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Review

Internalization and Mechanism of Action of Clostridial Toxins in Neurons

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Abstract

Botulinum toxins are metalloproteases that act inside nerve terminals and block neurotransmitter release via their activity directed specifically on SNARE proteins. This review summarizes data on botulinum toxin modes of binding, sites of action, and biochemical activities. Their use in cell biology and neuroscience is considered, as well as their therapeutic utilization in human diseases characterized by hyperfunction of cholinergic terminals.
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INTRODUCTION

Many pathogenic bacteria produce protein toxins that act inside cells. These toxins bind to the cell surface, become internalized in vesicular compartments, and translocate their catalytic subunit into the cytosol, where they exert their toxic activity by enzymatically modifying a protein substrate (Montecucco et al., 1994). Tetanus (TeNT) and botulinum (BoNT) neurotoxins, endowed with a metalloprotease activity, belong to this group of bacterial toxins. They are released from bacteria as single-chain polypeptides of about 150,000 Da and subsequently activated by proteases to generate dichain toxins formed by a heavy (H) and a light (L) chain, linked by a single disulfide bond (Lacy et al., 1998). Tetanus neurotoxin (TeNT), produced by *Clostridium tetani*, causes the spastic paralysis of tetanus, a disease well known from the beginning of medical literature (tetanos in greek means contraction), which is often fatal. Death occurs by respiratory failure or heart failure. Adult botulism, caused by intoxication with one of the seven botulinum

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neurotoxins (BoNTs, A-G serotypes) produced under anaerobic conditions by toxigenic strains of *Clostridium botulinum* or *Clostridium barati* or *Clostridium butirycum*, was first recognized and described much later than tetanus, possibly due to the less evident symptoms of botulism with respect to those of tetanus. Botulism is characterized by a generalized muscular weakness, which first affects ocular and throat muscles and extends later to the whole skeleton. In the more severe forms, a generalized flaccid paralysis may lead to death, which generally results from respiratory failure. After entering the general circulation, clostridial neurotoxins bind very specifically to the presynaptic membrane of motoneuron nerve endings. Intoxication then proceeds via a four-step mechanism, consisting of: (1) binding, (2) internalization, (3) membrane translocation, and (4) enzymatic target modification (see Schiavo et al., 2000, for a review). The COOH-terminal part (H_C) is mainly responsible for the neurospecific binding, whereas the NH_2-terminal 50-kDa domain of the H chain (H_N) is implicated in membrane translocation; the L chain of the toxin is responsible for the intracellular catalytic activity (see Schiavo et al., 2000, for a review). Clostridial neurotoxins exert their action by cleaving specific components of the synaptic vesicle (SV) fusion machinery: synaptobrevin/VAMP2, an integral protein of the SV membrane, is the substrate of TeNT and of BoNT/B, BoNT/D, BoNT/F, and BoNT/G; syntaxin and SNAP-25, two proteins located on the cytosolic face of the presynaptic membrane, are specifically cleaved by BoNT/C, BoNT/A, and BoNT/E, respectively (for reviews, see Jahn and Niemann, 1994; Montecucco and Schiavo, 1995). Therefore, all the toxins specifically attack protein components of the neuroexocytosis apparatus and end up blocking the neurotransmitter release.

Binding

Clostridial neurotoxins bind neuronal cells rapidly and with high affinity. After binding to the presynaptic membrane, BoNTs enter the neuronal cytosol and block the release of ACh, thus causing a flaccid paralysis. TeNT also binds to the motoneuron nerve terminal, but, differently from BoNTs which act locally, it is transported retrogradely inside the motoneuron axon and reaches the spinal cord, where it accumulates in the ventral horn of the gray matter (Stöckel and Thoenen, 1977; Halpern, 1995; Lalli et al., 2003). Within the spinal cord, TeNT migrates *trans*-synaptically from the dendrites of peripheral motoneurons into the inhibitory interneurons, eventually blocking the release of inhibitory neurotransmitters (Benecke et al., 1977; Lalli et al., 2003). The TeNT-induced blockade of inhibitory synapses impairs the neuronal circuits that ensure balanced voluntary muscle contraction, thus causing a spastic paralysis (Mellanby and Green, 1981; Wellhoner, 1982). Interestingly, the specificity of TeNT for inhibitory versus excitatory synapses is maintained when TeNT is applied to hippocampal slices (Calabresi et al., 1989) or injected into the hippocampus (Mellanby et al., 1977). Such specificity for inhibitory synapses of the central nervous system (CNS) also accounts for the neurodegenerative and epileptogenic effects of TeNT, which mainly result from excessive release of glutamate, not balanced by the action of inhibitory neurotransmitters (Bagetta et al., 1991).

In the last years, a considerable amount of effort has been focused on the identification of clostridial toxin receptors. A large number of studies have established that polysialogangliosides are involved in binding clostridial neurotoxins. In particular, it has been demonstrated that: (1) clostridial neurotoxins bind to polysialogangliosides, particularly to G_{D1b}, G_{T1b}, and G_{Q1b}, (2) preincubation of BoNT with polysialogangliosides partially prevents the poisoning of the NMJ, (3) pretreatment of cultured cells with polysialogangliosides increases their sensitivity to TeNT and BoNT/A, (4) treatment of membranes with neuraminidase, which removes sialic acid residues, decreases the binding of clostridial toxins (see Schiavo et al., 2000). However, it is unlikely that polysialogangliosides are the sole receptors of clostridial neurotoxins and it is instead widely accepted that proteins exposed to the cell surface are involved in toxin binding. Experiments carried out in the last years have provided evidence for an involvement of the synaptic vesicle proteins synaptotagmins I and II in the binding of different serotypes of BoNTs. In particular, it has been found that BoNT/B binds strongly to synaptotagmin II in the presence of polysialogangliosides and that Chinese hamster ovary cells transfected with the synaptotagmin II gene bind the toxin with low affinity and with a high affinity after membrane incorporation of gangliosides GT1b (Nishiki et al., 1994; Nishiki et al., 1996). This evidence has been confirmed by studies showing that synaptotagmins I and II mediate the entry of BoNT/B (but not BoNT/A or E) into PC12 cells. BoNT/B entry into PC12 cells and rat diaphragm motor nerve terminals was found to be activity-dependent and was blocked using fragments of synaptotagmin that contain the BoNT/B-binding domain (Dong et al., 2004). More recently, it has been shown that like

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