FEBS Letters 585 (2011) 199–206

journal homepage: www.FEBSLetters.org

Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1

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ARTICLE INFO

Article history:
Received 21 September 2010
Revised 23 November 2010
Accepted 25 November 2010
Available online 30 November 2010

Edited by Maurice Montal

Keywords:
Botulinum neurotoxin
BoNT/A1
BoNT/A2
Cell based assay
Cell entry
Neuron

ABSTRACT

Botulinum neurotoxins (BoNTs), the causative agent of human botulism, are the most potent naturally occurring toxins known. BoNT/A1, the most studied BoNT, is also used as an important biopharmaceutical. In this study, the biological activity of BoNT/A1 is compared to that of BoNT/A2 using neuronal cell models. The data obtained indicate faster and increased intoxication of neuronal cells by BoNT/A2 than BoNT/A1, and that the mechanism underlying this increased toxicity is faster and more efficient cell entry that is independent of ganglioside binding. These results have important implications for the development of new BoNT based therapeutics and BoNT countermeasures.

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1. Introduction

Botulinum neurotoxins (BoNTs), the most potent protein toxins known, cause botulism and are produced by a heterogenous group of neurotoxigenic bacteria including *Clostridium botulinum*, and select strains of *Clostridium butyricum* and *Clostridium baratii* [1,2]. Based on their antigenic specificity, BoNTs are classified into seven serotypes (A–G) [2], with BoNT/A, B, E, and F accounting for most recorded cases of human botulism [1]. BoNTs are important disease causing agents [1,3], potential bioterrorism agents [4], and pharmaceuticals for treating neuromuscular disorders and cosmetic use [5,6].

BoNTs are 150 kDa proteins consisting of a heavy chain (HC, ~100 kDa) and a light chain (LC, ~50 kDa) linked by a disulfide bond. BoNT intoxication of neuronal cells requires multiple steps (reviewed in [7]). The C-terminal domain of the HC (HCR/A) binds dual receptors, first a ganglioside on the cell surface and then a protein receptor upon neurotransmitter vesicle fusion with the plasma membrane [8]. The toxin–receptor complex then enters the neuron via endocytosis (reviewed in [9–11]), and acidification within the endosome leads to a conformational change and formation of a HC channel in the endocytic vesicle membrane [12,13]. The translocation domain of the HC then conducts the LC through the HC-channel into the cytosol and the disulfide bond linking the HC and LC is reduced. The released LC refolds to a catalytically competent conformation within the cytosol [12,13]. LCs are zinc endopeptidases that cleave and inactivate Soluble N-ethylmaleimide Sensitive Fusion Attachment Protein Receptors (SNARE), which are essential for neurotransmitter release [14–19].

Several subtypes of most BoNT serotypes have been identified by nucleotide sequence analysis (reviewed in [20]). BoNT/A has been divided into five subtypes (A1–A5) [21–28], but limited information is available on biological and functional characteristics among BoNT subtypes. Prior analyses of BoNT/A subtypes include studies using recombinant LC endopeptidases of BoNT/A1–A4 expressed in *Escherichia coli*, which indicate that all four subtypes bind SNAP-25 with similar affinity, but that rLC/A3 and rLC/A4 cleave SNAP-25 significantly less efficiently than rLC/A1 and rLC/A2 [29]. Sequence and structural analyses show that BoNT/A1 and A2 differ by ~10% at the amino acid level, with the greatest difference in the receptor binding domain. In

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0014-5793/$36.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2010.11.045

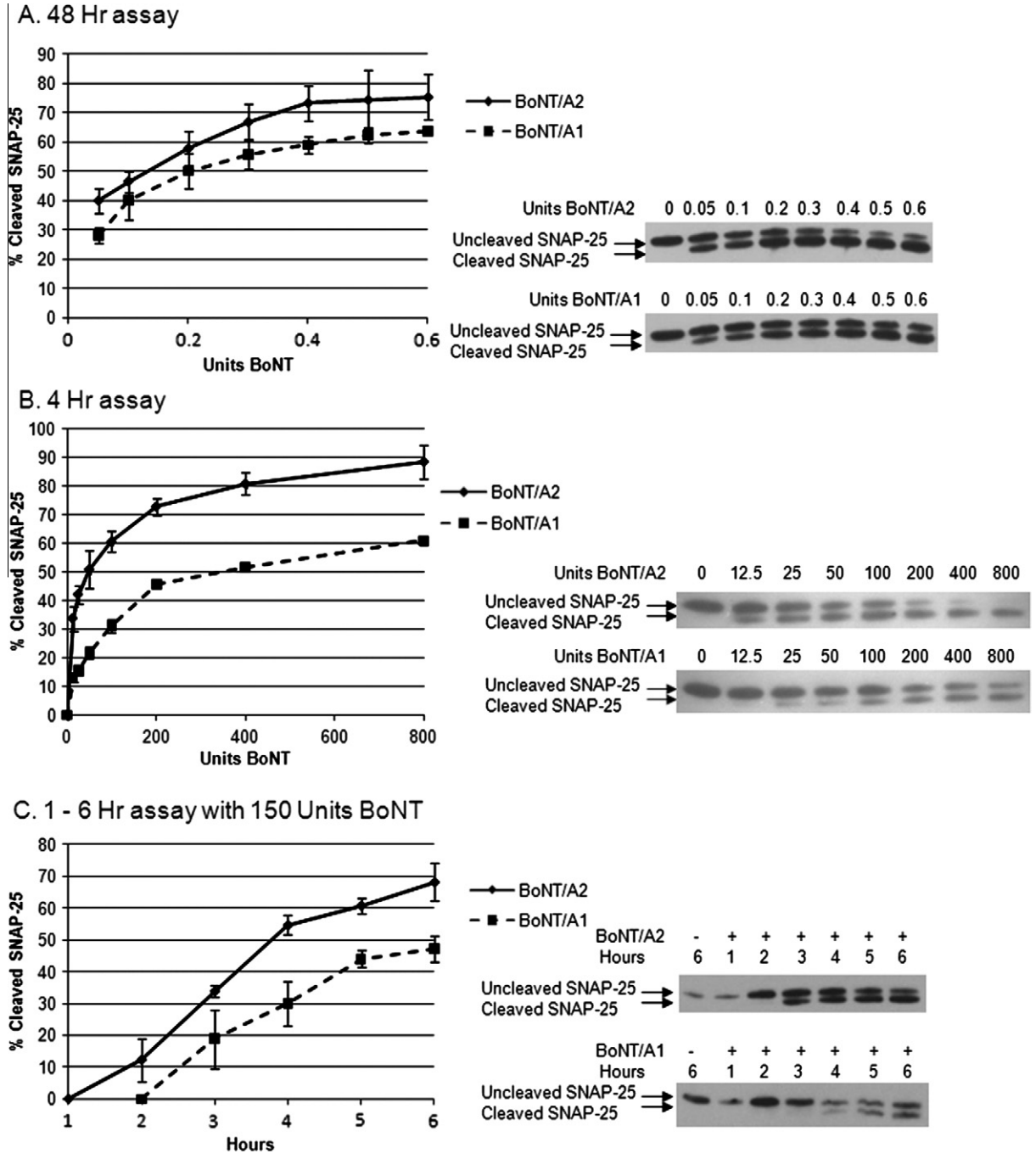

Fig. 1. Sensitivity of RSC cells to BoNT/A2 and A1. (A) 48 h exposure; (B) 4 h exposure; (C) exposure to 150 Units for 1–6 h. Representative Western blots are shown.

contrast, the ganglioside binding pocket and the catalytic LC domain are highly conserved [21,25]. BoNT/A1 and A2 have significantly altered monoclonal antibody binding and inhibition profiles by monoclonal antibodies [25], but no differences were observed in protection against the two subtypes by polyclonal antisera raised against BoNT/A1 [30].

Of the five BoNT/A subtypes identified, BoNT/A1, A2, and A5 have now been purified [31,32], enabling detailed studies which are needed to facilitate the increasing use of BoNTs for medicinal use and to develop effective countermeasures to intoxication by the various subtypes of BoNTs. A recent report suggests that BoNT/A2 is more potent than BoNT/A1 in vivo [33]. The data presented here demonstrate that BoNT/A2 is more potent than BoNT/A1 in neuronal cells due to faster cell entry.

2. Materials and methods

2.1. Botulinum neurotoxins

BoNT/A1, A2, and E were purified as previously described [34,32,35]. The specific activities were determined via the mouse bioassay [36,37] to be 1.3×10^8 LD_{50} Units/mg for BoNT/A1, 4.3×10^8 Units/mg for BoNT/A2, and 0.76×10^8 Units/mg for BoNT/E.

2.2. Reagents

Tissue culture reagents, media, and Western blot supplies were obtained from Invitrogen (Carlsbad, CA). Neuro-2a cells were pur-