoNT/A sequence published by Binz et al., 1990(a). The PCR product was resequenced and analyzed by combined in vitro transcription/translation. A single molecular species (Mr, 45,000) was obtained (data not shown). We then applied the corresponding mRNA in microinjection experiments, as shown in Fig. 9. One of the two identified presynaptic cholinergic neurons (pre 1) was first injected with the purified L chain of BoNT/A (20 nM calculated intracellular concentration). As expected, no inhibition of neurotransmitter release was observed within 90 min after injection (small filled squares). At this time point the Hc-specific mRNA was injected into the same neuron (pre 1). After a delay of about 35 min an onset of depression was observed indicating that, indeed, the Hc fragment mimicked the effects previously provided by the entire H chain. As a control, we first injected the second presynaptic neuron (pre 2) with Hc-specific mRNA. Evoked transmitter release remained unaltered for 2 h indicating that the Hc peptide by itself generated no neurotoxic effects (Fig. 8, large open squares). The subsequent injection of purified BoNT/A L chain into the same neuron caused an immediate onset of depression of the postsynaptic response.

Together, these data clearly demonstrate that in the Aplysia neurons the Hc domains of BoNT/A has to be exposed in the cytosol in order to allow the BoNT/A L chain to block neurotransmitter release. It has frequently been argued that the Aplysia synapses were too distant from vertebrate synapses on the evolutionary scale and that, therefore, the former synapses lacked a property or function being provided by the vertebrate nerve terminals. Indeed, the synergistic role of the Hc fragment increases a number of questions.

1. How does the Hc domain reach the cytosol? Does such translocation of Hc also occur in the neuron of higher organisms? At present, we can only speculate about such mechanism. If we assume that internalization of BoNT/A into Aplysia neurons takes place by receptor-mediated endocytosis, as documented for the uptake into motor nerves (Black and Dolly, 1986), the intact toxin molecule should then become delivered via an endosomal compartment into smooth-walled vesicles characterized by an internal low pH. Under these conditions the H chain of clostridial neurotoxins were reported to form pores (Boquet and Duflot, 1982; Boquet et
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