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Different mechanisms of inhibition of nerve terminals by botulinum and snake presynaptic neurotoxins

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ABSTRACT

The different mode of action on peripheral nerve terminals of the botulinum neurotoxins and of the snake presynaptic phospholipase A2 neurotoxins is reviewed here. These two groups of toxins are highly toxic because they are neurospecific and at the same time are enzymes that can modify many substrate molecules before being inactivated. The similarity of symptoms they cause in humans derives from the fact that both botulinum neurotoxins (seven serotypes named A–G) and snake presynaptic PLA2 neurotoxins block the nerve terminals and that peripheral cholinergic terminals are major targets. Given this general similarity of targets and clinical symptoms, the specific molecular and cellular mechanisms at the basis of their action are very different. This difference appears evident from the beginning of intoxication, i.e. neurotoxins binding to peripheral nerve terminals and proceeds with the different site of actions and molecular targets.

1. Nerve terminal binding of the botulinum neurotoxins

From the site of adsorption or injection, botulinum neurotoxins (BoNTs) diffuse in the body fluids and eventually bind very specifically to cholinergic terminals. These neurotoxins consist of three domains of 50 kDa each termed L, HN and HC. L is a metalloprotease, HN translocates L inside the cytosol and HC lays a major role in the neurospecific binding of BoNTs, which is mediated by two binding sites both present within the 25 kDa C-terminal sub-domain of HC: one for polysialogangliosides, which are highly enriched in nerve terminals, and one for the luminal domain of a synaptic vesicle (SV) protein (Montecucco et al., 2004; Chai et al., 2006; Jin et al., 2006; Rummel et al., 2007; Dong et al., 2006, 2008). This was demonstrated for BoNT/A, /B, /E and /G, but it is very likely to be true for all the other BoNTs. It is not clear if the SV protein receptors are made available only upon SV exocytosis with exposure of their lumen to the outside, or if some SV proteins are exposed on the presynaptic membrane following SV merging (Schiavo, 2006). In any case, the binding of BoNTs to the luminal domain of SV proteins accounts for the long known fact that the rate of BoNT intoxication increases with the rate of neuromuscular junction (NMJ) firing (Hughes and Whaler, 1962). This latter knowledge contributes to account for the apparent preferential activity of BoNT/A for hyperactive nerve terminals in the human therapy. BoNT/B and /G interact with the luminal domain of the SV proteins synaptotagmin-I and -II respectively. BoNT/B has a higher affinity for isoform II, whereas BoNT/G interacts preferentially with isoform I (Dong et al., 2003; Nishiki et al., 1996; Rummel et al., 2004). The crystal structures of BoNT/B in complex with the protein receptor shows that the protein receptor binding site is close, but not overlapping, to that of the ganglioside receptor (Chai et al., 2006; Jin et al., 2006). Mutations in one of the two receptor binding sites greatly reduce the toxicity of BoNT/B and /G, and double mutants at the two binding sites are non toxic (Rummel et al., 2007).

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At variance, BoNT/A and /E bind specifically to the luminal domain of the highly glycosylated SV2 protein (Dong et al., 2006, 2008; Mahrhold et al., 2006). Vertebrates express three similar isoforms of SV2: A, B and C. Motor neuron terminals express the isoform that binds BoNT/A and /B more strongly: SV2C and Synaptotagmin-II, whilst these high-affinity isoforms are poorly expressed in CNS neurons (reviewed in Rossetto and Montecucco, 2008). BoNT/E has a preference for SV2 isoforms A and B. Recently, Baldwin and Barbieri (2007) demonstrated that BoNT/A and /B binding domains associate with synaptic vesicle membrane proteins and suggested that BoNT protein receptor may be a component of a larger protein complex. It should be noted that all experiments are made with subcellular fractions of neurons in culture derived from the CNS (SV, synaptosomes etc.). As polysialoganglioside are similarly expressed in different peripheral nerve terminals, it is not clear at the moment if the different affinities for the different Syt and SV2 isoforms are sufficient to account for the remarkable specificity of all BoNTs for cholinergic terminals. Another aspect which should be investigated is the possibility that BoNTs bind to selected areas of the presynaptic membrane. Given that BoNTs bind to the luminal domain of SV proteins, it would make sense if the toxins would bind at, or close to, the sites of SV exocytosis, i.e. the active zones of the presynaptic membrane. We have preliminary evidence that this may be the case (Paoli et al., unpublished experiments).

2. Nerve terminal binding and activity of the snake presynaptic PLA2 neurotoxins

Contrary to BoNTs, and despite very active investigations, the receptor for none of the snake presynaptic PLA2 neurotoxins (SPANs) has been identified except for β-bungarotoxin, which interacts with K+ channels of the presynaptic membrane via an accessory subunit structurally resembling dendrotoxin (Black et al., 1988). Similarly to the BoNT, the application of SPANs to the neuromuscular junction leads to a progressive paralysis, and the paralysis time is greatly shortened by nerve stimulation (Chang et al., 1973; Kamenskaya and Thesleff, 1974), suggesting that their mode of action is linked to SV turn-over. Later on, the SPANs seem to disappear from the outside as the toxic effects cannot any longer be neutralized by anti-toxin antibodies, or by washings (Kamenskaya and Thesleff, 1974; Simpson et al., 1993).

The first step in SPAN action is the neurospecific binding, which has been studied with radioactively labelled toxins on the CNS tissue; the main outcome is that there are a limited number of high-affinity binding sites, mainly located at presynaptic nerve terminals. It was recently suggested that SPANs bind to selected portions of the presynaptic membrane corresponding to the active zones of neuroexocytosis, which face the Ach receptor-rich infoldings of the muscle membrane (Caccin et al., 2006). SPAN binding may cause by itself, or following a limited and local phospholipid hydrolysis, a structural alteration of these sites of neurotransmitter release resulting in a lowered probability of SV fusion. Toxin binding to the active zones of SV exocytosis would explain their presynaptic neurospecificity. The phospholipids hydrolysis by SPANs promotes the membrane fusion of ready-releasable SVs because lysophospholipids and fatty acids induce a positive curvature of the presynaptic membrane which favours SV fusion (Rigoni et al., 2005; Rossetto and Montecucco, 2008). In addition, we have also found that the SPAN catalyzed hydrolysis of phospholipids increases the probability of formation of membrane defects, i.e. transient membrane pores which allow the entry of external calcium (Rigoni et al., 2007). It is well established that a significant increase of the cytosolic Ca^{2+} triggers the exocytosis of all SVs present at a synapse (Ceccarelli et al, 1972; Rizzoli and Betz, 2005). If one then considers that the outward membrane bending caused by lysophospholipids and fatty acids (Chernomordik and Kozlov, 2008) inhibits membrane fission which is an essential step of SV endocytosis and recycling, one rationally explains the finding that SPAN-poisoned NMJs are largely depleted of SVs (Cull-Candy et al., 1976; Rossetto and Montecucco, 2008).

3. Internalization and membrane translocation of BoNTs

The BoNTs do not enter the cell directly from the plasma membrane, but are endocytosed inside the lumen of vesicular structures in a temperature- and energy-dependent process (reviewed in Schiavo et al., 2000). The finding of SV proteins as receptors of BoNTs supports the proposal (Montecucco and Schiavo, 1995) that BoNTs are endocytosed within SVs, an hypothesis originally advanced to account for the increased rate of poisoning with NMJ activity (Hughes and Whaler, 1962). This was later supported by the finding that tetanus neurotoxin is actually taken up via SV at the inhibitory interneurons of the spinal cord (Matteoli et al., 1996).

The L chains of BoNTs cleave in the cytosol the three SNARE proteins, and, in order to do so, the L chain must cross the hydrophobic barrier of the vesicle membrane; this translocation is driven by the luminal low pH generated by the vacuolar-type ATPase proton pump present on the SV membrane (Matteoli et al., 1996; Simpson et al., 1994). In fact, acidic pH induces a conformational change of BoNT with exposure of hydrophobic patches on the surface of both the H and L chains which then enter into the hydrocarbon core of the lipid bilayer (Montecucco et al., 1989; Puhar et al., 2004; Schiavo et al., 2000; Shone et al., 1987). Following this low pH-induced membrane insertion, the HN domain of BoNTs forms transmembrane ion channels and acts as a transmembrane chaperone for the L chain preventing its hydrophobicity-driven aggregation and maintaining it unfolded during translocation across the HN channel (Shone et al., 1987; Koriazova and Montal, 2003; Fischer and Montal, 2007a,b). The L chain is then released in the neutral cytosol where it refolds into its native enzymatically active conformation. It is possible that the cytosolic re-acquisition of the enzymatically active conformation of the L chain requires, or is facilitated by, cytosolic chaperones, but this remains to be investigated. This hypothesis is, however, supported by the recent finding that a complex of cytosolic chaperones and thioredoxin present on the cytosolic face of endosomes assists

the translocation of the enzymatic chain of other bacterial toxins acting in the cytosol (Haug et al., 2003; Ratts et al., 2003).

4. The entry of SPAN into nerve terminals

As discussed above, at least in the initial phase of their action, SPANs act on the outside of neurons. However, there is growing evidence that they also enter inside the paralyzed nerve terminal and contribute to further damage: a) β-bungarotoxin was detected within hippocampal neurons (Herkert et al., 2001); b) ammodytoxin A was found inside hippocampal neurons in culture and inside the NSC cell line (Petrovic et al., 2004; Pražnikar et al., 2008); c) taipoxin rapidly reached the cytosol of chromaffin cells (Neco et al., 2003); d) ammodytoxin A binds to cytosolic and mitochondrial proteins and taipoxin binds a 49-kDa calcium-binding protein located on the endoplasmic reticulum (reviewed in Pungercar and Krizaj, 2007). In addition, we recently found that enzymatically active fluorescent derivatives of notexin, β-bungarotoxin and taipoxin do enter nerve terminals in different types of neurons, including mouse spinal cord motoneurons. However, these SPANs do not disperse in the cytosol, but they rather bind specifically to mitochondria. The shape of the labelling changes with time. At the beginning it has the typical elongated appearance of mitochondria *in vivo*. Later on, the toxin-labelled mitochondria become rounded and localize in the plasma membrane bulges induced by SPANs in neurons in culture. These data indicate that SPANs do enter rapidly into nerve terminal in culture and that they bind specifically to mitochondria (Rigoni et al., 2008). It is not clear, at the moment, if mitochondrial rounding and loss of function is a direct consequence of the hydrolysis of mitochondrial lipids or if the entry of extracellular calcium is sufficient to cause mitochondria intoxication by calcium overloading or if both effects contribute to determine the experimental finding. We found that the SPANs very effectively induce the opening of the permeability transition pore of brain mitochondria in vitro (Rigoni et al., 2008). Thus SPANs can contribute to the patogenesis of nerve terminal degeneration by acting directly upon mitochondria, whose activity is essential for the physiology of nerve terminals.

How SPANs translocate from the outer layer of the presynaptic membrane inside the cytosol is not yet understood. However, it is clear that they do not follow the pathway of BoNTs because inhibitors of the transmembrane pH gradient of SV do not prevent their action as they do for BoNTs (Simpson et al, 1994; Williamson and Neale, 1994). However, SV endocytosis is not the only type of endocytosis taking place at the plasma membrane, and SPANs could use as "Trojan horses" other vesicles. The problem of translocating across the membrane remains, however, and would only be moved forward from the plasma membrane to an internal vesicle membrane. It is clear that low pH is not involved. In principle the SPANs could translocate by crossing the plasma membrane directly, as some viral proteins do. These proteins are characterized by the presence of clusters of positive residues, which are not present in any of the SPANs. Moreover, it is difficult to envisage how the large oligomeric taipoxin can cross the lipid bilayer. A more likely possibility is that the progressive enzymatic hydrolysis of the plasma membrane phospholipids creates high local concentrations of lysophospholipids and fatty acids that not only change the membrane curvature and increase the permeability to extracellular calcium, but could also mediate the entry of the SPANs. Fatty acid binding to the positive residues of the toxins could take place rendering them more hydrophobic. This effect together with the curvature change imposed by lysophospholipids could create conditions that promote the direct translocation of the SPAN across the lipid bilayer. It is possible that the definition of the molecular events leading to SPAN nerve terminal poisoning will allow to find novel therapies for motoneuron diseases.

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Conflict of interest

The author declares that there are no conflicts of interest.

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