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TALKING POINT

Tetanus and botulism neurotoxins: a new group of zinc proteases

Cesare Montecucco and Giampietro Schiavo

TETANUS AND BOTULINUM neurotoxins are the most potent toxins known (mouse lethal dose <0.1 ng kg⁻¹). They are released by bacteria of the genus *Clostridium* as a single polypeptide chain of 150 kDa, later cleaved to generate two disulfide-linked fragments. The heavy chain (H, 100 kDa) is involved in cell binding and penetration, while the light chain (L, 50 kDa) is responsible for the intracellular activity^{1,2}.

The tremendous potency of these toxins is primarily due to their absolute neurospecificity. They bind to the neuromuscular junction and are internalized inside vesicles³. Botulinum neurotoxin (BoNT, seven different serotypes: A–G) penetrates into the cytosol and blocks the release of acetylcholine, thus causing a flaccid paralysis. By contrast, tetanus neurotoxin (TeTx) migrates retroaxonally (in the reverse direction to nerve impulses) and, by transcytosis, reaches the spinal inhibitory interneurons, where it blocks neurotransmitter release with a consequent spastic paralysis⁴. Despite the opposing clinical symptoms of botulism and tetanus, these toxins act in a similar way at the cellular level. Since TeTx and BoNT are entirely responsible for the diseases tetanus and botulism, respectively, understanding their intracellular activity would also lead to the description of the molecular pathogenesis of these diseases. At the same time, we would expect to understand better the process of neuroexocytosis.

The active forms of tetanus and botulinum neurotoxins, released from the precursor molecule by specific proteolysis and reduction, block the release of neurotransmitters via a Zn²⁺-dependent protease activity. VAMP/synaptobrevin, an integral membrane protein of the synaptic vesicles, is cleaved at a single site by tetanus and botulinum B, D and F neurotoxins. The unique sequence, mechanism of activation and site of activity of clostridial neurotoxins mark them out as an independent group of Zn²⁺-endopeptidases.

Tetanus and botulinum neurotoxins are Zn²⁺ proteins

The enormous potency of these neurotoxins, and analogy with other bacterial toxins with intracellular activity, suggests that TeTx and BoNT possess or induce a catalytic activity inside cells.

Their amino acid sequences show a limited degree of similarity, concentrated mainly in the L chain. The segment with the closest homology is located in the central part and contains the Zn²⁺-binding motif, HEXXH, that is a characteristic feature of Zn²⁺ endopeptidases^{4–12}. Atomic absorption measurements showed that indeed TeTx and BoNT/A, B, C, E and F contain approximately one Zn²⁺ ion bound to the L chain^{5–7}. As with other Zn²⁺ proteases, the Zn²⁺ ion can be removed with EDTA or ortho-phenanthroline, thus forming

an apotoxin, and it can be replaced by incubation in Zn²⁺-containing buffers^{5–7}.

The three-dimensional structures of four Zn²⁺ endopeptidases have been solved by X-ray diffraction. In thermolysin¹³, neutral protease¹⁴ and *Pseudomonas aeruginosa* elastase¹⁵, the Zn²⁺ ion is at the center of a tetrahedron, coordinated by the two histidine residues of the motif, by one water molecule that is bound to the glutamate residue of the motif, and by the carboxylate group of another glutamic acid residue. By contrast, the recently reported structure of astacin¹⁶, a Zn²⁺ protease of the crayfish digestive tract, shows the Zn²⁺ ion penta-coordinated by the two histidine residues, the water molecule that is bound to the glutamate residue of the motif, an additional histidine residue, and a tyrosine residue. On the basis of these findings, Zn²⁺ endopeptidases

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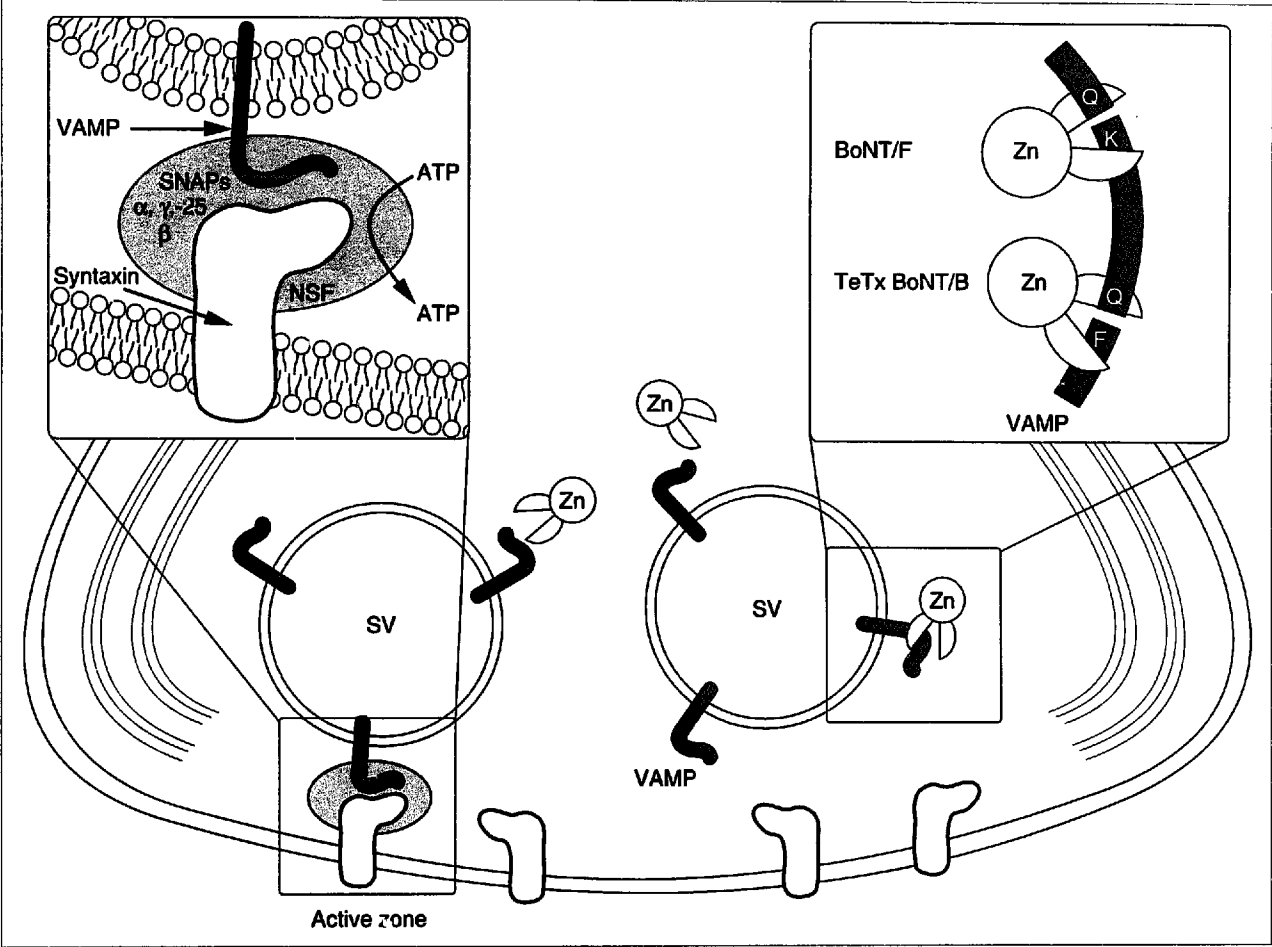


Figure 1

Scheme of the intracellular Zn^{2+} endopeptidase activity of tetanus and botulinum B, D and F neurotoxins. Docking of synaptic vesicles to the active zones of the presynaptic membrane results from the specific recognition between VAMP/synaptobrevin and syntaxin mediated via multiple SNAP factors and NSF. Zn^{2+} -dependent proteolytic cleavage of VAMP by the neurotoxins releases the amino-terminal part of VAMP to the cytosol and impairs docking and fusion of the vesicles with the presynaptic membrane. Neuroexocytosis is thus blocked. The toxin light chain is represented as scissors; SV, synaptic vesicles.

have been divided into two main categories: thermolysin-like and astacin-like. This latter group was further divided into four different subgroups on the basis of sequence similarities¹⁷.

Chemical modification with diethylpyrocarbonate (a specific modifier of histidine residues) indicates that two, rather than three, additional histidine residues are made available upon Zn^{2+} removal in TeTx and BoNT/A, B and E^{5,6}. Moreover, Co^{2+} -substituted TeTx shows spectroscopic properties more similar to those of Co^{2+} -thermolysin than to Co^{2+} -astacin (G. Schiavo *et al.*, unpublished). A comparison of the amino acid sequences of the neurotoxins with those of thermolysin and astacin reveals three segments of low but significant similarity between thermolysin and the clostridial neurotoxins, and the absence of the glycine residue

of astacin (Gly99) that is essential to bring the third histidine residue close to the Zn^{2+} ion. Although these data suggest that clostridial neurotoxins resemble thermolysin more than astacin, clearly more conclusive data are needed to establish their mode of Zn^{2+} coordination.

Blockage of neurotransmitter release

The best characterized and experimentally flexible neuronal system presently available to test the activity of clostridial neurotoxins is the buccal ganglion of the marine snail *Aplysia californica*^{18,19}. Toxin injection into selected neurons of this ganglion showed that TeTx-induced blockage of neurotransmitter release depends on the presence of a Zn^{2+} ion bound to the toxin. Moreover, phosphoramidon, a well-characterized inhibitor of Zn^{2+} endopepti-

dases²⁰, also inhibits the intraneuronal activity of TeTx and BoNT/A, B and E⁵. These results provide indirect but compelling evidence that clostridial neurotoxins block the neurotransmitter release via a Zn^{2+} -dependent endopeptidase activity. Moreover, they suggest that specific inhibitors might be found that are able to cross membranes; these would be potential therapeutic agents for tetanus and botulism.

Cleavage of VAMP/synaptobrevin

An intense search for a proteolytic substrate for clostridial neurotoxins among different subcellular fractions of nervous tissue and of chromaffin and PC12 cells has only identified one candidate. It was found that TeTx, BoNT/B and BoNT/F specifically cleave vesicle-associated membrane protein (VAMP)/synaptobrevin at a single site^{5,7,21}

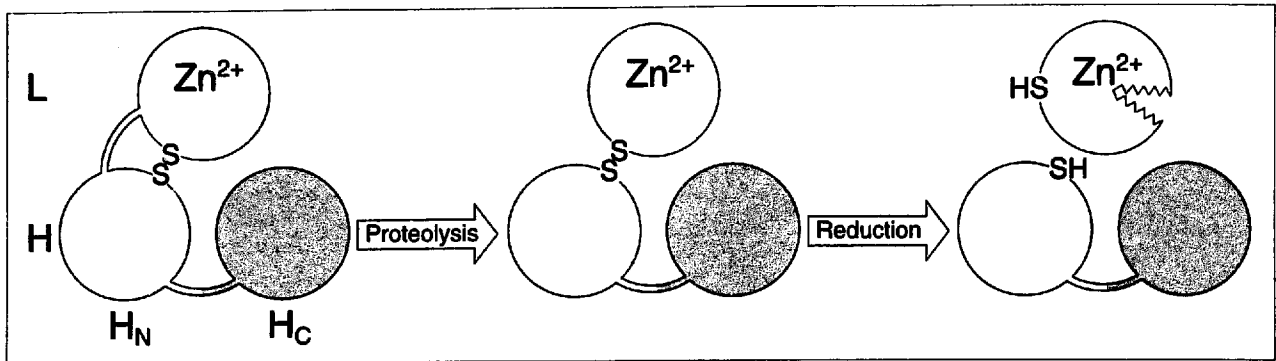


Figure 2

Scheme of the activation mechanism of the neurotoxins responsible for tetanus and botulism. The toxin is produced as a single polypeptide chain of 150 kDa which is later cleaved at a single site by tissue or bacterial proteases. The toxin is depicted as a tripartite structure with the different domains connected by exposed protease-sensitive loops on the basis of biochemical and structural data^{1,38}. The two chains are held together by a single interchain disulfide bond, which has to be reduced to free the Zn^{2+} endopeptidase activity of the light chain (L). The H_C domain of the H chain is mainly responsible for the neurospecific binding, while the H_N domain appears to be involved in the neuronal penetration of the L chain.

(Fig. 1). VAMP was first identified in the *Torpedo* electric organ²² and later cloned from a variety of species^{23–26}. It is a highly conserved, 13 kDa integral membrane protein of small synaptic vesicles with 90% of its mass exposed to the cytosol. There are two isoforms of VAMP in the rat CNS²³. TeTx and BoNT/E cleave only rat brain VAMP-2 at the Gln76–Phe77 peptide bond²¹, while VAMP-1 is resistant, presumably because it contains a valine residue in place of the glutamate residue of VAMP-2. BoNT/F cleaves both brain VAMP isoforms at their unique Gln–Lys peptide bond, while BoNT/D hydrolyses the adjoining Lys–Leu peptide bond (Ref. 7; G. Schiavo *et al.*, unpublished). This high specificity of action of TeTx, BoNT/B, BoNT/D and BoNT/F is unique among Zn^{2+} endopeptidases.

BoNT/A, BoNT/C and BoNT/E are ineffective on VAMP. This result is in keeping with previous electrophysiological data indicating a common site of action for TeTx, BoNT/B, D and F and a different one for BoNT/A and E^{27,28}.

VAMP is cleaved by TeTx, BoNT/B, BoNTD and BoNT/F only after specific proteolysis and reduction of the single-chain toxins²¹, as shown in Fig. 2. Hence, clostridial neurotoxins follow the general mechanism of activation of all bacterial protein toxins with intracellular targets: they need to be nicked at an exposed peptide segment connecting the catalytic protomer to the cell-binding protomer, and reduced to free the catalytic moiety.

Very recently Sollner *et al.*²⁹ have provided evidence that VAMP is implicated in the docking of synaptic ves-

icles via the specific recognition of syntaxin³⁰, an integral protein of the presynaptic membrane, mediated by a set of soluble factors (NSF, *N*-ethylmaleimide-sensitive factor and SNAPs, soluble NSF accessory proteins). Moreover, they have suggested that NSF, SNAPs, VAMP-like and syntaxin-like proteins may be involved in all intracellular vesicle recognition and fusion events^{29,31}. Hence it is not surprising that VAMPs are not only present in nerve and neuroendocrine tissues³², but also in exocytic vesicles of adipocytes³³, as well as in many other tissues (O. Rossetto *et al.*, unpublished). The findings of Sollner *et al.*²⁹ also suggest that the SNAPs or NSF are the target of the proteolytic action of BoNT/A, C and E.

Four pieces of evidence indicate that VAMP proteolysis is implicated in the blockade of neurotransmitter release by TeTx and BoNT/B: (1) the intraneuronal activity of the toxins is inhibited by highly specific inhibitors of Zn^{2+} endopeptidases; (2) only VAMP is cleaved in synaptosomes incubated with TeTx³⁴; (3) peptides spanning the cleavage site of VAMP inhibit TeTx and BoNT/B injected into *Aplysia* neurons²¹; and (4) an antibody highly specific for VAMP/synaptobrevin prevents the blockage of neurotransmitter release in injected *Aplysia* neurons of TeTx and BoNT/B, but not that of BoNT/A (B. Poulain *et al.*, unpublished). As summarized in the accompanying paper (see p. 327), Facchiano *et al.*^{35,36} have recently reported that TeTx binds and activates synaptic transglutaminase and that synapsin³⁷ is an excellent transglutaminase substrate.

These results clearly indicate that VAMP is a major target of the intraneuronal activity of TeTx, BoNT/B, BoNT/D and BoNT/F, although they do not rule out the presence of other protein substrates inside neurons.

Concluding remarks

Clostridial neurotoxins constitute a new group of Zn^{2+} endopeptidases with peculiar properties:

- (1) they are produced as inactive precursors which need proteolysis and reduction to display their proteolytic activity (see Fig. 2);
- (2) their amino acid sequence around the Zn^{2+} -binding motif does not resemble any of the various groups of Zn^{2+} endopeptidases¹⁶;
- (3) they are completely inactive outside cells, while the large majority of Zn^{2+} proteases are not;
- (4) they are very specific in terms of both protein target and peptide bond cleaved;
- (5) they act on an organelle on which no toxin had been shown to act before.

Acknowledgements

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THE STUDY OF the mechanism of action of toxins such as bungarotoxin, tetrodotoxin, cholera toxin and pertussis toxin has contributed significantly, and sometimes decisively, to the understanding of the cellular functions that these toxins disrupt. It is thus not surprising that the clostridial tetanus and botulinum neurotoxins, which very potently and selectively block exocytic neurotransmitter release, have attracted the attention of neurobiologists interested in exocytosis.

These toxins are synthesized as single 150 kDa polypeptides and are then proteolytically nicked to the active forms, in which the two resulting peptides (heavy and light chain) are held together by disulfide and noncovalent bonds¹. The action of tetanus toxin (TeTx) is initiated by injecting the light chain into the cytoplasm of nerve terminals, which then inhibits the exocytic fusion of synaptic vesicles with the plasma membrane (intoxication step)¹.

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The transglutaminase hypothesis for the action of tetanus toxin

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Tetanus toxin potently and almost irreversibly inhibits the release of neurotransmitters from nerve terminals. The toxin binds to and activates transglutaminase, a Ca^{2+} -dependent enzyme that can form stable crosslinks between substrate proteins. Transglutaminase is present in nerve terminals and recognizes synapsin I, an abundant synaptic vesicle phosphoprotein involved in neurotransmission, as an excellent substrate. The neuromuscular action of tetanus toxin might be due, at least in part, to the stimulation of synaptic transglutaminase and the consequent crosslinking of synapsin I.

The extreme selectivity of TeTx for nerve terminals is believed to be due to the presence of specific membrane receptors, and is lost when the toxin is administered intracellularly, for instance in chromaffin cells^{2,4} or PC12 cells⁵. Thus, the intracellular effect of TeTx appears to be similar in neuronal and non-neuronal secretory cells.

Specific features of the intoxication process, namely, its occurrence at extremely low concentrations of toxin (only a few light-chain molecules per synapse are sufficient for neuromuscular paralysis), its irreversibility, and the dependence of its onset on time, temperature and nerve stimulation (therefore possibly on $[\text{Ca}^{2+}]_i$), have suggested that the

toxin possesses or stimulates a Ca^{2+} -dependent enzymatic activity which would lead to inhibition of secretion¹. Clues as to the nature of this enzymatic activity have recently come from the inspection of the primary structure of TeTx. More than one hypothesis has, in fact, originated from this approach. The toxin's amino acid sequence contains, in addition to the signature motif of metalloendopeptidases^{6–9} (see the companion paper by Schiavo and Montecucco), a stretch of amino acids showing a relatively high degree of homology with known substrate sites of the enzyme transglutaminase (TGase)¹⁰. TGase complies with various requirements for an enzyme mediating the