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3,4-diaminopyridine reverses paralysis in botulinum neurotoxin-intoxicated diaphragms through two functionally distinct mechanisms

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ABSTRACT

Botulinum neurotoxins (BoNTs) are exceedingly potent neurological poisons that prevent neurotransmitter release from peripheral nerve terminals by cleaving presynaptic proteins required for synaptic vesicle fusion. The ensuing neuromuscular paralysis causes death by asphyxiation. Although no antidotal treatments exist to block toxin activity within the nerve terminal, aminopyridine antagonists of voltage-gated potassium channels have been proposed as symptomatic treatments for botulism toxemia. However, clinical evaluation of aminopyridines as symptomatic treatments for botulism has been inconclusive, in part because mechanisms responsible for reversal of paralysis in BoNT-poisoned nerve terminals are not understood. Here we measured the effects of 3,4-diaminopyridine (DAP) on phrenic nerve-elicited diaphragm contraction and end-plate potentials at various times after intoxication with BoNT serotypes A, B, or E. We found that DAP-mediated increases in quantal content promote neurotransmission from intoxicated nerve terminals through two functionally distinguishable mechanisms. First, DAP increases the probability of neurotransmission at non-intoxicated release sites. This mechanism is serotype-independent, becomes less effective as nerve terminals become progressively impaired, and remains susceptible to ongoing intoxication. Second, DAP elicits persistent production of toxin-resistant endplate potentials from nerve terminals fully intoxicated by BoNT/A, but not serotypes B or E. Since this effect appears specific to BoNT/A intoxication, we propose that DAP treatment enables BoNT/A-cleaved SNAP-25 to productively engage in fusogenic release by increasing the opportunity for low-efficiency fusion events. These findings have important implications for DAP as a botulism therapeutic by defining conditions under which DAP may be clinically effective in reversing botulism symptoms.

1. Introduction

Botulinum neurotoxins (BoNTs) are a family of highly potent bacterial proteins that block cholinergic release at peripheral nerve terminals, causing autonomic blockade and muscle paralysis. There are seven antigenically distinguishable BoNT serotypes, A-G, of which A, B, and E are associated with nearly all cases of clinical botulism in North America (Simpson, 2004; Centers for Disease Control and Prevention, 2004–2013). Each serotype contains a 100 kDa heavy chain (HC) and 50 kDa light chain (LC), which remain associated through a disulfide bond (Montal, 2010). HC mediates neuron-specific binding, presynaptic uptake via synaptic endocytosis and translocation into the presynaptic cytosol, while LC is a Zn^{2+}-dependent metalloprotease that specifically cleaves the neuronal SNARE proteins synaptosomal-associated protein 25 (SNAP-25; BoNT/A, /C, /E), synaptobrevin-1/2 (SYB1/2; BoNT/B, /D, /F, /G) or syntaxin-1 (STX1; BoNT/C). These three SNARE proteins are core elements of the presynaptic fusion complex and are essential for Ca^{2+}-activated synaptic neurotransmission. BoNT poisoning clinically manifests as dysfunction of autonomic synapses and paralysis of neuromuscular junctions (NMJs), which become lethal once respiratory muscles are impaired. Survival of lethal exposures requires rapid and sustained administration of supportive care, such as mechanical ventilation and parenteric nutrition. The combination of efficient neuronal targeting with the specific proteolysis of presynaptic SNARE proteins renders BoNTs the most potent toxins known, with human LD50 values estimated to range from 0.1–1.0 ng/kg (Cherington, 1998).

While the potential for mass casualty botulism outbreaks is high, particularly in cases of food-borne botulism, incidents are infrequent, and therefore it has been difficult to evaluate therapeutic approaches in a clinical setting (Kongsaengdao et al., 2006; Chalk et al., 2014).

Abbreviations: BoNT, botulinum neurotoxin; DAP, 3,4-diaminopyridine; HC, heavy chain; LC, light chain; NMJ, neuromuscular junction; EPP, endplate potential; mEPP, miniature endplate potential; TTX, tetrodotoxin; t_{50}, median paralysis; t_{100}, full paralysis; diEPP, DAP-induced EPP; RMP, resting membrane potential; E_{max}, peak contraction strength

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Currently, the only FDA-approved treatment for botulism is post-exposure immunization with equine-derived antitoxin antibodies (Centers for Disease Control and Prevention, 2010). Although antitoxins efficiently neutralize BoNT in circulation, they have no effect on toxin that is already bound to presynaptic receptors or internalized into nerve terminals, and therefore are ineffective in reversing botulism symptoms (Al-Saleem et al., 2011). Because the first clinical evidence of botulism poisoning is descending flaccid paralysis, which occurs subsequent to intracellular toxicity, the timeframe for effective antitoxin treatment is narrow. This critical limitation has driven a search for post-symptomatic countermeasures (Larsen, 2009; Smith et al., 2012).

Aminopyridine derivatives such as 3,4-diaminopyridine (DAP) represent potential symptomatic treatments for botulism (Lundh et al., 1977; Simpson, 1986). Aminopyridines block the intracellular domain of A-type voltage-gated K^+ channels, prolonging the duration of action potential-induced presynaptic depolarization and increasing neurotransmitter release (Taylor et al., 1972; Thomsen and Wilson, 1983; Lin-Shiau et al., 1991). Although administration of DAP to BoNT/A-intoxicated animals has been shown to enhance NMJ function under controlled conditions (Siegel et al., 1986; Adler et al., 2000), the use of DAP in clinical cases of botulism has resulted in highly variable and inconsistent outcomes (Davis et al., 1992; Dock et al., 2002; Friggeri et al., 2013). These studies involved multiple BoNT serotypes, with aminopyridines administered at different doses and at various stages of intoxication, rendering interpretation of clinical outcomes difficult. Thus, while both animal and clinical data suggest that DAP can provide partial relief of botulism symptoms, it is still unclear for which serotypes and under what conditions.

To elucidate the effects of aminopyridine treatment on intoxicated NMJs, nerve-elicited muscle contractions and endplate potentials were used to study neurotransmission in mouse phrenic nerve:hemidiaphragm preparations intoxicated with BoNT serotypes A, B, or E. The effects of DAP on NMJ function were characterized at various times after intoxication, allowing for a comparative analysis of DAP efficacy at defined stages of intoxication for each serotype. These studies reveal two functionally distinguishable mechanisms by which DAP enhances neuromuscular function in intoxicated tissues. Based on these results we develop models to explain DAP effects on intoxicated NMJs, and suggest parameters under which botulism patients are likely to benefit from DAP treatment.

2. Materials and methods

2.1. Reagents

Botulinum neurotoxin serotypes A1 (/A; 2.5×10^8 LD_{50}/mg), B1 (/B; 1.1×10^8 LD_{50}/mg), E1 (/E; 1.5×10^5 LD_{50}/mg) and formalin-inactivated BoNT/A1 dichain were obtained from Metabiologics (Madison, Wisconsin) at 1 mg/mL in Ca^{2+}/Mg^{2+}-free phosphate buffered saline, pH 7.4 (PBS) and stored in aliquots at −80 °C. Prior to storage, BoNT/E was activated by incubation in 0.05 M sodium phosphate buffer (pH 6.5) with 0.3 mg/mL TripLE trypsin (Sigma-Aldrich, St Louis, MO) at 37 °C for 60 min (Hubbard et al., 2012). Trypsin was neutralized by addition of 4 volumes of soybean trypsin inhibitor and 10% glycerol. The potency of the activated toxin was anticipated to be 3×10^7 LD_{50}/mg. DAP, tetrodotoxin (TTX) and all components of Tyrode's solution, containing (in mM) 137 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$, 24 $NaHCO_3$, 1 NaH_2PO_4 and 11 D-glucose, pH 7.4, were purchased from Sigma-Aldrich. μ-conotoxin GIIIB was purchased from Alomone Labs (Jerusalem, Israel). DAP, TTX and μ-conotoxin GIIIB were diluted to working concentrations in Tyrode's solution immediately prior to use.

2.2. Mouse phrenic nerve-hemidiaphragm muscle contraction studies

All procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89–544), as amended. Male C57BL/6 mice (6–10 weeks.; Jackson Labs, Bar Harbor, ME) were group-housed and provided a standard diet with regular enrichment and water ad libitum. Mice were thoroughly anesthetized using 5% isoflurane and euthanized by decapitation. Diaphragm muscles and corresponding phrenic nerves were isolated by dissection at 22–24 °C in Tyrode's solution. Hemidiaphragm preparations were mounted in a 50 mL organ bath system (Radnoti, Monrovia, CA) containing Tyrode's solution at 37 °C and continuously bubbled with 95% O_2/5% CO_2. The lateral side of the tissue, including ribs and intercostal muscles, was mounted with a stainless steel hook to an adjustable glass hook immersed and centered in the bath. The medial side with tendon was attached with a silk thread to an isometric force transducer (Radnoti) and resting tensions were set to 0.3–0.6 g. The phrenic nerve fiber was gently pulled through a custom-made bipolar-loop stainless steel stimulating electrode. The tissue was then allowed to equilibrate with stimulation (0.2 ms square voltage pulses at 0.05 Hz) at 110% supramaximal voltage, delivered via a bipolar stimulating electrode driven by a Powerlab stimulator (AD Instruments, Colorado Springs, CO) for at least 60 min before starting baseline measurements. Force measurements were continuously digitized at a sampling rate of 1000 Hz using Powerlab data acquisition system and recorded and visualized using Labchart version 8 (AD Instruments). Traces were filtered with a 50 Hz digital low-pass threshold to eliminate high frequency noise. Muscles were discarded as unstable if they displayed > 10% variability in tension amplitude during the initial hour of acclimation. Representative traces were median-smoothed in ± 40 s bins to mitigate high-frequency noise. Twitch amplitudes were not adjusted for the small changes (< 10%) that occurred in control muscles over the first 3 h, nor the slow decline in control muscle twitch amplitude seen during the remaining 6 h experimental window. Traces that did not fit a regression with $R^2 > 0.90$ were excluded from analysis.

Once muscles displayed a stable baseline for at least 30 min, treatments were applied and nerve-elicited twitch tensions were continuously measured at 0.05 Hz. Mouse diaphragm preparations typically produced stable twitch responses for at least 8 h. Median paralysis (t_{50}) was determined from nonlinear curve fitting against averaged muscle contraction data using Prism. Full paralysis (t_{100}) was defined as the time at which twitch tension amplitudes decreased below 0.020 g, corresponding to amplitudes measured after tetrodotoxin treatment (0.020 ± 0.007 g). Data were exported as raw tensions, or normalized peak parameters as necessary. If no peak was observed within 20 ms of a stimulus, height was recorded as 0 g.

2.3. Endplate potential recordings

Spontaneous miniature endplate potentials (mEPPs), evoked endplate potentials (EPPs) and resting membrane potentials (RMPs) were recorded from phrenic nerve–hemidiaphragm preparations pinned to a 10 mm dish filled with sylgard (Dow Corning, Midland, Michigan) and perfused with oxygenated Tyrode's solution at 22–24 °C. Recordings were performed on a HEKA Elektronik EPC10 patch clamp amplifier (Lambrecht, Germany) using sharp glass electrodes (10–20 MΩs) pulled with a Sutter Instrument P1000 (Novato, CA). Muscles fibers were impaled close to endplate junctions and recordings with starting RMP > −60 mV were rejected. Muscle contraction was selectively blocked by incubation with 1 μM μ-conotoxin GIIIB for 30 min, which preferentially blocks muscle-specific voltage-activated Na^+ channels (Alamone Labs) (Cruz et al., 1985). EPPs were elicited by stimulating the phrenic nerve using a bipolar stimulating electrode driven by a stimulation isolation unit (Digitimer, Fort Lauderdale, FL). For static EPP characterization experiments, phrenic nerves were stimulated with a square wave of constant current for 0.3 ms at 0.5 Hz at > 150% of the threshold stimulation required to reliably evoke EPPs. EPP amplitudes were averaged from 10 consecutive stimulations per muscle fiber and corrected for non-linear summation using the following conversion: