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Reversal of BoNT/A-mediated inhibition of muscle paralysis by 3,4-diaminopyridine and roscovitine in mouse phrenic nerve-hemidiaphragm preparations ☆

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ABSTRACT

Botulinum neurotoxins (BoNTs) comprise a family of neurotoxic proteins synthesized by anaerobic bacteria of the genus *Clostridium*. Each neurotoxin consists of two polypeptide chains: a 100 kDa heavy chain, responsible for binding and internalization into the nerve terminal of cholinergic motoneurons and a 50 kDa light chain that mediates cleavage of specific synaptic proteins in the host nerve terminal. Exposure to BoNT leads to cessation of voltage- and Ca^{2+}-dependent acetylcholine (ACh) release, resulting in flaccid paralysis which may be protracted and potentially fatal.

There are no approved therapies for BoNT intoxication once symptoms appear, and specific inhibitors of the light chain developed to date have not been able to reverse the consequences of BoNT intoxication. An alternative approach for treatment of botulism is to focus on compounds that act by enhancing ACh release. To this end, we examined the action of the K^+ channel blocker 3,4-diaminopyridine (3,4-DAP) in isolated mouse hemidiaphragm muscles intoxicated with 5 pM BoNT/A. 3,4-DAP restored tension within 1–3 min of application, and was effective even in totally paralyzed muscle. The Ca^{2+} channel activator (R)-roscovitine (Ros) potentiated the action of 3,4-DAP, allowing for use of lower concentrations of the K^+ channel blocker. In the absence of 3,4-DAP, Ros was unable to augment tension in BoNT/A-intoxicated muscle. This is the first report demonstrating the efficacy of the combination of 3,4-DAP and Ros for the potential treatment of BoNT/A-mediated muscle paralysis.

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1. Introduction

The seven serotypes of botulinum neurotoxin (BoNT) are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality (Arnon et al., 2001; Lindström and Korkeala, 2006; Rega et al., 2010). The neurotoxins are produced by spore forming anaerobic bacteria, chiefly *Clostridium botulinum*, and by a limited number of other clostridial strains (Simpson, 2004; Sobel, 2005). BoNTs are secreted initially as relatively inactive ~150 kDa protoxins (range 140–167 kDa), surrounded by a complex of neurotoxin-associated proteins that protect BoNT from degradation in the gastrointestinal tract (Kukreja and Singh, 2007; Gu et al., 2012). The protoxin is subsequently cleaved to form the active dichain neurotoxin, consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) (DasGupta and Sugiyama, 1972).

The BoNTs have three functional domains: binding, translocation and catalytic. Binding is mediated by the C-terminal region of the heavy chain, which interacts with gangliosides and protein receptors located on cholinergic nerve terminals (Dong et al., 2006; Benson et al., 2011); selective binding of BoNT to these receptors is responsible for the cholinergic selectivity of the BoNTs. The N-terminal region of the HC promotes translocation of the LC into the cytosol (Koriazova and Montal, 2003; Fischer and Montal, 2007). The LC is a Zn^{2+}-containing endopeptidase that cleaves specific sites on SNARE (soluble-*N*-ethylmaleamide sensitive factor attachment protein receptor) proteins, leading to a cessation of evoked transmitter release (Montecucco et al., 2005).

All seven serotypes of BoNT (A–G) inhibit release of acetylcholine (ACh) from cholinergic nerve terminals that innervate skeletal muscle, autonomic ganglia and post-ganglionic parasympathetic organs (Simpson, 2004). In skeletal muscle, inhibition of transmitter release leads to flaccid paralysis, which can progress to generalized

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muscle weakness and death when the muscles of respiration become sufficiently compromised.

Early signs of BoNT intoxication (botulism) include visual disturbances, difficulties in swallowing and impairment of speech (Sobel, 2005). At this stage, botulism can be treated by infusion of serotype specific antitoxin. However, when symptoms progress to generalized paralysis, antitoxin is no longer effective, and patients will need treatment in an intensive care facility (Tacket et al., 1984; Hatheway et al., 1984). The more severe cases may also require extensive periods of artificial ventilation, enteral feeding and physical therapy after discharge from intensive care (Shapiro et al., 1998; Robinson and Nahata, 2003; Marcus, 2009).

BoNT has numerous attributes that make it appealing to terrorists, including an unusually high potency, long duration of action and the potential to cause widespread panic with major social disruption (Arnon et al., 2001; Robinson and Nahata, 2003). Accordingly, BoNT has been classified as a Tier 1 select toxin by the U.S. Department of Health and Human Services, the only non-infectious agent to receive this designation.

Of the seven serotypes, BoNT/A has the highest potency and longest duration of action and it therefore represents the greatest bioterrorist threat (Arnon et al., 2001). A number of complementary approaches have been used to develop pharmacological antagonists for BoNT/A: these consist of synthesis and screening of small molecule inhibitors (SMIs) for their ability to inactivate the BoNT/A LC, strategies to enhance degradation of the LC from intoxicated nerve terminals and evaluation of physiological antagonists such as the K^+ channel blocker 3,4-diaminopyridine (3,4-DAP) and the Ca^{2+} channel activator (R)-roscovitine (Ros) that can overcome the inhibitory action of BoNT and restore ACh release. To date, SMIs have only been able to slow the rate of BoNT/A-mediated paralysis, but they have not proved effective in reversing the paralytic action of BoNT, and strategies to accelerate the removal of BoNT/A LC from the nerve terminal have not progressed beyond the proof-of-concept stage (Tsai et al., 2010). However, physiological antagonists have shown remarkable promise both in slowing the onset of muscle paralysis and in restoring tension in BoNT/A-paralyzed muscles (Molgó et al., 1980, 1987; Adler et al., 1995, 1996; Mayorov et al., 2010).

A notable advantage of developing 3,4-DAP and Ros for treatment of botulism is that both have an extensive history of clinical use: aminopyridines for the symptomatic treatment of multiple sclerosis, Lambert Eaton Myasthenic Syndrome (LEMS) and downbeat nystagmus (Shi and Sun, 2011; Kalla et al., 2011), and Ros for breast, ovarian and prostate cancer (Benson et al., 2007; Yarotsky and Elmslie, 2012). In addition, 3,4-DAP and other K^+ channel blockers have been investigated for treatment of human botulism, with some success (Puggiari and Cherington, 1978; Kalia and Swartz, 2011).

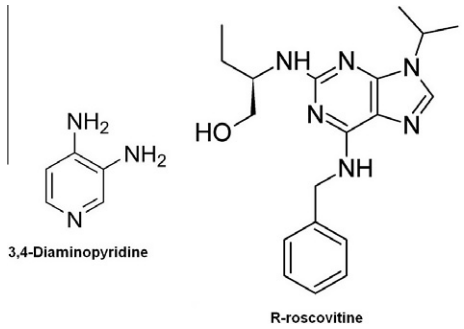

Fig. 1. Structure of the K^+ channel blocker 3,4-DAP and the Ca^{2+} channel activator Ros. 3,4-DAP (CAS No. 54-96-6) is a *N*-heterocyclic tertiary amine also known as amifampridine in clinical use. The phosphate salt was licensed in Europe as an orphan drug for treatment of rare muscle disorders such as LEMS in 2010. Ros (CAS No. 186692-46-6) is a 2,6,9-tri substituted purine analog that is marketed under the proprietary name Seliciclib primarily as an antitumor agent.

The current study was undertaken to examine the ability of 3,4-DAP and Ros (Fig. 1) to reverse muscle paralysis produced by BoNT/A in isolated mouse hemidiaphragm muscle. The results indicate that 3,4-DAP can restore muscle tension completely, and combinations of 3,4-DAP with Ros allow for use of lower, less toxic concentrations of the former. It is concluded that 3,4-DAP and Ros are promising lead compounds for the development of medical countermeasures for botulism. This is the first study examining the efficacy of Ros in BoNT intoxication, as well as the first showing efficacy of co-administering Ros and 3,4-DAP in reversing BoNT/A-mediated muscle paralysis.

2. Methods

2.1. Muscle preparation

Experiments were performed *in vitro* on isolated hemidiaphragm muscles dissected from adult male CD-1 mice (19–24 g on arrival; Charles River Laboratories, Wilmington, MA, USA). Mice were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) with food and water provided *ad libitum*. Animals were euthanized by decapitation following exposure to excess isoflurane. Hemidiaphragms with attached phrenic nerves were mounted in 20-ml tissue baths containing Tyrode's solution of the following composition (mM): NaCl, 137; KCl, 5; $MgSO_4$, 1; $NaHCO_3$, 24; NaH_2PO_4, 1; $CaCl_2$, 1.8, and glucose, 11. The solution was bubbled with a gas mixture of 95% O_2/5% CO_2 yielding a pH of 7.3–7.4. Resting tension was maintained at 0.7 g to generate optimal nerve-evoked contractions.

2.2. Tension recordings

Twitch tension was elicited by supramaximal stimulation of the phrenic nerve via bipolar, stainless steel electrodes (6.0–9.0 V, 0.2 ms duration) at 0.033 Hz. Tetani were elicited by repetitive stimulation at 30 Hz for 1 s, with 1-min intervals between stimulus trains. Muscle tensions were measured using Grass FT03 force displacement transducers (West Warwick, RI, USA), digitized and analyzed offline using pClamp software v. 10.1 (Molecular Devices, Sunnyvale, CA, USA). Following a 15- to 20-min equilibration, muscles were exposed to 5 pM of pure BoNT/A (Metabiologics, Inc., Madison, WI, USA) for 30 min at room temperature (18–22 °C) in the absence of nerve stimulation. The muscles were then washed with control Tyrode's solution to remove unbound BoNT/A, warmed to 36 °C and monitored for development of paralysis 3,4-DAP (Sigma–Aldrich, St. Louis, MO, USA) and Ros (A.G. Scientific, Inc., San Diego, CA, USA) were generally added to the bath when muscle tensions declined to ~50% of their initial values. Tensions were recorded for 1–3 h after drug addition to determine the time course for reversal of muscle paralysis.

2.3. Drug preparation

Stock solutions of 3,4-DAP were prepared in deionized water at concentrations of 20 mM. Stock solutions of Ros were prepared in dimethylsulfoxide (DMSO) at a concentration of 50–100 mM and stored in opaque vials to limit their photoreactivity. Stock solutions were stored at 4 °C.

2.4. Data analysis

Unless stated otherwise, all data are expressed as means ± SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison