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Therapeutic effectiveness of botulinum neurotoxin A: Potent blockade of autonomic transmission by targeted cleavage of only the pertinent SNAP-25

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ABSTRACT

In search of a basis for the impressive potency of an endoprotease that cleaves SNAP-25, botulinum neurotoxin type A (BoNT/A), in treating numerous diseases due to hyper-active autonomic nerves, truncation of its target and inhibition of neurotransmission were studied in rat sympathetic neurons. Tetrodotoxin-sensitive spontaneous cholinergic neurotransmission was blocked >80% by 1 pM BoNT/A despite cleaving <20% of the SNAP-25. A maximum cleavage of ~60% SNAP-25 could be achieved with >1 nM BoNT/A, despite an absence of non-cleavable SNAP-25 in the detergent-solubilised neurons. In contrast, BoNT/E (100 nM) truncated nearly all the SNAP-25 in the intact cells, but was unable to block neurotransmission at low concentrations like BoNT/A. Chimeras created by inserting the acceptor-binding H_C domain of BoNT/A into BoNT/E still cleaved all the SNAP-25, indicating ubiquitous expression of BoNT/A acceptors. Accordingly, SV2 and SNAP-25 were found to be co-expressed and broadly co-localised in neurons, but absent from non-neuronal cells. On the other hand, partial cleavage by the BoNT/A protease persisted upon replacing its H_C with counterparts from BoNT/E or BoNT/B. Moreover, limited cleavage of SNAP-25 was conferred onto the protease from BoNT/E when fused to the N-terminus of BoNT/A. Thus, the BoNT/A protease is uniquely well-adapted for selectively inactivating the SNAP-25 directly involved in neurotransmission; this together with the toxin's acceptor and its target being localised on the peri-somatic boutons likely contribute to its exceptional therapeutic utility in the clinic.

1. Introduction

Botulism is a life-threatening neuroparalytic disease caused by botulinum neurotoxins (BoNTs), a group of 7 serotypes (A–G) produced by *Clostridium botulinum*, that represent the most lethal substances known (Montecucco and Molgo, 2005). These neurotoxic proteins potently and selectively block the quantal release of acetylcholine and other cell mediators. The dominant, immediate symptoms of human botulism result from the toxins' inhibition of neuro-transmission in skeletal muscles; these usually require intensive care treatment but eventually recover over several months (Sobel, 2005). Although additional abnormalities of autonomic transmission persist even longer (up to ~2 years), there has only been limited research on the basis of this extraordinarily prolonged blockade.

On the other hand, astounding clinical success has been achieved by local injection of botulinum neurotoxin type A (BoNT/A) to relax hyper-active skeletal (e.g. in dystonia, spasticity) or smooth muscles (e.g. over-active bladder, abnormal gastrointestinal sphincters). It is also used for attenuating secretory disorders (including hyper-hidrosis and sialorrhoea) (Evidente and Adler, 2010). More recently, BoNT/A has proven useful in ameliorating the symptoms of chronic pain/migraine in certain patient groups (Jackson et al., 2012). The clinical effectiveness of BoNT/A results from a potent, specific and persistent blockade of neuro-exocytosis (Dolly et al., 2009). This di-chain, Zn^{2+}-dependant protease selectively enters nerve terminals upon binding to neuronally-enriched gangliosides and the lumenal domains of synaptic vesicle protein 2 (SV2) (Dong et al., 2006;

Abbreviations: ACSF, artificial cerebrospinal fluid; BoNT, botulinum neurotoxin; BoNT/A – G, BoNT type A – G; DAPI, 4′,6-diamidino-2-phenylindole; H_C and H_N, C- and N-terminal halves of the toxins' heavy chains; LC, light chain; nAChR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; SCGNs, superior cervical ganglion neurons; sEPSCs, spontaneous excitatory post-synaptic currents; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, soluble N-ethyl-maleimide-sensitive factor attachment protein receptor; SV2, synaptic vesicle protein 2; TTX, tetrodotoxin; vAChT, vesicular acetylcholine transporter; VAMP, vesicle-associated membrane protein.

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Mahrhold et al., 2006). The latter also acts as an acceptor for BoNT/E (Dong et al., 2008) whereas synaptotagmin I/II is used by BoNT/B (Chai et al., 2006). After internalisation by endocytosis, endosome acidification triggers BoNTs to transfer to the cytosol. Their enzymic moieties then cleave and inactivate soluble N-ethyl-maleimide-sensitive factor-attachment protein receptors (SNAREs) that are essential for neurotransmitter release (Schiavo et al., 2000; Simpson, 2004). These are synaptosomal-associated protein of 25 kDa (SNAP-25; BoNT/A,/E and C1), vesicle-associated membrane protein (VAMP, also known as synaptobrevin; BoNT/B,/D,/F and G) and syntaxin (BoNT/C1). BoNT/A is the clinicians' preferred serotype due to a very high potency and the remarkable persistence of its light-chain (LC) protease inside neurons (Foran et al., 2003; Keller et al., 1999). Such features translate into a prolonged relief of symptoms (Dolly et al., 2011) e.g. focal dystonias (3–6 months), over-active bladder (6–9 months) and hyper-hidrosis (6 months to >>1 year). With the exception of BoNT/C1 (Morbiato et al., 2007), the enzymic domains of other serotypes are not as stable and, consequently, they have proven to be less effective in providing either long-term relief in clinical trials (Eleopra et al., 2004, 1998) or prolonged muscle weakening in animal models (Meunier et al., 2003).

From both clinical and scientific viewpoints, there is an unmet need to gain insight into why the symptoms of botulism affect autonomic nerves for much longer than motor neurons, and the related more persistent therapeutic benefit of BoNT/A on smooth muscles and secretory glands. Thus, a basis was sought for the remarkable pharmacological performance of BoNT/A relative to other serotypes, noted above, using cultures of sympathetic superior cervical ganglion neurons (SCGNs); these innervate many cranial organs (e.g. salivary gland and smooth muscle) (Gibbins, 1991). They are known to express cholinergic markers *in vivo* and, in culture, form contacts mediating synaptic interactions via nicotinic acetylcholine receptors (nAChR) (Cuevas et al., 2000; Ko et al., 1976). Their susceptibilities to BoNT/A and BoNT/E were quantified, and the features of BoNT/A identified that contribute to its potency in blocking autonomic neurotransmission. Proteolysis of <20% of the SNAP-25 with as little as 1 pM BoNT/A inhibited cholinergic neurotransmission between SCGNs by 80–90%, whereas more than one third of the SNAP-25 evaded proteolysis by even very high concentrations of BoNT/A. Paradoxically, BoNT/E was much less potent than BoNT/A in blocking neurotransmission but at high concentrations it truncated virtually all of the SNAP-25. Use of recombinant BoNT chimeras, created by swapping functional domains between serotypes, excluded the possibility of a BoNT/A-insensitive sub-population of neurons. Furthermore, in this way it was established that incomplete proteolysis of SNAP-25 is an intrinsic, transferrable and dominant property of the BoNT/A LC. Our findings reveal that this protease has developed selectivity for cleaving the fraction of SNAP-25 that directly participates in synaptic vesicle fusion, a property that would contribute to its potency and, possibly, persistence in suppressing autonomic neurotransmission.

2. Materials and methods

2.1. Materials

Natural BoNTs, purchased from Metabiologics Inc. (Madison, WI), were added directly into the cell growth medium; their specific neurotoxicities in mice (after nicking, where required) were determined by the manufacturer ($\times 10^8$ median lethal doses [MLD_{50}] per mg); A (2.5); B (0.9), and E (0.6). The production of recombinant BoNT/A (rBoNT/A) and chimeras is described elsewhere: EA (Wang et al., 2008); AB and BA (Wang et al., 2012); rBoNT/A, rBoNT/A_{AA}, LC_E-BoNT/A and LC_E-$BoTIM_A$ (Wang et al., 2011). Antibodies with the noted specificities were purchased: SNAP-25 (mouse monoclonal SMI-81 [Cambridge Bioscience, Cambridge, U.K.] and rabbit polyclonal serum [Sigma, Arklow, Ireland]), syntaxin (mouse monoclonal HPC1; Sigma), VAMP2 or VAMP1/2/3 (rabbit polyclonal antibodies from Synaptic Systems, Goettingen, Germany). A monoclonal antibody recognising all three SV2 isoforms developed by K. M. Buckley was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242. Alkaline phosphatase-conjugated secondary antibodies were bought from Sigma and Alexa-fluor labelled IgGs purchased from Jackson Immunoresearch Inc. (Newmarket, U.K.).

2.2. Neuron culture, exposure to BoNTs and harvesting for analysis by SDS-PAGE plus Western blotting

Protocols employed for the isolation of rat superior cervical ganglia, enzymatic dissociation of their neurons and maintenance in culture were as described (Mahanthappa and Patterson, 1998). The neurons isolated from ~10 ganglia were seeded into a 48-well culture dish, and exposed to BoNTs or chimeras in the growth medium (Mahanthappa and Patterson, 1998), as detailed in figure legends. The cells were then dissolved in SDS sample buffer and heated (80 °C, 5 min) before SDS-PAGE on NuPAGE 12% acrylamide, Bis-Tris gels (Biosciences, Dun Laoghaire, Ireland). Proteins were transferred to polyvinylidene fluoride (Merck Millipore, Cork, Ireland) for immunoblotting with a monoclonal antibody to syntaxin or rabbit polyclonal antibodies to VAMP2 or SNAP-25. Bound IgGs were detected with alkaline-phosphatase conjugates of species-reactive secondary antibodies, visualised by development of the coloured product (Lawrence et al., 2012). Images of the Western blots were captured with a digital camera (G:Box; Syngene, Cambridge UK). Cleavage of SNAP-25 by BoNT/A or BoNT/E each resulted in the appearance of a second immunoreactive band below the original; the extents of SNAP-25 proteolysis were calculated by quantification of both signals using Image J (NIH, USA), as described previously (Lawrence et al., 2012). In the case of VAMP2 proteolysis by BoNT/B, immuno-signals were expressed relative to the values for syntaxin (insensitive to this toxin) in the same sample lane, to adjust for any possible well-to-well variation in protein loading, before calculating their intensities as a % of the signal in toxin-free control lanes.

2.3. Immuno-isolation of SNAP-25 and proteolysis by BoNT/A or BoNT/E in vitro

SNAP-25 was isolated with a monoclonal antibody (clone SMI-81) linked to Sepharose-4B, as described (Bajohrs et al., 2004), and incubated with 50 nM BoNT/A or BoNT/E in 150 mM NaCl, 20 mM Hepes pH 7.4, 50 μM $ZnCl_2$ and 5 mM dithiothreitol for 30 min at 37 °C before adding SDS-sample buffer and performing SDS-PAGE and Western blotting before probing for cleaved and intact SNAP-25.

2.4. Immuno-cytochemistry and fluorescence microscopy

SCGNs were grown for seven days on glass coverslips or in plastic-bottomed dishes optimised for microscopy (μdish; ibidi, Martinsreid, Germany) and rinsed with phosphate-buffered saline (PBS) before fixation with 4% paraformaldehyde and permeabilisation using 0.1% (v/v) Triton X-100 in PBS, as outlined before (Meng et al., 2007). After blocking with 1% (v/v) foetal bovine serum in PBS, the cells were exposed to primary antibodies, then washed with PBS before detection with Alexa fluor (488 or 594)-conjugated secondary antibodies. Images were captured on an Olympus IX71 inverted microscope equipped with a digital camera.

2.5. Electrophysiological measurements

Cultures of SCGNs (8 weeks old), untreated or exposed to BoNT/A or BoNT/E (for 18–20 h), were used for electrophysiological assessment of spontaneous synaptic currents. Whole-cell recordings of synaptic activity were obtained from individual neurons, visualised with an Olympus BX51WI microscope, under continuous perfusion with oxygenated ACSF [artificial cerebrospinal fluid (in mM); 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 3.5 $CaCl_2$, 1.2 $MgCl_2$ and 25 glucose, pH 7.3, 36–37 °C] at a rate of ~2–3 ml/min. An EPC10USB amplifier controlled with PatchMaster software (HEKA, Lambrecht, Germany) was used for data collection, with current- and voltage-clamp recordings made from membrane potential at or close to −65 mV. Recording electrodes were filled with a potassium methyl sulphate-based internal solution [(mM); 140 KCH_3O_3S, 5 KCl, 5 NaCl, 2 MgATP, 0.01 EGTA and 10 HEPES, pH 7.3; in-bath resistance was 3–5 MΩ]. After establishing whole-cell configuration in the current-clamp mode, membrane responsiveness and impedance were evaluated with small hyperpolarizing (−10 pA) rectangular current pulses; bridge balance was implemented digitally. Only neurons with input resistance exceeding 100 MΩ and generating over-shooting action potentials were included in the current analysis. Values presented are not corrected for liquid junction potential. Following the current-clamp recordings, the amplifier was switched to voltage-clamp mode with C_m (membrane capacitance) cancelled and series resistance compensated (~80%). The readouts of C_m, input resistance (R_{input}) and holding current (I_{hold}) values obtained in these experiments are similar to those reported for SCGNs (Lawrence et al., 2012). Spontaneous excitatory post-synaptic currents (sEPSCs) were recorded in continuous mode. Analogue signals were sampled at 20 kHz, digitised at 10 kHz and analysed offline (FitMaster, HEKA; pClamp9). Stretches of sEPSC recordings (at least 3 min) were utilised for assessments of the