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Exploration of endogenous substrate cleavage by various forms of botulinum neurotoxins

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ABSTRACT

Botulinum neurotoxins are the most potent protein neurotoxin known to human. The dual roles of BoNTs as both the causative agent of human botulism and a widely used protein-based therapeutic agent for treatment of numerous neuromuscular disorders/cosmetic uses make it an extremely hot topic of research. Biochemical characterization of these toxins was mainly confined to the recombinant light chains and substrate and little is known about their efficiency on the cleavage of endogenous substrates. In the present study, we showed that BoNTs exhibited variable activities on their endogenous substrates and that their efficiency to cleave recombinant and endogenous substrate was not consistent, presumably due to the differential recognition of their respective substrates in the natural SNARE complex format. Through testing the combinatorial effects of different BoNTs on cleavage of endogenous substrates, we showed that the combinations of LC/A and LC/B, as well as LC/A and LC/F, could enhance the activity of each individual BoNT. This finding may shed light on the future development of new BoNT serotypes for clinical application, and formulation of combinatorial uses of different BoNTs to minimize the development of immuno-resistance by using a lower amount of individual type.

1. Introduction

Botulinum neurotoxins (BoNTs) are produced by the anaerobic bacteria *Clostridium botulinum, Clostridium butyricum, Clostridium baratii* and *Clostridium argentinense*. These compounds affect human muscular activity by imposing a presynaptic block on the release of neurotransmitters (Schantz and Johnson, 1992). To date, seven serotypically distinct BoNTs (BoNT/A-G) and more than 40 subtypes have been identified and characterized (Rossetto et al., 2014). Recently, an eighth serotype (designated as BoNT/H) produced by a *C. botulinum* type Bh strain, IBCA10-7060, was proposed (Barash and Arnon, 2014; Dover et al., 2014). Botulism mostly affects wild and domesticated animals; human botulism is much rarer and mostly caused by BoNT/A,/B,/E, but rarely by/F (Rossetto et al., 2014). BoNTs specifically target neurons at neuromuscular junctions and cleave the soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) complex, which is composed of syntaxin 1, SNAP25 (synaptosomal-associated protein 25) and VAMP2 (vesicle-associated membrane protein 2, also known as synaptobrevin) (Pantano and Montecucco, 2014). These components are specific targets of different BoNT serotypes (Montecucco and Schiavo, 1994). BoNT proteins are initially synthesized as single polypeptide chains of ~150 kDa, then maturated by proteolysis to form a di-chain protein linked by a disulphide bond, which consists of a ~50 kDa light chain (LC, a zinc-dependent metalloprotease) and a ~100 kDa heavy chain (HC) (Montecucco and Schiavo, 1994; Davletov et al., 2005).

The extreme toxicity of BoNTs infers that these compounds can be used as a potential biological warfare agent; however, the reversible intoxication process triggered by these compounds has, in the past two decades, enabled them to be transformed from deadly agent to effective therapeutic for treatment of a wide range

Abbreviations: BoNTs, Botulinum Neurotoxin; LC, light chain; LC/T, light chain of Tetanus neurotoxin; VAMP2, vesicle associated membrane protein-2; SNAP25, Synaptosomal-associated protein 25; SNARE, soluble NSF attachment receptor.

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of common neuromuscular disorders (Davletov et al., 2005; Klein, 2004; Chen, 2012; Glogau, 2002). In addition, BoNT/A has been proven to be effective and safe in treatment of several less common conditions such as vaginism (Bertolasi et al., 2009; Fageeh, 2011; Patti et al., 2005). However, immuno-resistance development is a major concern during BoNT-based therapies. In some reported cases, patients who receive treatment for cervical dystonia or spasticity, which requires larger doses and periodic administration of the toxin, developed blocking antibodies, thereby reducing the effectiveness of future treatment (Atassi, 2004; Goschel et al., 1997; Zesiewicz et al., 2004; Atassi and Dolimbek, 2004). Nevertheless, the expanding clinical usage of BoNTs in treatment of overt diseases will undoubtedly increase the number of immuno-resistance cases. Unfortunately, there is no ideal solution to this problem to date. Attempts which have been made to overcome this obstacle include blocking the epitopes of BoNTs by conjugating them with monomethoxypolyethylene glycol (mPEG) (Dolimbek et al., 2011), engineering the BoNTs HC receptor binding motif to enhance their binding capacity (Foster, 2009), or modifying LC to elevate their protease activity (Guo et al., 2013), so as to reduce the dosage required for treatment and reduce the chance for occurrence of immuno-resistance.

Different serotypes and subtypes have been reported to exhibit differential enzymatic rates *in vivo* (Guo et al., 2013; Whitemarsh et al., 2013), yet data regarding the *in vitro* cleavage activity of specific serotypes of BoNTs on their respective endogenous substrate, and the efficacy of substrate cleavage through combinatorial use of different serotypes of BoNTs, are currently not available. In the present study, we reported the comparative activities of BoNT serotypes, both individually and in various combinations, on the cleavage of recombinant and endogenous substrates. Our results showed that certain combinations of BoNTs exhibit synergistic effect on endogenous substrate cleavage, providing a novel formulation for minimizing the development of immuno-resistance through reducing the amounts of individual BoNT used in therapy.

2. Methods

2.1. Recombinant protein expression and purification

Recombinant LC/B (1–430), LC/A (1–425), LC/E (1–408), LC/F (1–446), LC/D (1–430), LC/TeNT-(1–436), VAMP2 (1–97) and SNAP25 (141–206) were expressed and purified as described previously (Chen et al., 2007; Chen and Barbieri, 2007; Chen and Wan, 2011; Chen et al., 2008; Guo and Chen, 2013).

2.2. Neuro-2A cell culture

Neuro-2A cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% newborn calf serum, 1.4% sodium bicarbonate, and 1% penicillin-streptomycin at 37 °C in 5% CO_2. Confluent cells were harvested and either stored at −80 °C for future use or freshly lysed by RIPA cell lysis buffer (SIGMA, USA), which was then supplemented freshly with 1 mM PMSF on ice for 10 min, spun down for 10 min at 14,000 rpm at 4 °C; the supernatant was retained for the hydrolysis assays.

2.3. Hydrolysis of LCs on recombinant VAMP2 or SNAP25

Linear velocity reactions were performed as previously described (Chen et al., 2007). In a 10 μl reaction system, a desired amount of recombinant VAMP2 or SNAP25 (designated as rVAMP2 or rSNAP25 respectively) proteins was incubated with varying concentrations of LCs in reaction buffer of 10 mM Tris–HCl (pH 7.6), 20 mM NaCl (10:20 buffer) at 37 °C for 20 min. Reactions were stopped by adding SDS-PAGE sample buffer; cleaved and uncleaved substrate were resolved by running SDS-PAGE. The amount of substrate cleaved was determined by densitometry. All assays were repeated in three independent experiments.

2.4. Hydrolysis of LCs on endogenous VAMP2 or SNAP25

The assays of the *in vitro* cleavage of endogenous VAMP2 or SNAP25 (designated as eVAMP2 or eSNAP25 respectively) by different LCs were performed as previously described (Guo et al., 2013). Briefly, the Neuro-2A cell supernatant was prepared as mentioned above, the total protein in the supernatant was quantified by performing the Bradford assay (Bio-Rad, USA), then an equal amount of total protein was mixed with the desired amount of LCs in reaction buffer in a 10 μl reaction volume. All reactions were then incubated at 37 °C for 20 min, stopped by SDS-PAGE sample buffer, then analysed by SDS-PAGE and western blotting analysis. The remaining un-cleaved VAMP2 was probed with anti-VAMP2 antibody (ab3347, Abcam, UK) and both cleaved and uncleaved SNAP25 were probed with anti-SNAP25 antibody (SMI 81, Abcam, UK).

For the combinatorial cleavage analysis on endogenous VAMP2 or SNAP25 by different LC combinations, assays were performed as described above, except that two indicated LCs were mixed together with the Neuro-2A cell supernatant in the reaction buffer, followed by analysis by SDS-PAGE and western blotting, probed with anti-VAMP2 or anti-SNAP25 antibodies respectively.

3. Results and discussion

The activity of different LCs on their respective recombinant or endogenous substrates was determined. Surprisingly, the efficiency by which LCs cleave recombinant substrates was not consistent with that for cleavage of the endogenous substrates. The most dramatic difference was seen in LC/A, which cleaved its endogenous substrate at a dramatically slower rate than its recombinant substrate (>20,000-fold lower efficiency). LC/T also exhibited ~16-fold reduced efficiency in cleaving the endogenous substrate as compared to cleavage of recombinant VAMP2. However, LC/B and LC/D respectively exhibited about 16-fold and 5-fold higher efficiency in cleavage of endogenous substrates when compared to that of the recombinant substrates (Table 1). These data suggest that formation of SNAP25-syntaxin 1 complexes within neuronal cells affects the efficiency of substrate cleavage by LC/A and LC/E. However, this reason cannot be used to explain the increased efficiency of LC/B and LC/D in cleaving endogenous VAMP2, and vice versa in the case of LC/T and LC/F, since VAMP2 can be in a free form in the neuronal cells most of the time.

Then combinatorial uses of different LCs to cleave their endogenous substrates were investigated. The combination of LC/A with

Table 1
Individual activity of LCs on cleavage of recombinant or endogenous substrate.

LCs	Amount of different LCs required for cleavage of 50% of substrate (nM)			
	rSNAP25	rVAMP2	eSNAP25	eVAMP2
LC/A	2	–	>40,000	–
LC/E	3	–	30	–
LC/B	–	8	–	0.5
LC/T	–	45	–	750
LC/D	–	7	–	1.5
LC/F	–	25	–	125

Note: rSNAP25/rVAMP2, recombinant SNAP25 and VAMP2 respectively; eSNAP25/eVAMP2, endogenous SNAP25 and VAMP2 respectively.