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Botulinum Neurotoxins Are Metalloproteases Specific for SNARE Proteins Involved in Neuroexocytosis

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The 7 botulinum neurotoxins (BTX, serotypes indicated with letters from A to G) and the single tetanus neurotoxin (TTX) constitute a group of metalloproteases endowed with several unique properties. They are very specific for presynaptic nerve terminals because they only bind to receptors localized on the presynaptic membrane. TTX blocks neuroexocytosis from the inhibitory interneurons of the spinal cord causing the spastic paralysis of tetanus. At variance, BTXs block the release of acetylcholine from both skeletal and autonomic cholinergic nerve terminals causing the flaccid paralysis of botulism. These bacterial metalloproteases do not act on the cell surface, but exert their enzymatic action in the cell cytosol on selected proteins which form the core of the neuroexocytosis machinery. As a net result of such a double specificity of binding and of substrate and of their catalytic activity, these neurotoxins are the most poisonous substances known with a mouse LD_{50} of few picograms [1].

Their structural architecture in 3 domains (L, H_N and H_C) endowed with different functions is strictly linked to their mechanism of cell intoxication which consists of 4 steps [2, 3]. Nerve cell binding mediated by H_C (step 1) is followed by internalization inside vesicles (step 2), which are endowed with an ATPase proton pump. The acidification of the vesicular lumen causes a rearrangement of the toxin structure which inserts H_N in the membrane and translocates L in the cytosol (step 3), where it displays its proteolytic activity (step 4).

Neurospecific Binding

Available evidence indicates that the H_C domain of these neurotoxins plays a major role in neurospecific binding [4, 5]. However, it appears that additional

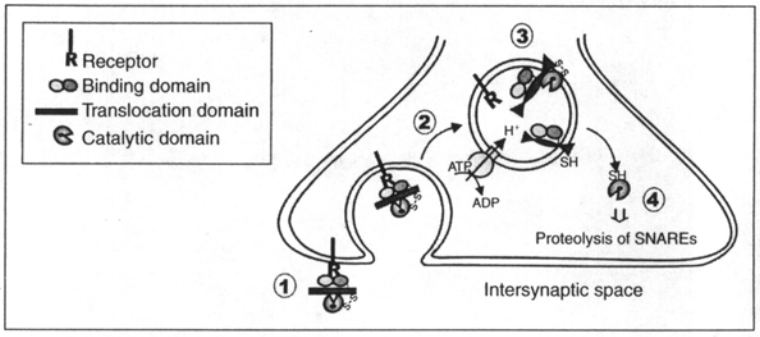

Fig. 1. Entry of BTXs inside nerve terminals. 1 = BTXs bind to the presynaptic membrane at as yet unidentified receptors of peripheral nerve terminals; 2 = binding is followed by internalization inside endocytic vesicles, whose lumen becomes acid following the activity of an ATPase proton pump; 3 = at low pH, BTXs change conformation, insert themselves into the lipid bilayer of the vesicle membrane and translocate the L chain into the cytosol; in contrast, not shown here, TTX is internalized and transported retroaxonally to the spinal cord where it enters the inhibitory interneuron terminals as depicted here for the BTXs at cholinergic peripheral terminals; 4 = inside the cytosol, the L chain catalyzes the proteolysis of 1 of the 3 SNARE proteins.

toxin regions are also involved in binding in as much as H_C provides only a partial protection from intoxication with the intact molecule.

The molecular structure of the presynaptic receptor(s) of BTXs is not known. Polysialogangliosides are certainly involved in binding, but there is evidence that unidentified protein(s) of the cell surface play a role in the process [4, 6, 7]. The presence of both lectin-like and protein-binding subdomains in the H_C domain of BTXs [8] supports the suggestion that clostridial neurotoxins bind strongly and specifically to the presynaptic membrane because they display multiple interactions with sugar and protein binding sites, as previously suggested [9] .

Internalization Inside Neurons and Entry in the Neuronal Cytosol

Binding is followed by endocytosis inside intracellular vesicular compartments via a temperature- and energy-dependent process [10]. The nature of these compartments has not been established, but there is evidence that their lumen is acidic and that low pH is essential for the translocation of the L chain across the vesicle membrane, as depicted in figure 1 [11–13]. BTXs undergo a low-pH-driven conformational change from a water-soluble 'neutral' structure to an 'acid' structure capable of inserting itself in the hydrocarbon core of the lipid bilayer [8].

Botulinum Neurotoxins Are Metalloproteases Specific for the SNARE Proteins

The 7 BTXs are remarkably specific proteases. Among the many proteins and synthetic substrates assayed so far, only 3 targets, the so-called SNARE proteins (soluble n-ethylmaleimide-sensitive fusion protein accessory protein receptor; SNARE defines proteins involved in vesicular trafficking and fusion with target membranes within the cell) have been identified (fig. 2). BTX types B, D, F and G cleave vesicle-associated membrane protein (VAMP)/synaptobrevin, each at a single site (table 1). At variance, BTX-A and -E cleave a 25-kD synaptosome-associated protein (SNAP-25) each at a single site while BTX-C cleaves both syntaxin and SNAP-25 [8]. Strikingly, TTX and BTX-B cleave VAMP at the same peptide bond (Gln76–Phe77) and yet when injected into an animal they cause the opposite symptoms of tetanus and botulism, respectively [14], conclusively demonstrating that the different symptoms of the two diseases derive from different sites of intoxication rather than from different molecular targets.

The 3 SNARE proteins form a heterotrimeric coiled-coil SNARE complex which induces the juxtaposition of the neurotransmitter-containing vesicle to the cytosolic face of the presynaptic membrane and which is involved in their fusion [15, 16], as depicted in figure 2a. VAMP forms a family of vesicular SNAREs which comprises proteins composed of about 120 residues. They have a short C-terminal tail facing the vesicle lumen, a single transmembrane domain and the remaining N-terminal part exposed to the cytosol (fig. 2b). Different VAMP isoforms are located on different cell vesicles and contribute to address each vesicle to its appropriate target membrane with which it will fuse; VAMP-1 and -2 are the isoforms mainly involved in the binding and fusion of neurotransmitter-containing synaptic vesicles with the presynaptic membrane (neuroexocytosis). Syntaxin is about twice the size of VAMP and it is anchored to target membranes via a C-terminal hydrophobic tail. The cytosolic part consists of two domains and only the one closer to the membrane participates in complex formation. Of the many syntaxin isoforms presently known, syntaxin 1A, 1B and 2 are the isoforms mainly involved in neuroexocytosis. SNAP-25 (few isoforms) are 25-kD SNARE proteins bound to the target membrane via fatty acids covalently linked to cysteine residues present in the middle of the polypeptide chain (fig. 2b).

The SNARE complex is insensitive to BTX proteolysis [17], as expected on the basis of the fact that proteases are known to attack predominantly unstructured exposed loops. Only the non-complexed SNAREs can be cleaved by the BTXs. Once cleaved by BTX, the SNARE proteins cannot become part of the complex capable of mediating the vesicle-target membrane fusion. At cholinergic nerve terminals, this specific proteolytic cleavage mediated by the

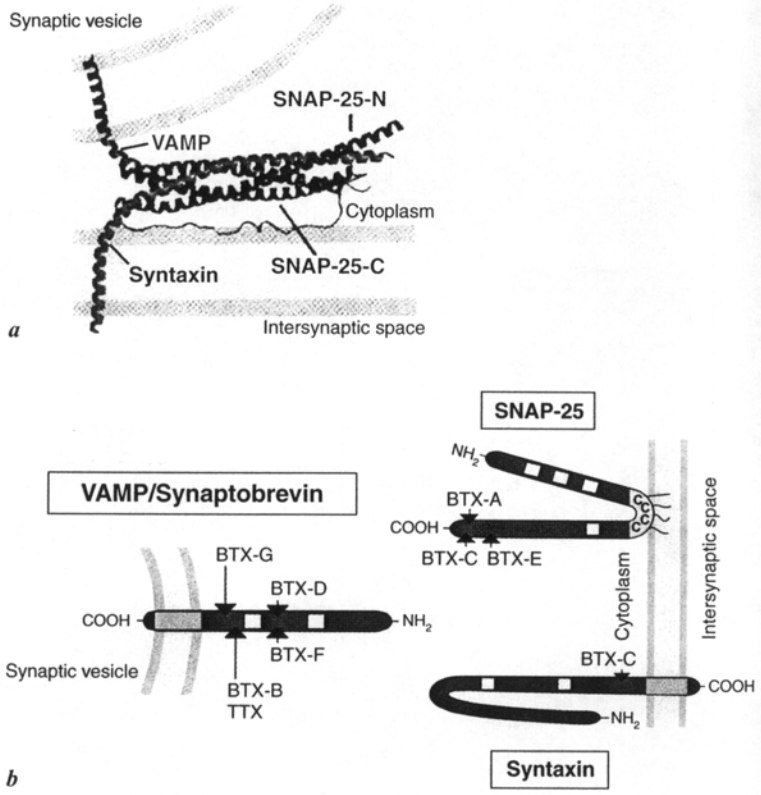

Fig. 2. Structure of the neuronal SNARE complex and diagram of SNAREs with cleavage sites of TTX and BTXs. ***a*** Ribbon diagram of the 4-helix bundle of the neuronal core complex with syntaxin in red, VAMP in blue and SNAP-25 C-terminal coil (SNAP-25-C) and SNAP-25 N-terminal coil (SNAP-25-N) in green. ***b*** VAMP has a short C-terminal tail protruding into the vesicle lumen and a transmembrane segment (gray), followed by a 95-residue-long cytosolic part. This portion is highly conserved among isoforms and species, whereas the N-terminal portion is poorly conserved and rich in prolines. Syntaxin is inserted in the plasma membrane and projects most of its mass into the cytosol. SNAP-25 is bound to the cytosolic face of the presynaptic membrane via palmitoylated cysteine residues located in the middle of the polypeptide chain and via interactions with syntaxin. Arrows indicate the sites of cleavage of BTXs while the SNARE motifs required for protease-substrate interaction are shown by small yellow squares.

BTXs in the synaptic cytosol prevents the release of acetylcholine and hence the transmission of the nerve impulse. A remarkable finding is that the proteolysis of a small part of the total SNARE present within a synapse is sufficient to block the neuroexocytosis in vivo [18] indicating that there are active and inactive pools of SNARE proteins within a synapse.

Table 1. Tetanus and botulism neurotoxins: target and peptide bond specificities (sequences refer to human SNAREs)

Toxin type	Intracellular target	Peptide bond cleaved P4-P3-P2-P1–P1′-P2′-P3′-P4′
TTX	VAMP	Gly-Ala-Ser-Gln–Phe-Glu-Thr-Ser
BTX-A	SNAP-25	Glu-Ala-Asn-Gln–Arg-Ala-Thr-Lys
BTX-B	VAMP	Gly-Ala-Ser-Gln–Phe-Glu-Thr-Ser
BTX-C	Syntaxin	Asp-Thr-Lys-Lys–Ala-Val-Lys-Phe
BTX-C	SNAP-25	Ala-Asn-Gln-Arg–Ala-Thr-Lys-Met
BTX-D	VAMP	Arg-Asp-Gln-Lys–Leu-Ser-Glu-Leu
BTX-E	SNAP-25	Gln-Ile-Asp-Arg–Ile-Met-Glu-Lys
BTX-F	VAMP	Glu-Arg-Asp-Gln–Lys-Leu-Ser-Glu
BTX-G	VAMP	Glu-Thr-Ser-Ala–Ala-Lys-Leu-Lys

BTX-poisoned and electrically silent synapses show an increased number of docked vesicles, as judged from electron microscopy [19–23], suggesting that SNAREs are involved in steps subsequent to docking of vesicles to the target membrane. It was thus discovered that the C-terminal region of SNAP-25 plays a key role in exocytosis [24, 25]. In particular, the region between the cleavage sites of BTX-A and -E is critical for conferring calcium sensitivity to the process [26–32]. Consequently, SNAP-25-derived peptides, SNAP-25 mutants truncated at the C-terminal, and novel pinpointed uses of BTX-A and -E allowed to demonstrate the existence in exocytosis of an initial postdocking step sensitive to low concentrations of BTX-A and of a later prefusion step which is sensitive to very high toxin concentrations [29–35]. This later step is inhibited by an antibody against a region of SNAP-25 which includes two copies of the SNARE motif [36] (see below), in agreement with the possibility that the SNARE motif is conserved because it is involved in an essential prefusion step of exocytosis. The C-terminal part of SNAP-25 comprised between the BTX-A and -E cleavage sites is also essential for vesicle endocytosis [21].

Molecular Basis of the SNARE Specificity

The structural basis of the specificity of BTXs for the 3 SNAREs is only partially known. As shown in table 1, the sequences flanking the cleavage sites of VAMP, SNAP-25 and syntaxin are not similar and thus cannot account for such a specificity. On the other hand, BTXs are highly similar at both primary and tertiary structural levels [37] suggesting that a common pattern of recognition

of the SNARE substrates exists. Sequence comparison of the SNARE proteins involved in neuroexocytosis revealed the unique presence of a 9-residue-long motif, characterized by 3 carboxylate residues alternated with hydrophobic and hydrophilic residues, termed thereafter SNARE motif [38]. These segments are contained within regions adopting a helical conformation [16], and when the motif is plotted as an α-helix, the 3 negatively charged residues cluster on one face, adjacent to a hydrophobic face of the helix [38]. The findings that only protein segments including at least one SNARE motif are cleaved by the toxins and that the motif is exposed at the protein surface [39–45] clearly indicate the involvement of the SNARE motif in the specificity of action of BTXs.

Duration of the Effect of Botulinum Neurotoxins

If they do not die from paralysis of the respiratory muscles or other reasons linked to the loss of functionality of cholinergic terminals, botulism patients fully recover over a period of months depending on the type of BTX. Translated at the cellular level, a BTX-poisoned nerve cell does not die and will completely recover its neurotransmitter release ability with time. As a consequence of the enzymatic nature of the activity of the L chain, as long as one (or few) molecule(s) of toxin is present in the synapse the newly synthesized SNARE molecule will be cleaved and neuroexocytosis will continue to be prevented. What determines the lifetime of the L chains and the kinetics of their inactivation is not known, but it is not surprising that they may persist for many days since in general bacterial toxins are very stable molecules. Moreover, there is evidence that the SNAP-25 molecule deprived of its C-terminal by the BTX-A L chain persists at the synapse and assembles into a nonfunctional SNARE complex [34]. These cumulative effects determine the time required by the neuron to fully recover the activity of its BTX-intoxicated synapse. In addition, there are no data on the time required by the neuronal cell to synthesize a SNARE and to deliver it from the cell body to the synapse. It is expected that the longer the axon, the longer it takes (1) for the synapse to signal the inactivation of the neuroexocytotic machinery and (2) to replace the cleaved SNARE with a newly made copy.

Because of these uncertainties, the duration of the effect of BTXs cannot be anticipated and has to be determined in each nerve, in each animal and for each serotype of toxin. In the clinical experience with BTX-A, it has been determined that the duration of the beneficial effects of neuroparalysis varies even from patient to patient. A striking difference exists between skeletal and autonomic cholinergic nerve terminals. The former recover within few months whereas in the latter inactivation persists for a year or more. This aspect of BTX action on the SNARE proteins is clearly one of the least understood and one

requiring a major effort to be clarified. On the other hand, it is a very relevant point with respect to the clinical use of BTXs. The possibility of modulating the duration of the paralytic effect of BTXs will improve the current therapeutic value and will extend its applications.

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