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In vitro potency determination of botulinum neurotoxin B based on its receptor-binding and proteolytic characteristics

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ABSTRACT

Botulinum neurotoxins (BoNTs) are the most potent toxins known. However, the paralytic effect caused by BoNT serotypes A and B is taken advantage of to treat different forms of dystonia and in cosmetic procedures. Due to the increasing areas of application, the demand for BoNTs A and B is rising steadily. Because of the high toxicity, it is mandatory to precisely determine the potency of every produced BoNT batch, which is usually accomplished by performing toxicity testing (LD_{50} test) in mice. Here we describe an alternative *in vitro* assay for the potency determination of the BoNT serotype B. In this assay, the toxin is first bound to its specific receptor molecules. After the proteolytic subunit of the toxin has been released and activated by chemical reduction, it is exposed to synaptobrevin, its substrate protein. Finally the proteolytic cleavage is quantified by an antibody-mediated detection of the neoepitope, reaching a detection limit below 0.1 mouse LD_{50}/ml. Thus, the assay, named BoNT/B binding and cleavage assay (BoNT/B BINACLE), takes into account the binding as well as the protease function of the toxin, thereby measuring its biological activity.

1. Introduction

Botulinum neurotoxins (BoNTs) produced by the anaerobic bacterium *Clostridium botulinum* are the most potent toxins known, with a lethal dose of approximately 1 ng per kg body weight in humans (Gill, 1982). BoNTs cause paralysis through an inhibiting effect on efferent nerves preceding neuromuscular junctions. Among the seven BoNT serotypes known to date (A to G) (Rossetto et al., 2014), the serotypes A and B are most effective in humans and are therefore used for a broad range of medical and cosmetic applications (Abrams and Hallett, 2013). Different forms of dystonia such as spasmodic torticollis and blepharospasm are treated with BoNTs. Other fields of BoNT application are strabismus or hyperhidrosis. In addition, wrinkles can be diminished by paralyzing the muscles of the respective skin region.

BoNTs aggregate with neurotoxin-associated proteins (NAPs) to form the progenitor toxin complex. In the typical intoxication process via the oral route, these enveloping NAPs protect the toxin from degradation in the stomach and intestine, and are also involved in the translocation of the BoNT across the intestinal epithelium. After this translocation step, a pH-driven separation of the NAPs and BoNTs takes place (Gu and Jin, 2013; Sugii and Sakaguchi, 1975; Wagman and Bateman, 1953). The non-complexed BoNT, which consists of a heavy chain (H-chain) connected by a disulfide bond to a light chain (L-chain), reaches its target neurons at neuromuscular junctions via the blood stream. Here the binding region on the H-chain interacts with its receptors on the presynaptic surface of the neurons. The distinct BoNT serotypes recognize different receptors. BoNT serotype B, which is the focus of this study, is able to bind simultaneously to synaptotagmin, its specific protein receptor, and to gangliosides (Berntsson et al., 2013). The bound toxin is subsequently internalized into endosomes. Upon acidification in the endosomal compartment, the translocation domain on the H-chain mediates the transfer of the L-chain into the cytosol (Donovan and Middlebrook, 1986; Finkelstein, 1990; Fischer and Montal, 2013). The cleavage of the inter-chain disulfide bond by the cytosolic thioredoxin reductase leads to the release of the L-chain into the cytoplasm and to the concomitant activation of its proteolytic domain (Pirazzini et al., 2014, 2013). The active protease cleaves proteins involved in the exocytosis of acetylcholine, thus inhibiting the

Abbreviations: BINACLE, binding and cleavage assay; BoNT, botulinum neurotoxin; BSA, bovine serum albumin; GT1b, ganglioside GT1b; H-chain, heavy chain; HCc, C-terminal region of the H-chain; L-chain, light chain; LD_{50}, lethal dose for 50% of test animals; NAP, neurotoxin-associated protein; PBS, phosphate-buffered saline; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); Syt, synaptotagmin peptide; TCEP, tris(2-carboxyethyl)phosphine; TMAO, trimethylamine N-oxide; TMB, 3,3′,5,5′-tetramethylbenzidine.

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release of this neurotransmitter. For BoNT/B, the specific substrate is synaptobrevin (Montecucco and Schiavo, 1993).

The range of applications of BoNTs A and B for pharmaceutical and cosmetic purposes is broad and steadily growing, leading to an increasing market for botulinum neurotoxins. Before the finished product is released onto the market, the potency of each batch has to be precisely determined. For this purpose the European Pharmacopoeia mandates the LD_{50} test in mice, which measures the toxin dose that is lethal for 50% of the animals treated. However, other *ex vivo* or *in vitro* methods for the determination of the specific activity of BoNTs may be used if they have been successfully validated against the LD_{50} test as the current gold standard (Council of Europe, 2016a, 2016b). Due to the lack of reliable and freely available alternative methods, the LD_{50} test is still the most widely used method for determining the specific activity and the potency of BoNTs. In 2010, at least 600,000 mice were used worldwide for the LD_{50}-based potency determination of BoNT/A products (Bitz, 2010). Approximately 60,000 mice were used for the potency determination of BoNT/B in Germany in 2014 (German Federal Institute for Risk Assessment, 2016). In the future, this number is expected to further increase as the demand for BoNT products is growing. There is thus a strong need for robust and reliable alternative *in vitro* methods.

2. Material and methods

2.1. Reagents

BoNT/B (1×10^8 LD_{50}/mg, molecular weight 150 kDa) and BoNT/B with associated complex proteins (1.2×10^7 LD_{50}/mg, average molecular weight 475 kDa) were obtained from Metabiologics (Madison, WI, USA). The BoNT/B L-chain comprising amino acids 1 to 436 and the C-terminal region of the BoNT/B H-chain comprising amino acids 858 to 1291 (HCc) were obtained from List Biological Laboratories (Campbell, CA, USA). A sample of a pharmaceutical BoNT/B complex preparation (Myobloc®, 1.14×10^8 LD_{50}/mg, molecular weight 700 kDa) was kindly provided by US WorldMeds (Louisville, KY, USA).

Transparent 96 well microtiter plates type MaxiSorp were purchased from Thermo Fisher (Braunschweig, Germany). The synaptotagmin peptide Syt (acetyl-GESQEDMFAKLKEKFFNEINKC) comprising the toxin binding site of murine synaptotagmin-2 with a C-terminal cysteine residue and a control version of this peptide with a scrambled amino acid sequence (Dong et al., 2003) were synthesized by GeneCust (Dudelange, Luxembourg). Biotinylated goat-anti-rabbit IgG and horseradish peroxidase conjugated to streptavidin were from Dianova (Hamburg, Germany). Recombinant synaptobrevin (amino acids 1–97 of rat synaptobrevin-2 with an N-terminal histidine tag) and the monospecific polyclonal rabbit antibody specifically recognizing cleaved synaptobrevin were produced as described previously (Kegel et al., 2007). Bovine serum albumin (BSA) and sodium acetate were ordered at Serva (Heidelberg, Germany). Trimethylamine *N*-oxide (TMAO) was obtained either from Sigma-Aldrich (Taufkirchen, Germany) or from Tokyo Chemical Industry UK Ltd (Oxford, United Kingdom). All other reagents were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich. A 40 mg/ml stock solution of asolectin in phosphate buffered saline (PBS) was prepared by sonication and stored at −20 °C until usage.

2.2. BoNT/B BINACLE

Unless otherwise indicated, the volumes added to each well of the microtiter plate were 100 µl for incubation and coating steps, 300 µl for washing steps and 250 µl for blocking steps.

The receptor plates were prepared by coating MaxiSorp microtiter plates with 5 µg/ml GT1b and 2.5 µg/ml Syt in PBS for 2 h at 37 °C and 250 rpm in a microplate thermoshaker, unless otherwise specified. Wells coated with PBS without receptor molecules were used as controls for non-specific binding. All wells were washed four times with washing buffer (PBS, 0.05% Tween 20). Then blocking buffer A (PBS, 1% BSA) was added for 2 h at 37 °C and 250 rpm, and the receptor plates were washed again. Afterwards BoNT/B diluted in PBS with 1% BSA was added in the indicated concentrations and incubated at 4 °C overnight to enable the toxin to bind to the receptor molecules. For the tests described in Section 3.5, the BoNT/B L-chain or the HCc fragment was added to the receptor plates instead of the BoNT/B holotoxin, where indicated.

In parallel to the binding step, the substrate plates were prepared by coating fresh MaxiSorp microtiter plates with 1.5 µM recombinant synaptobrevin in PBS for 2 h at 37 °C and 250 rpm. These substrate plates were subsequently incubated with blocking buffer B (PBS, 5% sucrose, 0.5% BSA, 100 µg/ml asolectin) overnight at 4 °C.

The following day, the receptor plates were washed four times with washing buffer and once with piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (100 mM, pH 6.4). Then a reduction/cleavage buffer containing 2.5 mM *tris*(2-carboxyethyl)phosphine (TCEP), 1.5 M TMAO and 5% sucrose in 100 mM PIPES, pH 6.4 was added to the receptor plates and incubated for 30 min at 37 °C and 250 rpm in order to separate the L-chain from the receptor-bound H-chain. In the meantime the substrate plates were washed four times with washing buffer and once with PIPES buffer. Then the supernatants from the receptor plates were transferred to the substrate plates and incubated for 6 h at 37 °C and 250 rpm. The substrate plates were washed four times with washing buffer before the rabbit antibody against cleaved synaptobrevin diluted in PBS with 0.5% BSA was added to the plates for an overnight incubation at 4 °C.

All following steps were performed at room temperature. First the substrate plates were washed, and a biotin-conjugated goat-anti-rabbit antibody diluted in PBS with 0.5% BSA was added to the plates and incubated for 45 min with gentle agitation. After the next washing procedure, a solution of peroxidase-conjugated streptavidin was incubated on the plates for 45 min under slight shaking. The plates were washed again before the development buffer (0.11 M sodium acetate pH 5.5, 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (TMB), 0.006% H_2O_2) was added and the plates were left in darkness for 25 min. The color reaction was stopped by adding 50 µl of 1 M H_2SO_4 per well. The absorbance was measured at 450 nm against a reference wavelength of 620 nm.

2.3. Determining the binding capability of the receptor plates

In order to characterize the capability of the receptor plates for toxin binding, microtiter plates were coated with the receptor molecules or with PBS alone (negative control), blocked and incubated with BoNT/B overnight as described in Section 2.2.

Afterwards the residual amount of non-bound, active BoNT/B in the supernatants of this binding incubation was quantified. For this, a complete BoNT/B BINACLE as described in Section 2.2 was performed using (1.) the supernatants of the extra binding step as test samples and (2.) a freshly prepared concentration series of BoNT/B as standard preparation. The residual toxin content in the supernatants of the first receptor plate was finally estimated by comparing the signals obtained with these supernatants to those obtained with the fresh BoNT/B solutions.

2.4. Statistical methods

All samples were measured in triplicate within each assay, and the mean absorbance was determined. Outliers determined by the Dixon's Q test were removed from further analysis (Dean and Dixon, 1951). All experiments were performed at least twice. Error bars represent the standard deviation.

Nonlinear response curves in Sections 3.4 and 3.7 were fitted with four-parameter logistic functions using the R package drc (R Core Team, 2013; Ritz and Streibig, 2005). The used parameters are lower and upper limit, slope and reversal point, which represents the half