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Effects of hydroxamate metalloendoprotease inhibitors on botulinum neurotoxin A poisoned mouse neuromuscular junctions

Baskaran Thyagarajan [a], Joseph G. Potian [a], Carmen C. Garcia [a], Kormakur Hognason [a], Kateřina Čapková [b], Scott T. Moe [c], Alan R. Jacobson [c], Kim D. Janda [b], Joseph J. McArdle [a,*]

[a] Department of Pharmacology and Physiology, UMDNJ – New Jersey Medical School, Newark, NJ 07103, USA
[b] Department of Chemistry, The Skaggs Institute for Chemical Biology and the Worm Institute of Research and Medicine (WIRM), The Scripps Research Institute, La Jolla, CA 92037, USA
[c] Absolute Science, Inc., Lexington, MA 02421, USA

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ABSTRACT

Currently the only therapy for botulinum neurotoxin A (BoNT/A) poisoning is antitoxin. Antidotes that are effective after BoNT/A has entered the motor nerve terminals would dramatically benefit BoNT/A therapy. Inhibition of proteolytic activity of BoNT/A light chain by metalloendoprotease inhibitors (MEIs) is under development. We tested the effects of MEIs on *in vitro* as well as *in vivo* BoNT/A poisoned mouse nerve-muscle preparations (NMPs). The K_i for inhibition of BoNT/A metalloendoprotease was 0.40 and 0.36 μM, respectively, for 2,4-dichlorocinnamic acid hydroxamate (DCH) and its methyl derivative, ABS 130. Acute treatment of nerve-muscle preparations with 10 pM BoNT/A inhibited nerve-evoked muscle twitches, reduced mean quantal content, and induced failures of endplate currents (EPCs). Bath application of 10 μM DCH or 5 μM ABS 130 reduced failures, increased the quantal content of EPCs, and partially restored muscle twitches after a delay of 40–90 min. The restorative effects of DCH and ABS 130, as well as 3,4 diaminopyridine (DAP) on twitch tension were greater at 22 °C compared to 37 °C. Unlike DAP, neither DCH nor ABS 130 increased Ca^{2+} levels in cholinergic Neuro 2a cells. Injection of MEIs into mouse hind limbs before or after BoNT/A injection neither prevented the toe spread reflex inhibition nor improved muscle functions. We suggest that hydroxamate MEIs partially restore neurotransmission of acutely BoNT/A poisoned nerve-muscle preparations *in vitro* in a temperature dependent manner without increasing the Ca^{2+} levels within motor nerve endings.

1. Introduction

The anaerobic bacterium *Clostridium botulinum* produces a family of seven immunologically distinct botulinum neurotoxins (BoNTs) A through G. All BoNTs bind to ectoreceptors on the surface of motor nerve endings which allow endocytosis of holotoxin into the cytoplasm. After entering the nerve terminal cytosol, the heavy chain of BoNT/A is believed to form a pore which translocates the Zn-endoprotease light chain (Lc) into the cytosol (Montal et al., 1992). There, the Lc selectively cleaves the SNARE protein SNAP-25 to inhibit acetylcholine (ACh) release (Montecucco, 1986; Montecucco et al., 1994). This effect results in skeletal muscle paralysis which can persist for several months.

Active and passive immunization are currently used to protect against and treat BoNT poisoning. However, the increasing use of BoNTs to treat human diseases makes active immunization less desirable. At the same time, available antitoxins do not have access to BoNTs which have entered nerve endings. Thus, survival of severely poisoned patients depends upon long periods of artificial ventilation (Souayah et al., 2006). Following the survival phase, patients may continue to exhibit unexplained neuromuscular dysfunction. Thus, widespread BoNT/A poisoning of a diverse human population (Arnon et al., 2001) would present acute and long term challenges to current medical infrastructures. Therefore, it is important to develop effective therapies for patients with BoNT/A-induced muscle paralysis.

Inhibition of BoNT/A Lc endoprotease may provide a therapy for BoNT/A poisoning. High affinity peptide based competitive inhibitors were first shown to inhibit BoNT/A activity in a cell free system (Schmidt and Stafford, 2002). Extension of the cell free studies to small molecules identified additional inhibitors of BoNT/A Lc metalloendoprotease (Boldt et al., 2006; Eubanks et al., 2007; Fischer et al., 2009). One of these, 2,4 dichlorocinnamic hydroxamate (DCH) protected mice from BoNT/A lethality

* Corresponding author. Tel.: +1 973 972 4428; fax: +1 973 972 4554.
E-mail address: mcardle@umdnj.edu (J.J. McArdle).

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(Eubanks et al., 2007). Therefore, we examined the effect of DCH and a methyl analog, ABS 130, on ACh release and neurally-evoked muscle twitches of isolated nerve-muscle preparations (NMPs) acutely poisoned with BoNT/A.

2. Methods

2.1. In vitro studies

All animal procedures were approved by the Institutional Animal Care and Use Committee. *Triangularis sterni* (TS) or hemidiaphragm nerve-muscle preparations were removed from adult Swiss Webster mice anesthetized with isoflurane. The TS preparation was studied because of its suitability for electrophysiologic experiments (McArdle et al., 1981 #5052). However, its delicate morphology makes the TS unfavorable for study of force generation. Therefore, studies of neurally-evoked twitches were made for the hemidiaphragm preparation.

TS nerve-muscle preparations were pinned to a Sylgard-lined Plexiglass chamber and bathed in HEPES Ringer solution (HRS, 22 °C) containing (mM) NaCl (135), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), dextrose (5.5), and HEPES (5). For control preparations, 0.75 μM μ-conotoxin GIIIB (Alamone Labs, Israel) was added to the bath to inhibit muscle action potentials and mechanical responses to nerve stimulation. Electrical activity of the motor endplate was recorded with two electrode voltage clamp. The electrodes were inserted into the endplate membrane at an interelectrode distance of approximately 50 μm. One electrode filled with 4 M potassium acetate delivered current. The other electrode filled with 3 M KCl recorded membrane voltage. Endplate currents (EPCs) were elicited by placing the muscle nerve into a suction electrode and stimulating at 1 Hz. EPC and miniature endplate currents (mEPCs) were amplified (Axoclamp-2B, Axon Instruments, Foster City, CA, USA), digitized (Digidata 1200, Axon Instruments), acquired and analyzed with PCLAMP software (version 9.2, Axon Instruments). All currents were recorded at a holding potential of −75 mV. To load neurotoxin, TS preparations were bathed (22 °C) in physiologic solution containing 10 pM BoNT/A plus 40 mM KCl (osmolarity adjusted by reducing Na^+ to 102 mM). After 90 min of BoNT/A loading, preparations were washed 3 times with BoNT/A-free physiological solution. EPC and mEPC recordings were acquired before and after exposure of control and BoNT/A poisoned TS preparations to physiological solution containing either DCH or ABS 130.

Hemidiaphragm preparations were mounted in a glass chamber (Rodnoti Glass Technology, Inc., Monrovia, CA) filled with oxygenated (95% O_2–5% CO_2) normal Ringer solution (pH 7.4, 22 °C or 37 °C) containing (mM) NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), $NaHCO_3$ (15), glucose (5.5). The phrenic nerve was drawn into a suction electrode for indirect activation of muscle twitches. One tendon of the muscle was tied to a Grass Force transducer connected to a Digidata 1440A. This enabled acquisition and analysis of muscle twitches with PCLAMP software. Isolated preparations were adjusted to optimal length for force generation and equilibrated for 15 min prior to stimulation at 1 Hz in the presence of 10 pM BoNT/A. Twitch tension declined to less than 10% of the initial value at 90 min after exposure to BoNT/A. At this time, nerve-muscle preparations were washed 3 times with toxin-free Ringer solution (22 °C or 37 °C). After the preparation equilibrated for 15 min, twitches were recorded for 15 min prior to adding DCH, ABS 130, and/or DAP directly to the muscle chamber.

2.2. In vivo studies

Adult Swiss Webster mice were anesthetized with an intraperitoneally injected mixture of ketamine (100 mg/kg) and xylazine (9 mg/kg) prior to surgical exposure of the peroneal nerve's entry into the *Extensor digitorum longus* (EDL) muscle. Three μl of 6.67 pM BoNT/A in HEPES buffer was injected bilaterally into the space surrounding the innervation site of the EDL with a 26 Gauge Hamilton syringe. The skin incision was then surgically closed with aseptic technique. For experiments with DCH or ABS 130, each MEI was bilaterally injected (3 μl of 1 mM stock solution) either 2 h before the injection of BoNT/A to test for the protective effect against the inhibitory effects of BoNT/A or 12 h following BoNT/A injection; at 12 h after BoNT/A, the toe spread reflex was suppressed. The toe spread reflex was scored from 1 to 5 depending on the number of toes that the mouse extended from the midline of the foot when lifted by the tail. Daily scoring began at 24 h and continued for 3 weeks after treatment with BoNT/A alone or with DCH or ABS 130.

2.3. Cell culture and intracellular Ca^{2+} imaging

Mouse cholinergic neuroblastoma (Neuro 2a) cells were cultured in DMEM-F12 medium (pH 7.4) supplemented with 10% Fetal Bovine Serum and antibiotics. For fluorescent analysis of intracellular Ca^{2+}, Neuro 2a cells were grown on 25 mm circular coverslips and incubated with 2 μM fura-2 AM for 30–40 min at room temperature in HRS. The coverslips were then washed in HRS, placed in a 0.8 ml stainless steel holder (Molecular Probes), and viewed with a Zeiss Axiovert 100 microscope coupled to an Attofluor digital imaging system. Results were presented as the ratio (R) of fluorescence intensities at excitation wavelengths of 334 and 380 nm. Cells were continuously superfused (22 °C) with HRS, and Ca^{2+} entry due to addition of either 10 μM DCH, 5 μM ABS 130, 50 μM DAP, DAP plus DCH or DAP plus ABS 130 was measured.

2.4. Data analyses

The K_i for inhibition of BoNT/A Lc endoprotease by DCH and ABS 130 was determined as previously described (Capkova et al., 2008).

Quantal content of EPCs was calculated with the direct method as the ratio of mean EPC amplitude to mean mEPC amplitude. The failure of transmitter release was calculated for 1 min trains of supramaximal 1 Hz stimulation. The ratio of the number of EPCs divided by 60 (number of expected EPCs in the 1 min train) was subtracted from 1 and multiplied by 100 to calculate the percent failure of neuromuscular transmission. Failure of EPCs contributed values of 0 to the calculation of mean EPC amplitude and accounts for mean quantal content values of less than 1.

All data were expressed as mean ± S.E.M. Student's t test evaluated the statistical significance of population mean differences with ** indicating $p < 0.01$.

2.5. Chemicals and drugs

BoNT/A and μ Conotoxin GIIIB were purchased from Metabiologics Inc. (WI) and Alamone Labs (Israel), respectively. All other chemicals were purchased from Sigma (USA).

3. Results

DCH and ABS 130 (Fig. 1) were evaluated *in vitro* for inhibitory activity against BoNT/A Lc endoprotease. The K_i values were determined with a Liquid Chromatography-Mass Spectrometry based assay (Capkova et al., 2008). DCH and ABS 130 were assayed at a final concentration of 10 μM, with 10 μM SNAP-25 as the substrate. The obtained velocities were fitted with the following equations:

$$IC_{50} = \frac{[I]}{\frac{V_0}{v} - 1} \qquad K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

K_i for DCH and ABS 130 was 0.40 μM and 0.36 μM, respectively.

To facilitate toxin uptake, TS nerve-muscle preparations were bathed in physiological solution containing 40 mM KCl plus 10 pM BoNT/A. Control preparations were exposed to physiological buffer solution containing 40 mM KCl. After 90 min, preparations were washed thrice with toxin-free physiological solution and allowed to equilibrate for 30 min prior to recording mEPCs and EPCs. mEPC amplitudes were equivalent for BoNT/A treated and control preparations (Fig. 2). In contrast, EPC amplitude was 0.64 ± 0.09 nA and 64.17 ± 13.97 nA for BoNT/A treated and control preparations. The ratio of mean EPC to mean mEPC amplitude indicated that the BoNT/A treated and control preparations had quantal contents of 0.53 ± 0.14 ($n = 6$ muscles, 25 endplates) and 59.97 ± 9.43 ($n = 5$, 18), respectively. These data indicate that 40 mM KCl stimulated BoNT/A loading into mature motor nerve endings acutely inhibits stimulus-evoked transmitter release *in vitro* to less than 95% of control (Thyagarajan et al., 2009).

EPC amplitude of BoNT/A poisoned TS preparations began a gradual increase at 40–50 min after application of either 10 μM DCH or 5 μM ABS 130 to the bathing solution (Fig. 3). At longer times after exposure to either DCH or ABS 130, the BoNT/A poisoned TS preparations began to twitch in response to nerve stimulation (see below). The recovery of EPC amplitude reversed upon washout of DCH but not for ABS 130. EPC amplitude increased when DCH was reapplied to the BoNT/A treated preparations. Neither DCH nor ABS 130 altered the EPC decay time of control preparations (Fig. 3D).

Neither DCH nor ABS 130 altered the amplitude, frequency, or 90–10% decay time of mEPCs for BoNT/A poisoned preparations (Fig. 4). However, 10 μM DCH or 5 μM ABS 130 increased the quantal content of evoked transmitter release for BoNT/A treated preparations from 0.53 ± 0.14 to 35.71 ± 12.43 and 62.35 ± 14.28,

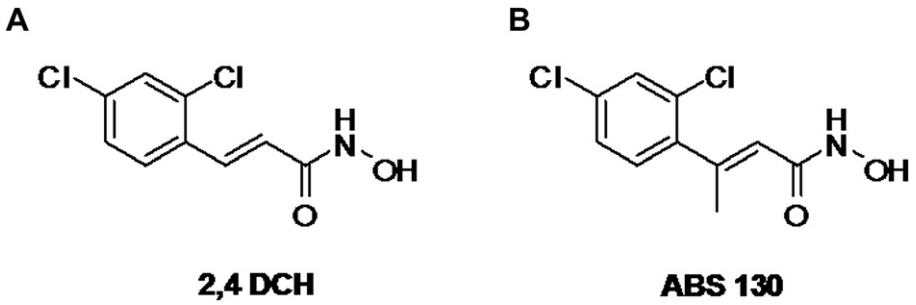

Fig. 1. Structure of hydroxamate metalloendoprotease inhibitors investigated: 2,4-dichlorocinnamic acid hydroxamate (DCH) and a methyl derivative and ABS 130.

respectively, while reversing BoNT/A-induced failures of EPCs (Fig. 5). In the absence of BoNT/A pretreatment, 10 μM DCH or 5 μM ABS 130 did not change quantal content (Fig. 5A). However, 10 μM ABS 130 alone inhibited EPC generation and mEPCs for 3 TS preparations. This depressant action of ABS 130 was not further investigated. In contrast, quantal content was not further increased, and the delayed onset of effect persisted, when BoNT/A poisoned preparations were exposed to 20 μM DCH.

Analysis of nerve-evoked muscle twitches provided a quantitative indication of the magnitude of the DCH and ABS 130 effects. As summarized in Fig. 6, the average twitch tension of control hemidiaphragm preparations was 3908.22 ± 33.9 mg ($n = 6$) at 22 °C. This value declined to 0 mg ($n = 6$) after stimulation at 1 Hz for 90 min in the presence of 10 pM BoNT/A. Application of 10 μM DCH or 5 μM ABS 130 enhanced the average twitch tension to 2372.43 ± 11.38 mg ($n = 3$) and 2324.36 ± 15.64 mg ($n = 3$) respectively. This effect is equivalent to the restoration of muscle twitches for the TS muscle in the electrophysiologic studies made at 22 °C. Addition of 50 μM DAP after DCH or ABS 130 treatment further increased the average twitch tension of the BoNT/A intoxicated diaphragm preparation to 3249.98 ± 59.48 mg ($n = 3$) and 3179.26 ± 64.28 mg ($n = 3$), respectively.

In contrast to the robust restoration of neuromuscular transmission for preparations treated with BoNT/A at 22 °C, DCH and ABS 130 were less effective for nerve-muscle preparations paralyzed with 10 pM BoNT/A at 37 °C (Fig. 8). To follow the time course of DCH and ABS 130 effects at 37 °C, we recorded nerve-evoked muscle twitches of hemidiaphragm NMPs which were paralyzed after stimulating at 1 Hz (37 °C) in physiologic solution containing 10 pM BoNT/A. The graph of Fig. 7A shows that the twitch tension of control preparations gradually declined from 2405.19 ± 2.05 mg to 2348.89 ± 5.42 mg after 60 min (Control; $n = 12$). As expected, the twitch tension of preparations exposed to 10 pM BoNT/A (+BoNT/A; $n = 14$) underwent a more dramatic decline. That is, twitch tensions for control and BoNT/A treated preparations were 2383.93 ± 1.43 mg and 1945.01 ± 3.96 mg at 40 min. A further decline to 72.77 ± 2.86 mg was observed for BoNT/A treated preparations at 80 min. At 120 min, the respective twitch tensions were 2372.31 ± 3.31 mg and 0 mg. Preparations were then washed several times with toxin-free physiological solution and allowed to

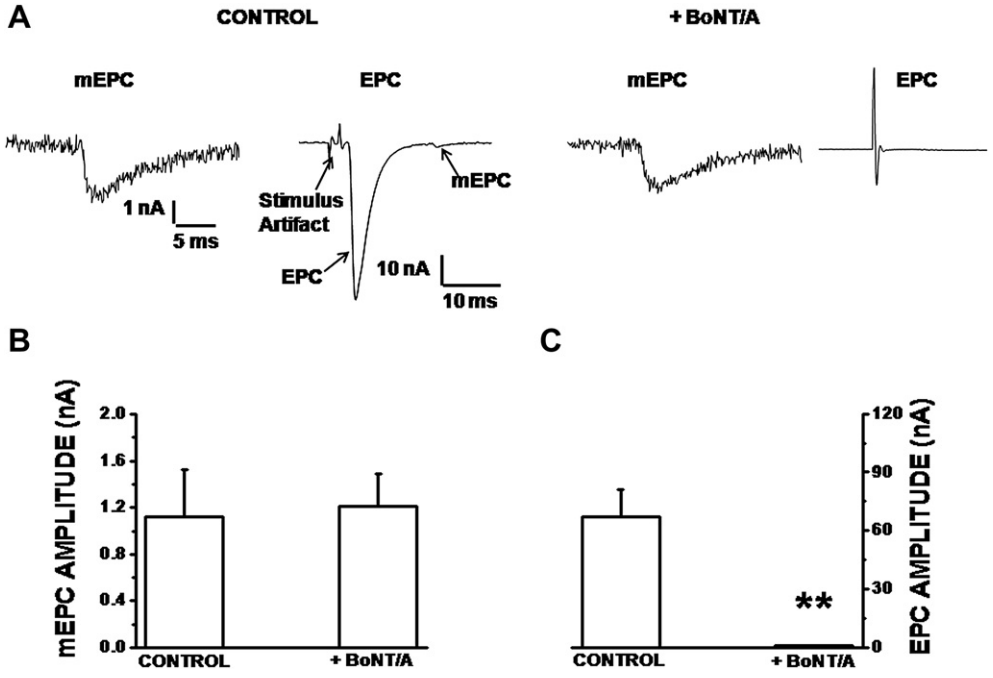

Fig. 2. Acute BoNT/A treatment decreases the amplitude of EPCs but not mEPCs of nerve-mucle preparations *in vitro*. A. Representative traces of mEPCs and EPCs of control and BoNT/A (10 pM for 90 min, 22 °C) treated *Triangularis sterni* nerve-muscle preparations. Nerve terminal uptake of BoNT/A was stimulated by co-incubation in 40 mM KCl containing HRS. Control preparations were treated with 40 mM KCl containing HRS for 90 min followed by wash with HRS for 30 min. B and C summarize mEPC and EPC amplitudes of control ($n = 5$ muscles, 18 endplates; 48 EPCs) and BoNT/A ($n = 6$ muscles, 25 endplates; 101 EPCs) treated preparations, respectively.

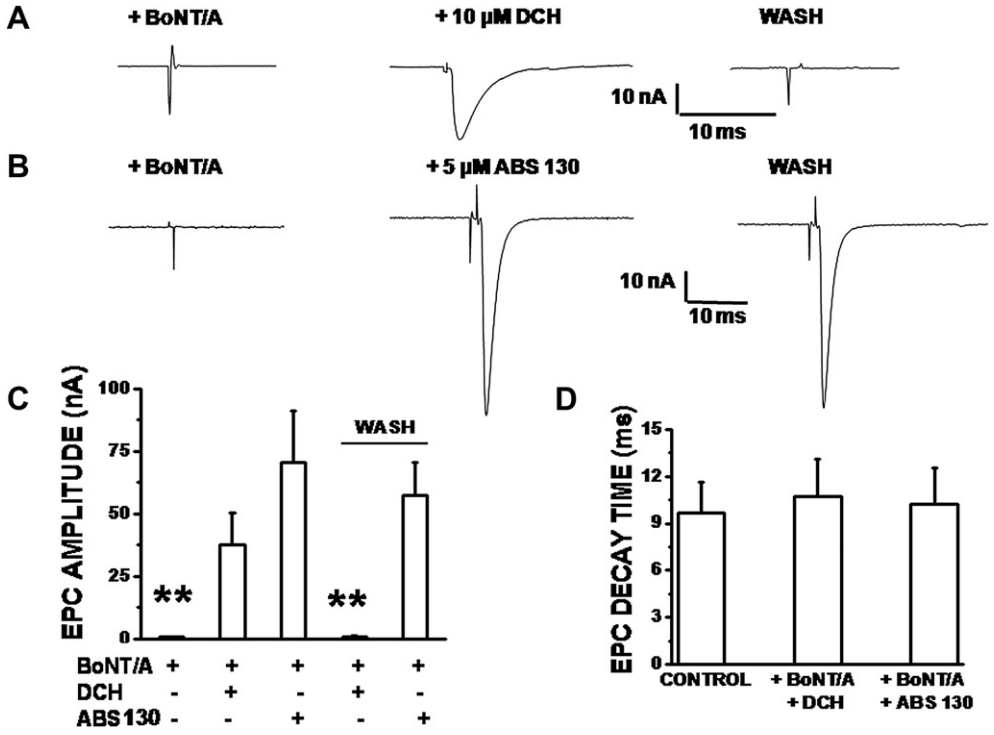

Fig. 3. DCH and ABS 130 reverse BoNT/A mediated inhibition of acetylcholine release. A and B. Representative traces of EPCs following exposure of *Triangularis sterni* nerve-muscle preparations to BoNT/A (10 pM for 90 min in 40 mM KCl containing physiological solution, 22 °C; left) and at 40–90 min after exposure to 10 μM DCH or 5 μM ABS 130. Wash with hydroxamate-free solution reversed the effect of DCH but not ABS 130. C. EPC amplitudes of BoNT/A alone ($n = 6$ muscles, 25 endplates, 101 EPCs), DCH following BoNT/A ($n = 6$, 28, 142), ABS 130 following BoNT/A ($n = 3$, 12, 34), and after the washout of DCH or ABS 130. D. Decay time of EPCs in control and +BoNT/A + DCH or +BoNT/A + ABS 130 treated preparations.

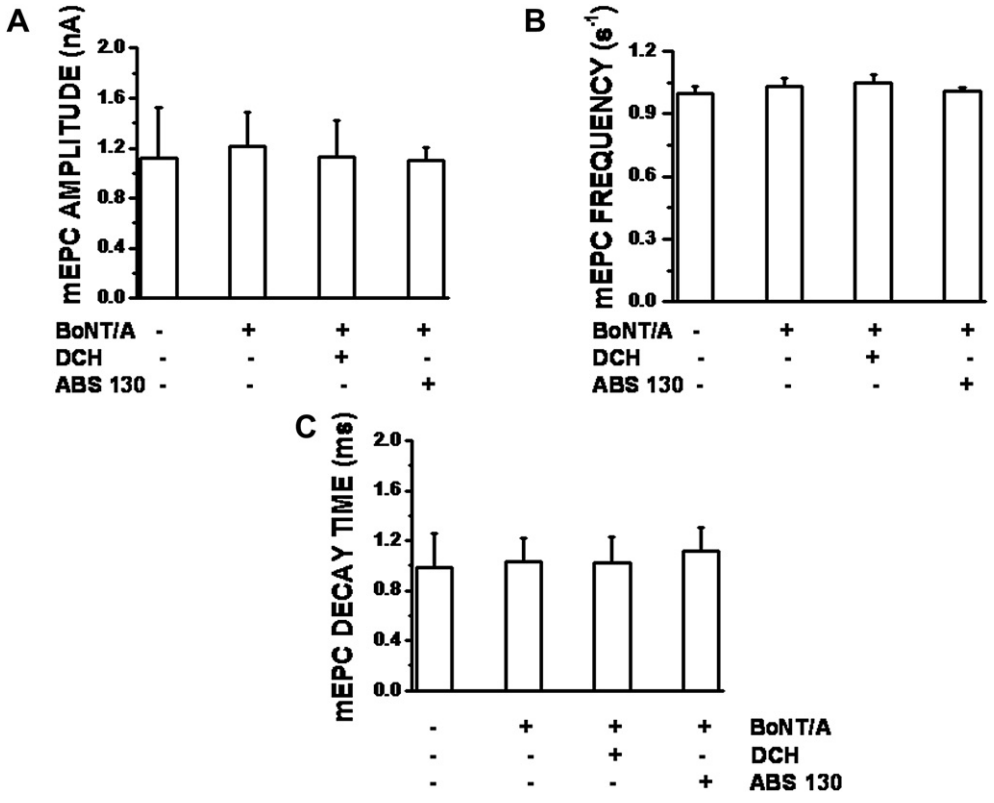

Fig. 4. Neither DCH nor ABS 130 affects amplitude (A), frequency (B), and decay time (C) of mEPCs of nerve-muscle preparations acutely treated with BoNT/A *in vitro*. For each condition, the number of muscles, endplates, mEPCs measured was: no treatment (−BoNT/A, −DCH, and−ABS 130), 5, 18, 660; BoNT/A alone (BoNT/A+), 6, 25, 1175; BoNT/A + DCH, 6, 28, 1204; BoNT/A + ABS 130, 3, 12, 404.

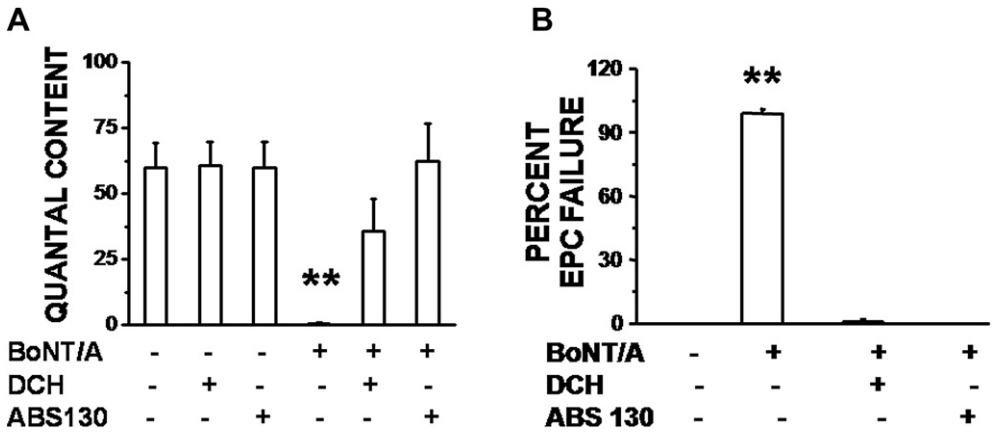

Fig. 5. DCH and ABS 130 significantly increase stimulus-evoked release of acetylcholine from motor nerve endings acutely poisoned with BoNT/A *in vitro*. A. Neither DCH nor ABS 130 alone altered the mean quantal content of EPCs. However, both DCH and ABS 130 increased quantal content for TS NMPs acutely poisoned with BoNT/A *in vitro*. The number of muscles and endplates examined in each condition was: control (−BoNT/A, −DCH, −ABS 130) 5, 18; DCH alone, 3, 20; ABS 130 alone, 3, 18; BoNT/A alone, 6, 25; DCH after BoNT/A, 6, 28; ABS 130 after BoNT/A, 3, 12. Bars represent mean ± S.E.M. B. Both DCH and ABS 130 significantly reduce the incidence of EPC failures in response to repetitive nerve stimulation. The number of muscles and endplates examined in each condition was: control (−BoNT/A, −DCH, −ABS 130) 5, 18; after BoNT/A alone, 6, 25; DCH after BoNT/A, 6, 25; ABS 130 after BoNT/A, 3, 12.

equilibrate at 22 °C for 30 min prior to stimulation at 1 Hz. The muscle twitch was recorded for 15 min, prior to adding 10 μM DCH or 5 μM ABS 130 to the muscle bath. Both DCH and ABS 130 produced a gradual recovery of twitch tension beginning at 90 min. At 120 min after DCH or ABS 130 application the average twitch tension increased to a steady state value of 68.67 ± 0.72 mg ($n = 4$) and 72.89 ± 5.05 mg ($n = 5$), respectively (Fig. 8A and B). Exposure of these preparations to 50 μM DAP did not further increase twitch tension (Fig. 8B). That is, the average twitch tension after adding 50 μM DAP to nerve-muscle preparations already treated for 90 min with either DCH or ABS 130 was 57.12 ± 3.26 mg ($n = 3$). However, the response to direct stimuli was not altered in these preparations either after BoNT/A or after DCH, ABS 130 or DAP. In separate studies at 37 °C, twitch tension of BoNT/A poisoned preparations increased to a steady state level within 10 min after the application of 50 μM DAP alone. However, this steady state level of twitch tension began to decline at 20 min after DAP application.

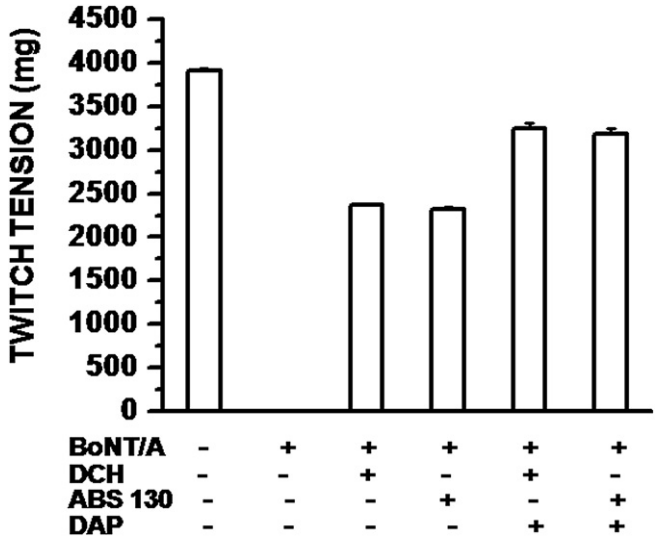

Fig. 6. DCH and ABS 130 significantly restore twitch tension to hemidiaphragm nerve-muscle preparations acutely poisoned with BoNT/A at 22 °C. Twitch tension (mg) ± S.E.M. for control ($n = 6$), 90 min after BoNT/A ($n = 6$), 90 min after 10 μM DCH or 5 μM ABS 130 following BoNT/A ($n = 3$), and at 50 min after additional application of 50 μM DAP to the DCH and ABS 130 treated preparations ($n = 3$).

Fig. 8C indicates that neither 10 μM DCH nor 5 μM ABS 130 alone altered peak twitch tension of control hemidiaphragm preparations. This agrees with the lack of effect of DCH and ABS 130 alone on stimulus-evoked transmitter release (Fig. 5).

Since DAP treatment after DCH or ABS 130 did not further increase the twitch tension, we compared the effect of DAP alone in preparations that were paralyzed with BoNT/A at 22 °C and 37 °C. As summarize in Fig. 6, the average twitch tension of control hemidiaphragm preparation was 3908.22 ± 33.9 mg ($n = 6$) at 22 °C. This value declined to 0 mg ($n = 6$) at 90 min after application of 10 pM BoNT/A. Fig. 9 A and C show that addition of 50 μM DAP alone increased the twitch tension of preparations paralyzed with BoNT/A at 22 °C to 80 ± 12% (3126.24 ± 46 mg; $n = 3$) of control.

Control preparations had an initial twitch tension of 3646.15 ± 23.94 mg at 37 °C. After 90 min of 1 Hz stimulation in the presence of 10 pM BoNT/A, twitch tension declined to 0 mg. Addition of 50 μM DAP restored twitch tension to only 4.5 ± 0.54% (125.42 ± 3.32 mg; $n = 3$) of control (Fig. 9B). Increase of the DAP to 100 μM did not produce an additional restoration of twitch tension for preparations poisoned with BoNT/A at 22 °C or 37 °C (Fig. 9D).

Regardless of the temperature, twitch tension was partially restored by DAP alone with a half time of 6 min. DAP induced twitches increased to a steady state which lasted for as long as 4 h in preparation treated with BoNT/A at 22 °C while the less dramatic facilitatory effect of DAP at 37 °C lasted for only 20 min.

DAP enhances synaptic transmission at the neuromuscular junction by blocking K^+ channels and prolonging Ca^{2+} influx *via* voltage dependent Ca^{2+} channels (Cho and Meriney, 2006). Since DAP enhances neurotransmitter release in BoNT/A poisoned nerve-muscle preparations (Adler et al., 2000, 1996, 1995), we compared the effects of DAP, DCH, and ABS 130 on Ca^{2+} influx into cholinergic Neuro 2a cells continuously superfused with HRS. At 50 s after beginning to record fluorescent intensity, we changed the superfusion solution to one containing 10 μM DCH, 5 μM ABS 130 or 50 μM DAP. Neither 10 μM DCH ($n = 117$) nor 5 μM ABS 130 ($n = 84$) increased intracellular Ca^{2+}. In contrast, 50 μM DAP ($n = 74$) significantly increased the intracellular Ca^{2+} level (Fig. 10).

To test whether DAP can increase intracellular Ca^{2+} after DCH or ABS 130 treatment, we perfused Neuro 2a cells with 10 μM DCH or 5 μM ABS 130 in the presence of 1.8 mM extracellular Ca^{2+} before

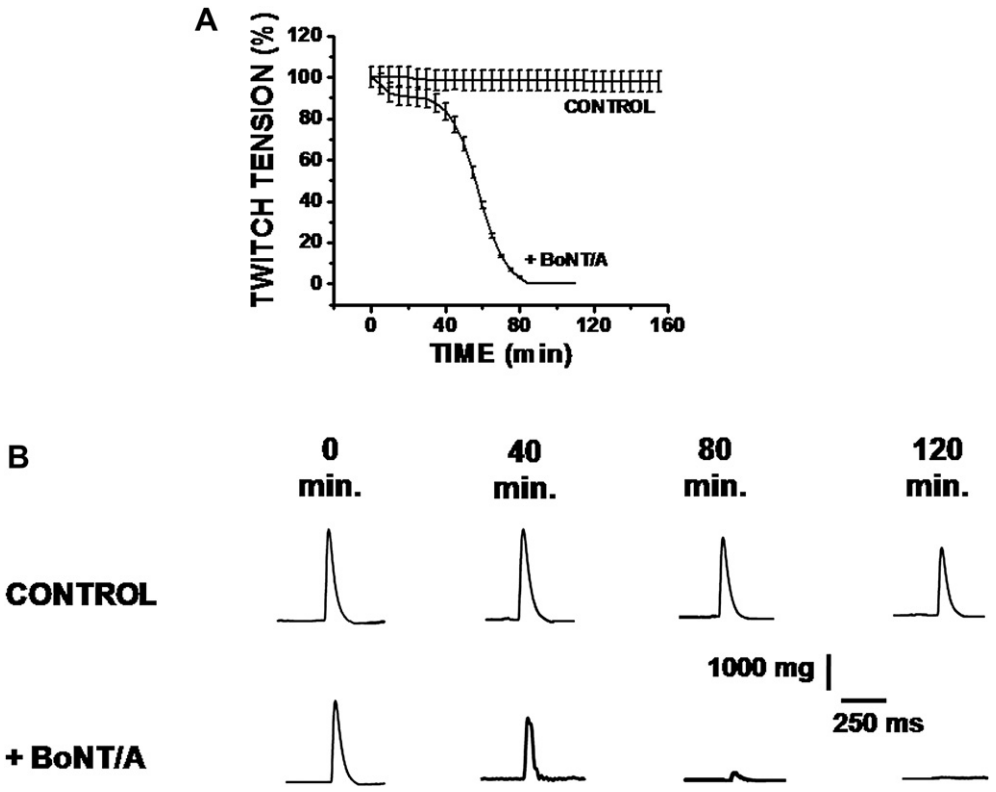

Fig. 7. Acute BoNT/A treatment inhibits twitch tension of nerve-muscle preparations *in vitro*. A. Twitch tension, as percent of the initial value, plotted against time after addition of 10 pM BoNT/A to the bathing solution (37 °C); the control curve represents the mean ± S.E.M for 12 preparations and the +BoNT/A curve represents the mean ± S.E.M. for 14 preparations. A supramaximal stimulus applied to the phrenic nerve at a frequency of 1 Hz initiated muscle twitches. B. Representative twitches of control and BoNT/A poisoned preparations at 0, 40, 80, 120, and 160 min, respectively, after application of 10 pM BoNT/A.

the addition of DAP. While neither DCH ($n = 36$) nor ABS 130 ($n = 33$) increased intracellular Ca^{2+}, addition of 50 μM DAP after DCH ($n = 38$) or ABS 130 ($n = 42$) treatment significantly increased intracellular Ca^{2+} levels (Fig. 11).

Next we tested the ability of DCH or ABS 130 to restore neurotransmitter release in NMPs isolated from animals that were poisoned with BoNT/A *in vivo*. For this, we injected BoNT/A into mice at the innervation region of the EDL muscle. EDL muscle. EDL NMPs were harvested 24 h post BoNT/A exposure and used for electrophysiologic study. Neither DCH (10 μM) nor ABS 130 (5 μM) application restored EPCs.

Finally, we tested for *in vivo* prophylactic and therapeutic effects of DCH and ABS 130 by injecting DCH or ABS 130 (3 μl of 10 mM stock) either 2 h before or 12 h after BoNT/A injections (3 μl of 6.67 pM stock) in a region close to nerve entry into the EDL (Table 1). Control mice received only vehicle (buffer) devoid of BoNT/A. Toe spread reflex scoring (Thyagarajan et al., 2009) at 48 h post BoNT/A injection was measured to evaluate the effects of DCH and ABS 130. Mice ($n = 12$) were unable to spread their toes at 12 h after injection of BoNT/A alone. Injection of either DCH ($n = 4$) or ABS 130 ($n = 5$) before BoNT/A did not protect the toe spread reflex. Similarly, DCH ($n = 6$) or ABS 130 ($n = 4$) injected 12 h after BoNT/A did not antagonize, restore or prevent inhibition of the toe spread reflex.

4. Discussion

Cleavage of SNARE proteins is widely regarded as the mechanism whereby BoNTs inhibit stimulus-evoked ACh release from motor nerve endings (Adler et al., 2001; Dolly, 2003; Keller and Neale, 2001). Therefore, MEIs may serve as antidotes to BoNTs. To test this hypothesis, several laboratories have prepared small peptides as well as organic molecules that prevent BoNT/A cleavage of purified SNAP-25 (Aiken et al., 1992; Boldt et al., 2006; Eubanks et al., 2007; Schmidt and Stafford, 2002). Preliminary data suggest that peptide inhibitors of BoNT/A endoprotease have limited *in vivo* prophylactic or therapeutic efficacy for muscles paralyzed with BoNT/A (McArdle et al., 2007). Furthermore, protection of mice against the lethal action of BoNT/A was most pronounced for DCH, the least potent member of a series of small molecule BoNT/A MEIs (Eubanks et al., 2007). The present study derives from the hypothesis that MEIs, which may be useful in the prevention or therapy of BoNT/A-induced muscle paralysis, will restore functional synaptic transmission. Therefore, we examined the effect of DCH and its methyl derivative, ABS 130, on transmission across neuromuscular junctions acutely poisoned *in vitro* with BoNT/A as well as on the function of muscles paralyzed with BoNT/A *in vivo*.

For *in vitro* poisoning at 22 °C, isolated nerve-muscle preparations were exposed to 40 mM KCl to enhance spontaneous vesicular release of ACh and facilitate endocytosis of BoNT/A. When BoNT/A-free normal physiologic solution was reapplied at 90 min after exposure to the neurotoxin loading solution, EPCs failed, quantal content was dramatically reduced, and twitches were prevented. While inhibition of neuromuscular transmission persisted for the lifetime of BoNT/A treated *in vitro* preparations, EPC amplitude began to increase at 40–90 min after application of either 10 μM DCH or 5 μM ABS 130. The restoration of transmission progressed so that twitches resulted from nerve stimulation.

The delayed increase of EPC amplitude after DCH and ABS 130 was not accompanied with a change in the amplitude or the decay

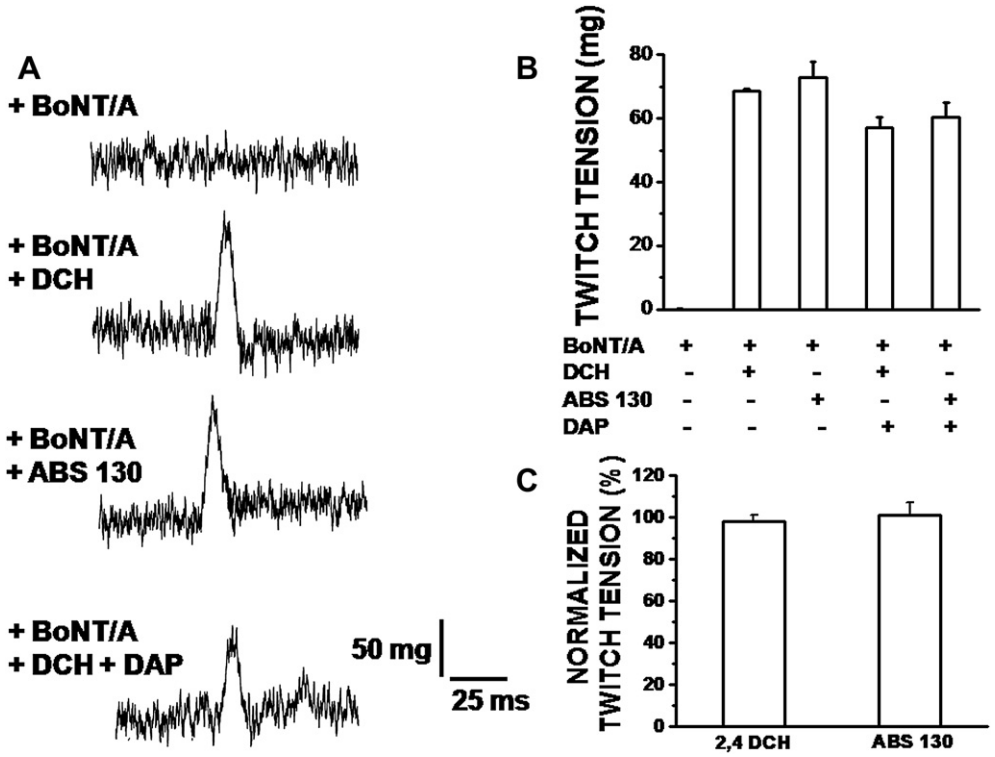

Fig. 8. DCH and ABS 130 partially restore twitch tension to hemidiaphragm nerve-muscle preparations acutely poisoned with BoNT/A at 37 °C. A. Representative twitches for control (BoNT/A), 10 μM DCH after BoNT/A, 5 μM ABS 130 after BoNT/A, and 50 μM 3,4 diaminopyridine (DAP) after DCH and BoNT/A or ABS 130 and BoNT/A. B. Mean ± S.E.M. of twitch tension at 90 min after BoNT/A for: BoNT/A alone ($n = 14$), 90 min after DCH following BoNT/A ($n = 4$), 90 min after ABS 130 following BoNT/A ($n = 5$), at 50 min after additional application of DAP to DCH treated preparations ($n = 3$) and at 50 min after additional application of DAP to ABS 130 treated preparations ($n = 3$). C. Normalized twitch tension for control preparations at 90 min after application of DCH or ABS 130 alone.

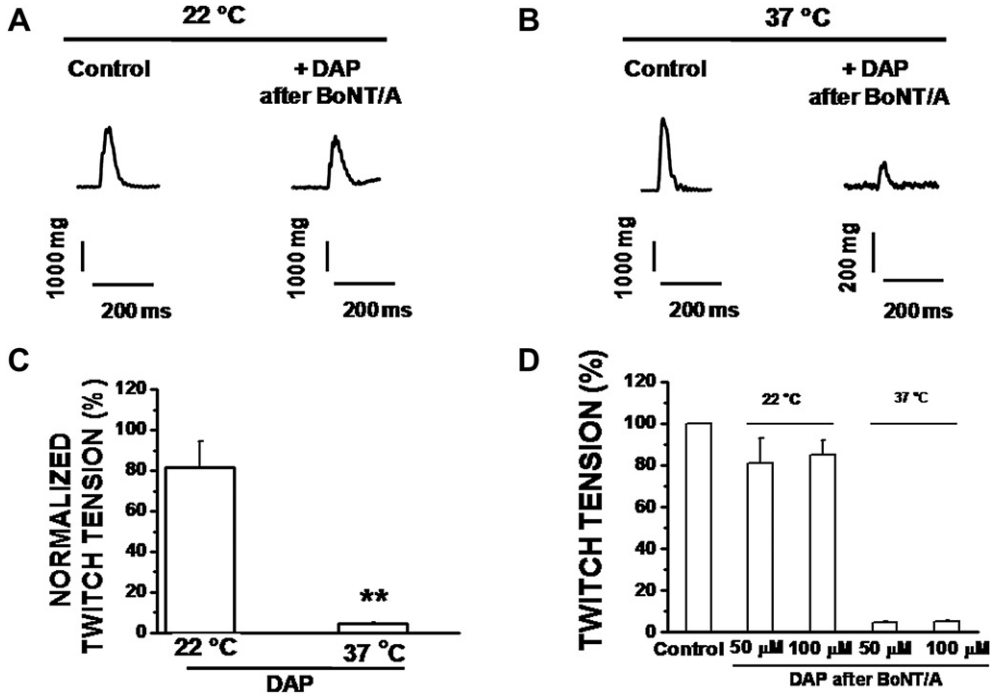

Fig. 9. DAP significantly restores twitch tension to hemidiaphragm nerve-muscle preparations at 22 °C but not at 37 °C. A and B. Representative twitches for control (no BoNT/A) and 50 μM DAP after BoNT/A ($n = 3$) at 22 °C and 37 °C respectively. C. Normalized twitch for nerve-muscle preparations treated with DAP at 90 min after BoNT/A at 22 °C (or at 37 °C). D. Twitch tension normalized to control for nerve-muscle preparations treated with 50 μM or 100 μM DAP at 90 min after BoNT/A treatment at 22 °C or 37 °C.

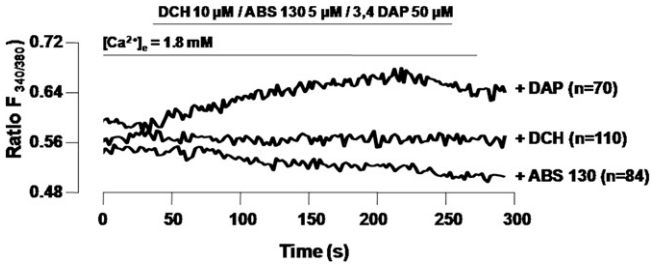

Fig. 10. Unlike DAP, neither DCH nor ABS 130 increase intracellular Ca^{2+} levels of Neuro 2a cells. Each trace represents the time course of average change in fluorescence ratio before and after exposure to 50 μM DAP ($n = 70$ cells), 10 μM DCH ($n = 110$) or 5 μM ABS 130 ($n = 84$).

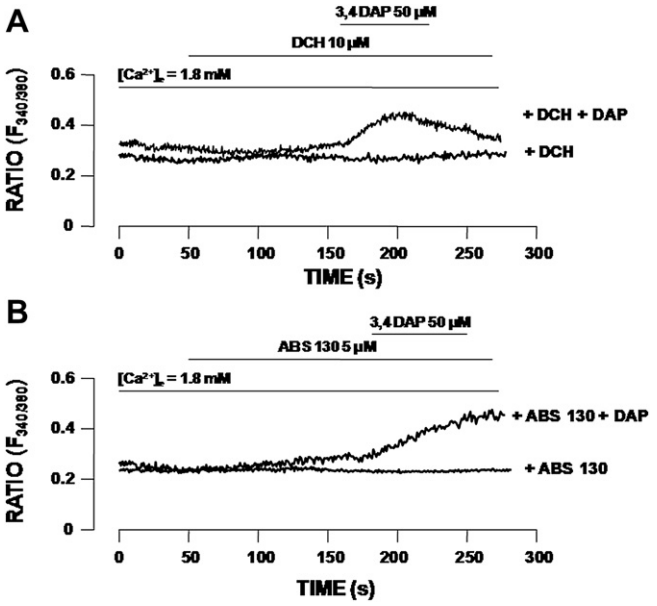

Fig. 11. DAP increases intracellular Ca^{2+} levels in DCH or ABS 130 treated Neuro 2a cells. Each trace represents the time course of average change in fluorescence ratio before and after the treatment of 10 μM DCH (A; $n = 38$) or 10 μM DCH + 50 μM DAP (A; $n = 36$) or 5 μM ABS 130 (B; $n = 42$) or 5 μM ABS 130 + 50 μM DAP (B; $n = 33$).

time of mEPCs. Thus, the hydroxamates do not alter acetylcholinesterase activity. This apparent presynaptic action of DCH and ABS 130 was unique to BoNT/A intoxicated motor nerve terminals since neither compound altered the quantal content of control preparations. However, increasing the ABS 130 concentration to 10 μM greatly decreased EPC amplitude so that mEPCs and EPCs were no longer observed at 3 h after treatment. On the other hand, increasing the concentration of DCH from 10 μM to 20 μM neither inhibited the EPCs nor decreased the time lag prior to an increase of EPC amplitude after acute BoNT/A poisoning.

The 40–50 min delay prior to an increase of transmitter release can be attributed to slow distribution of DCH and ABS 130 into the motor nerve terminal compartment. Once the MEIs inhibit ongoing SNAP-25 cleavage, several processes may restore transmitter release. For example, the peripheral nerve stump may deliver SNAP-25 to active zones. This would require that the peripheral stump of the isolated nerve-muscle preparation contains SNAP-25 which it can traffic to appropriate nerve terminal sites. Alternatively, inhibition of further BoNT/A endoprotease activity may facilitate an exocytic event which is less dependent upon intact SNAP-25.

The restorative effects of DCH, ABS 130 or DAP on twitch tension inhibited by BoNT/A depend on the temperature at which BoNT/A uptake occurs. That is, for preparations that were treated with BoNT/A, at 22 °C, DCH, ABS 130 or DAP treatment significantly increased the twitch tension. In striking contrast, the restorative effects of DCH, ABS 130 or DAP were much less at 37 °C. As suggested in the model of Fig. 12, the rates of internalization of BoNT/A, translocation of BoNT/A Lc, and cleavage of SNAP-25 may be slower at 22 °C (Simpson, 1982). At 37 °C, the rates of internalization, translocation and SNAP-25 cleavage are enhanced and this may significantly reduce the restorative effects of DCH, ABS 130 or DAP. However, antagonism of BoNT/A inhibition of neurotransmission at 22 °C suggests an unknown signaling molecule that is essential for neurotransmitter release and, like SNAP-25, is more sensitive to BoNT/A at 37 °C. Previous observations demonstrating that the extent of synaptic blockade produced by BoNT/A is greater than the extent of SNAP-25 proteolysis (Keller and Neale, 2001) further supports the existence of another, yet unidentified, target of BoNT/A.

For nerve-muscle preparations paralyzed with BoNT/A at 22 °C, DCH and ABS 130 initiated an increase of quantal content before recovery of the muscle twitch. This is not surprising since microelectrode recording samples a small population of surface muscle fibers which respond quickly to bath applied substances. Since the recovery of the twitch prevented further intracellular recording, we did not estimate the percentage of BoNT/A poisoned nerve terminals capable of stimulus-evoked transmitter release after treatment with DCH or ABS 130. Treatment with MEIs following BoNT/A exposure at 22 °C resulted in restoration of muscle twitch up to 50% of the controls. However, the fact that these MEIs restored only a fraction of the initial twitch tension of the MNEs poisoned with BoNT/A at 37 °C suggests the need for caution in extrapolating an anti-BoNT/A effect observed at a single neuromuscular junction to an entire muscle at physiological temperature.

In contrast to the slow onset of action for DCH and ABS 130, DAP quickly but partially restored neurotransmission to BoNT/A poisoned neuromuscular junctions. This can be attributed to the greater accessibility of motor nerve terminal K^+ channels which DAP must inhibit, as compared to the intracellular BoNT/A Lc metalloendoprotease which DCH and ABS 130 presumably inhibit in order to restore neurotransmitter release. A mechanism of DCH and ABS 130 distinct from K^+ channel block is further indicated by the fact that the hydroxamates, unlike DAP, did not increase Ca^{+2} levels within cholinergic Neuro 2a cells. This study also indicates that DAP applied after DCH or ABS 130 did not produce a further restoration of twitch tension when BoNT/A treatments were made at 37 °C. Thus, although MEIs and K^+ channel blockers act by distinct mechanisms, they did not produce additive or synergistic restoration of neuromuscular transmission. These findings suggest that restoration of ACh release from motor nerve endings which are acutely poisoned with BoNT/A has a limit which cannot be

Table 1
Effect of DCH and ABS 130 injected before (column A) or after (column B) BoNT/A on the toe spread reflex (TSR).

	PRE – BoNT/A TREATMENT (A)	POST – BoNT/A TREATMENT (B)
Control (No BoNT/A)	6/6 (60/60)	6/6 (60/60)
+BoNT/A (3 μl of 6.67 pM)	12/12 (0/120)	12/12 (0/120)
DCH (3 μl of 1 mM)	4/4 (0/40)	6/6 (0/60)
ABS 130 (3 μl of 1 mM)	5/5 (0/50)	4/4 (0/40)

Numbers in columns A and B represent the number of mice treated as indicated in the column to the left. Numbers in parentheses are the total number of toes that mice could spread for control (vehicle injected), BoNT/A alone, DCH or ABS 130 treated mice. DCH or ABS 130 was injected either 2 h before (A) or 12 h after (B) BoNT/A injection.

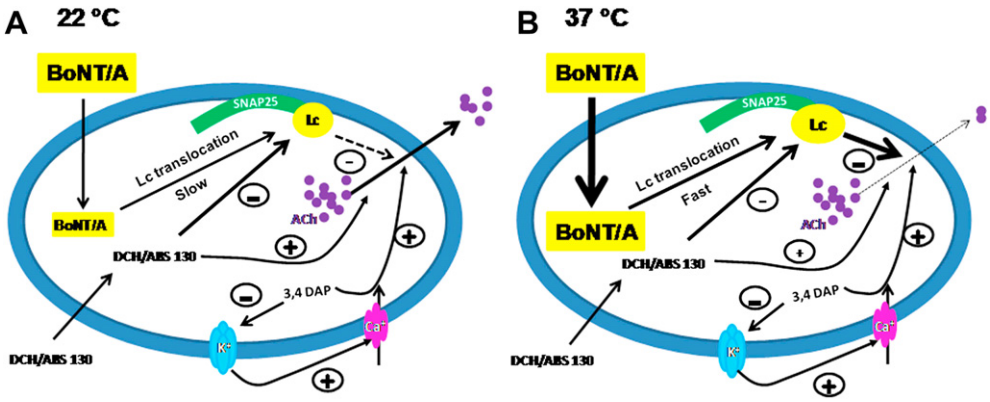

Fig. 12. Hypothetical model representing the effects of MEIs (DCH and ABS 130), 3,4 DAP, and temperature on the inhibition of acetylcholine release by BoNT/A. A, at 22 °C, internalization of BoNT/A Lc causes cleavage of SNAP-25. This reduces exocytosis of acetylcholine (ACh). Treatment with MEIs inhibits BoNT/A Lc cleavage of SNAP-25 and enables ACh release. Addition of DAP inhibits K^+ channels which increases influx of Ca^{2+}. This results in restoration of ACh release. B, at 37 °C, more efficient uptake of BoNT/A occurs into the motor nerve terminals. This facilitates faster translocation of BoNT/A Lc and more efficient cleavage of SNAP-25 which in turn inhibits ACh release. Treatment with MEIs only partially restores ACh release and this restoration is substantially lower at 37 °C than at 22 °C since SNAP-25 cleavage by BoNT/A is more efficient at the more physiological temperature. Similarly, addition of DAP either alone or after MEIs results in partial restoration of ACh release.

overcome by drugs with distinct pharmacological actions. As a result, the benefit derived from drugs which activate ACh release from acutely BoNT/A poisoned motor nerve endings may be limited in magnitude under physiologic conditions.

Conversely, in preparations that were treated with BoNT/A at 22 °C, both DCH and ABS 130 enhanced the stimulus-evoked twitches and further treatment with DAP increased the amplitude of twitch tension. The differences between the effects of DAP at 22 °C and at 37 °C can be attributed to a greater proteolytic activity of BoNT/A Lc at the more physiologic temperature (Fig. 12). The greater SNAP-25 proteolysis at 37 °C may limit the restorative effects of DAP.

This temperature effect may account for the inability of DCH and ABS 130 to restore neuromuscular transmission to EDL preparations which were isolated at 24 h after *in vivo* BoNT/A injection. In contrast, DAP did reactivate neuromuscular transmission for such EDL preparations. Thus, a relatively brief window of opportunity may exist during which MEIs can restore neuromuscular transmission. In contrast, the transmitter release process remains responsive to K^+ channel block with DAP for longer times after poisoning with BoNT/A (Adler et al., 2000, 1996, 1995). The prolonged capacity for reactivation of transmitter release suggests that DAP can restore neurotransmission even after cleavage of a critical quantity of SNAP-25. In contrast, MEIs may have limited effectiveness once the critical level of SNAP-25 is digested. However, MEIs may facilitate neuron-mediated recovery (Rogozhin et al., 2008). That is, motor neuron replacement of SNAP-25 will be delayed as long as endoprotease remains active in the motor nerve ending. As a consequence, inhibition of long lived intraterminal endoprotease activity may enhance the rate at which the motor neuron restores terminal levels of SNAP-25 as well as neuromuscular transmission.

The tests for *in vivo* prophylactic and therapeutic effects of DCH or ABS 130 were negative. That is, injection of these MEIs at 2 h prior to BoNT/A failed to prevent inhibition of the toe spread reflex. Similarly, DCH or ABS 130 injected 12 h after BoNT/A did not prevent reduction or initiate restoration of the TSR (Thyagarajan et al., 2009). These data suggest that the *in vitro* benefit of DCH and ABS 130 does not translate into a prophylactic or therapeutic effect. A simple explanation for this discrepancy is that the dose or timing of administration of hydroxamates was not appropriate to achieve an *in vivo* pharmacological effect. The issue of pharmacokinetics complicates the study of all pharmacologic treatments for BoNT/A and remains to be examined for MEIs.

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References

Adler, M., Scovill, J., Parker, G., Lebeda, F.J., Piotrowski, J., Deshpande, S.S., 1995. Antagonism of botulinum toxin-induced muscle weakness by 3,4-diaminopyridine in rat phrenic nerve-hemidiaphragm preparations. Toxicon 33, 527–537.

Adler, M., Macdonald, D.A., Sellin, L.C., Parker, G.W., 1996. Effect of 3,4-diaminopyridine on rat extensor digitorum longus muscle paralyzed by local injection of botulinum neurotoxin. Toxicon 34, 237–249.

Adler, M., Capacio, B., Deshpande, S.S., 2000. Antagonism of botulinum toxin A-mediated muscle paralysis by 3,4-diaminopyridine delivered via osmotic minipumps. Toxicon 38, 1381–1388.

Adler, M., Keller, J.E., Sheridan, R.E., Deshpande, S.S., 2001. Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle. Toxicon 39, 233–243.

Aiken, S.P., Sellin, L.C., Schmidt, J.J., Weinstein, S.A., McArdle, J.J., 1992. A novel peptide toxin from *Trimeresurus wagleri* acts pre- and post-synaptically to block transmission at the rat neuromuscular junction. Pharmacol. Toxicol. 70, 459–462.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L., Tonat, K., 2001. Botulinum toxin as a biological weapon: medical and public health management. JAMA 285, 1059–1070.

Boldt, G.E., Eubanks, L.M., Janda, K.D., 2006. Identification of a botulinum neurotoxin A protease inhibitor displaying efficacy in a cellular model. Chem. Commun. (Camb.), 3063–3065.

Capkova, K., Hixon, M.S., McAllister, L.A., Janda, K.D., 2008. Toward the discovery of potent inhibitors of botulinum neurotoxin A: development of a robust LC MS based assay operational from low to subnanomolar enzyme concentrations. Chem. Commun. (Camb.), 3525–3527.

Cho, S., Meriney, S.D., 2006. The effects of presynaptic calcium channel modulation by roscovitine on transmitter release at the adult frog neuromuscular junction. Eur. J. Neurosci. 23, 3200–3208.

Dolly, O., 2003. Synaptic transmission: inhibition of neurotransmitter release by botulinum toxins. Headache 43 (Suppl. 1), S16–S24.

Eubanks, L.M., Hixon, M.S., Jin, W., Hong, S., Clancy, C.M., Tepp, W.H., Baldwin, M.R., Malizio, C.J., Goodnough, M.C., Barbieri, J.T., Johnson, E.A., Boger, D.L., Dickerson, T.J., Janda, K.D., 2007. An *in vitro* and *in vivo* disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. Proc. Natl. Acad. Sci. USA 104, 2602–2607.

Fischer, A., Nakai, Y., Eubanks, L.M., Clancy, C.M., Tepp, W.H., Pellett, S., Dickerson, T.J., Johnson, E.A., Janda, K.D., Montal, M., 2009. Bimodal modulation of the botulinum neurotoxin protein-conducting channel. Proc. Natl. Acad. Sci. USA 106, 1330–1335.

Keller, J.E., Neale, E.A., 2001. The role of the synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. J. Biol. Chem. 276, 13476–13482.
McArdle, J.J., Angaut-Petit, D., Mallart, A., Bournaud, R., Faille, L., Brigant, J.L., 1981. Advantages of the triangularis sterni muscle of the mouse for investigations of synaptic phenomena. J. Neurosci. Methods 4, 109–115.
McArdle, J.J., Potian, J.G., Hognason, K., Lebeda, F. and Schmidt, J.J., 2007. Peptide inhibitors of botulinum neurotoxin A zinc metalloendoprotease as therapeutic agents. Abstract presented at Interagency botulinum Research Coordinating Committee Meeting, Oct 14–18, 2007, Asilomar Conference Ground, CA.
Montal, M.S., Blewitt, R., Tomich, J.M., Montal, M., 1992. Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins. FEBS Lett. 313, 12–18.
Montecucco, C., Papini, E., Schiavo, G., 1994. Bacterial protein toxins penetrate cells via a four-step mechanism. FEBS Lett. 346, 92–98.
Montecucco, C., 1986. How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem. Sci. 11, 314–317.
Rogozhin, A.A., Pang, K.K., Bukharaeva, E., Young, C., Slater, C.R., 2008. Recovery of mouse neuromuscular junctions from single and repeated injections of botulinum neurotoxin A. J. Physiol. 586, 3163–3182.
Schmidt, J.J., Stafford, R.G., 2002. A high-affinity competitive inhibitor of type A botulinum neurotoxin protease activity. FEBS Lett. 532, 423–426.
Simpson, L.L., 1982. The interaction between aminoquinolines and presynaptically acting neurotoxins. J. Pharmacol. Exp. Ther. 222, 43–48.
Souayah, N., Karim, H., Kamin, S.S., McArdle, J., Marcus, S., 2006. Severe botulism after focal injection of botulinum toxin. Neurology 67, 1855–1856.
Thyagarajan, B., Krivitskaya, N., Potian, J.G., Hognason, K., Garcia, C.C., McArdle, J.J., 2009. Capsaicin protects mouse neuromuscular junctions from the neuroparalytic effects of botulinum neurotoxin A. J. Pharmacol. Exp. Ther. 331, 361–371.