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Nerve cell-mimicking liposomes as biosensor for botulinum neurotoxin complete physiological activity

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ABSTRACT

Botulinum neurotoxins (BoNT) are the most toxic substances known, and their neurotoxic properties and paralysing effects are exploited for medical treatment of a wide spectrum of disorders. To accurately quantify the potency of a pharmaceutical BoNT preparation, its physiological key activities (binding to membrane receptor, translocation, and proteolytic degradation of SNARE proteins) need to be determined. To date, this was only possible using animal models, or, to a limited extent, cell-based assays. We here report a novel in vitro system for BoNT/B analysis, based on nerve-cell mimicking liposomes presenting motoneuronal membrane receptors required for BoNT binding. Following triggered membrane translocation of the toxin's Light Chain, the endopeptidase activity can be quantitatively monitored employing a FRET-based reporter assay within the functionalized liposomes. We were able to detect BoNT/B physiological activity at picomolar concentrations in short time, opening the possibility for future replacement of animal experimentation in pharmaceutical BoNT testing.

1. Introduction

Botulinum Neurotoxin (BoNT) is a highly toxic bacterial toxin. In its physiologically active form it is known to be a potent inhibitor of cholinergic motoneuron activity. By blocking the secretion of acetylcholine neurotransmitter, the signal transmission is impeded and the target muscle is paralysed. If administered in minute doses, a constrained paralysing effect can be achieved, which is employed for the treatment of an increasing number of diseases, disorders and in aesthetic surgery (Wheeler and Smith, 2013). While Botox® may be the best known pharmaceutical BoNT-preparation, there is a range of related products, which contain either BoNT type A (BoNT/A) or BoNT/B as active compound (Bigalke, 2013). To gain approval, and alongside production, every batch of pharmaceutical BoNT needs to be tested for its specific overall toxic activity, termed potency (EDQM, 2011). This directly correlates to the different physiological activities of the toxin at the motoneuron, which can be divided into three key steps: 1) binding to specific nerve-cell receptors via the toxin's heavy chain (HC), 2) translocation of the toxin's light chain (LC) across the plasma membrane, induced by a decrease in pH, and 3) proteolytic cleavage of SNARE proteins by the catalytically active LC (Binz, 2013; Rummel, 2013).

Currently, the mouse bioassay is still considered to be the "gold standard" for determination of BoNT potency (e.g. AOAC Official Method 977.26). For this, mice are injected intraperitoneally with dilutions of the respective pharmaceutical preparation, monitored for typical symptoms of botulism for up to 96 h, and the LD50 determined accordingly. Depending on the exact BoNT type, the test exhibits a detection limit between 5 and 100 pg (Dorner et al., 2013; Weingart et al., 2010). It was estimated that in the year 2008, batch potency controls of BoNT-pharmaceuticals required approximately 600,000 mice (Bitz, 2010). However, not only the high number of animals, but also the required facilities and personnel result in enormous costs. Above all, due to the severity of the experimental conditions and the resulting distress for the animals, the mouse LD50 test is widely criticized. With respect to in vitro test systems, a few cell-based assays have been reported to monitor the key steps that govern the toxin's physiological activity (Pellett, 2013). Recently, a relatively sensitive testing scheme based on cultured neuroblastoma cells gained FDA-approval for testing of the manufacturer's own BoNT product line (Fernández-Salas et al., 2012). However, the need for expensive lab equipment, specifically trained personnel, and lengthy assay times present considerable shortcomings. Furthermore, most cell-based assays require additional methods to measure the actual toxic effect caused by BoNT, e.g. quantification of intracellularly cleaved SNARE-proteins, thus adding further hands-on time and need for accurate and reproducible protocols (Dong et al., 2004; Fernández-Salas et al., 2012; Shi et al., 2009). Also, in spite ongoing research efforts, there is no validated, cell-based method for BoNT/B commercially available to date (Dong et al., 2007; Whitemarsh et al., 2012). In order to completely replace animal testing of BoNT pharmaceuticals, development of alternative and easy-to-use in vitro methods is highly desirable (Adler et al., 2010).

Towards this aim, we here describe a novel in vitro system to assay the function of BoNT/B. We employed fully assembled and

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functionalized liposomes as nanometre-sized reaction compartments providing a nerve-cell mimicking system that allows detection of the toxin's three physiological key activities in a single assay format.

2. Material and methods

2.1. Toxins

The study was performed using an analytical grade, un-nicked 150 kDa BoNT type B1 preparation (strain Okra) with a potency of 1.1×10^8 MLD_{50} mg^{-1} from a commercial manufacturer (Metabiologics Inc., Madison, WI, USA). When appropriate, the toxin was diluted in assay buffer (12.5 mM HEPES pH 7.4; AppliChem GmbH, Darmstadt, Germany).

2.2. Test for endopeptidase activity

PL150 peptide substrate (Pharmaleads, Paris, France) was used for the detection of BoNT/B-LC endopeptidase activity. The peptide substrate consists of an oligopeptide with an N-terminally linked Pyrenylalanine fluorophore (Pya), and a C-terminally coupled Para-nitro-phenylalanine quencher molecule (Nop). The oligopeptide contains the specific cleavage site for BoNT/B-LC. Förster resonance energy transfer (FRET) between fluorophore and quencher impedes emission of fluorescence in the intact molecule. When the oligopeptide chain is cleaved, fluorophore and quencher are spatially separated and, if excited, the fluorophore emits a quantifiable fluorescence signal (Anne et al., 2001). Lyophilized PL150 was first dissolved in ultrapure H_2O at 200 μM and diluted for experiments to the desired concentrations in PL150-specific reaction buffer, constituting of 10 mM HEPES pH 7.4, 75 μM $ZnSO_4$, and 2.5 mM TCEP pH 7.0 (Sigma-Aldrich, Buchs, Switzerland). Unless stated otherwise, PL150 was used at a concentration of 10 μM, and the reaction took place in a final volume of 100 μL. Fluorescence was measured in non-binding, black 96 half-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Fluorescent signals were recorded every 30 s for 180 min with a spectrofluorometer (SpectraMax GeminiXS or M2e; Molecular Devices, Sunnyvale, CA, USA), at excitation and emission wavelengths of 341 and 397 nm, respectively. Data were further analysed using Prism Version 6.0 (GraphPad Inc., La Jolla, CA, USA). All measurements were performed in duplicates, i.e. two independent experiments were performed concurrently with individual substrate and toxin dilutions. Substrate turnover kinetics, i.e., half-time values, were calculated from the curve progressions using one-phase association non-linear regression models. To calculate SD values for half-times, curves were fitted with the described models onto the measured signal intensities from each replicate measurement. The half-time value derived from each fit was then combined with the half-time from its replicate measurement and standard deviation calculated accordingly.

2.3. Preparation of functionalized liposomes

For production of fully assembled and functionalized liposomes, purified Trisialoganglioside GT1b (Matreya LLC, Pleasant Gap, PA, USA) was used as lipid receptor for BoNT/B. As the toxin's protein receptor, a GST-SytII fusion protein (toxogen GmbH, Hannover, Germany) was used (Rummel et al., 2004). The protein receptor consists of an N-terminal Glutathione-*S*-Transferase (GST) bound to the luminal and transmembrane domain of rat Synaptotagmin II (SytII; amino acid residues 1 to 90), and was produced in *Escherichia coli*. For purification, the protein receptor was N-terminally coupled to GST without hindering binding to BoNT/B-HC. GST-SytII was purified and stored until use in phosphate-buffered saline (PBS), and 0.5% Triton X-100. Large unilamellar vesicles (LUV) were prepared by hydration of a thin film of dried lipids and ganglioside receptor molecules. Unless otherwise stated, the lipids used for assembly of functionalized liposomes consist of a commercially available, native lipid mixture, i.e. total lipid extract derived from porcine brain (Avanti Polar Lipids Inc., Alabaster, AL, USA). As such, the extract contains numerous components including phospholipids (approximately 41%), e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, as well as other chloroform soluble compounds such as lipoproteins, sterols, cerebrosides, ceramides, sphingolipids, glycolipids, and other neutral lipids (approximately 59%). To match GT1b concentrations found in native neuronal membranes, 0.1 mM GT1b was used, which corresponds to 0.5% of approximately 20 mM total lipid content (Breckenridge et al., 1972; Kozaki et al., 1998). For production of 500 μL LUV, 3.5 mg of dry brain lipid extract, and 109 μg of GT1b were dissolved in 200 μL chloroform and methanol (50:50) in a glass vial by gentle agitation. Subsequently, the solvent was evaporated by applying a vacuum using a Eppendorf concentrator 5301 (Eppendorf, Hamburg, Germany) to create a dry lipid film. Unless stated otherwise, the lipid film was reconstituted in 500 μL PL150-specific reaction buffer supplemented with GST-SytII and PL150 at concentrations of 1.8–3.6 μM and 20 μM, respectively. Shaking at 4 °C for 60 min resulted in a turbid vesicle emulsion. Triton X-100 content in the vesicle emulsion was below 0.1%. GT1b integration into vesicle membranes takes place via spontaneous incorporation of its ceramide chain during reconstitution of the lipid film (Shen et al., 2013). The apolar transmembrane domain of GST-SytII and associated Triton X-100 molecules facilitate integration of the molecule into the vesicular membranes (Rigaud and Lévy, 2003). It should be noted that due to the production method, GT1b and GST-SytII may possibly be present in an orientation facing either outside or inside from the liposome membrane (Girard et al., 2004). Following lipid film hydration, unilamellar LUV with uniform size distribution were produced by repeated extrusion of the vesicle emulsion through nucleopore track-etched polycarbonate membranes of 400 nm, 200 nm, and finally 100 nm pore size, flanked by a double stack of polyethylene drain discs on each site (Whatman plc, Kent, UK) assembled in a Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA). Remaining Triton X-100 from supplementation of protein receptor was removed by incubation of the emulsion with 40 mg of Bio-Beads SM-2 Adsorbent (Bio-Rad Laboratories, Hercules, CA, USA) on an overhead rotator for 90 min at 4 °C. Then, non-encapsulated substrate molecules and components of the cleavage buffer were removed by a minimum of 12 h of dialysis at 4 °C against 12.5 mM HEPES pH 7.4 using SpectraPor Dialysis Membranes type 7 with a molecular weight cut-off of 25 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Liposomes were used directly after production or stored until use at 4 °C. Storage did not have a significant effect on liposome integrity (Fig. S1). Liposomes devoid of receptors were produced in the same manner, without supplementation of neither GT1b nor GST-SytII receptor molecules.

2.4. Liposome characterization

2.4.1. Size exclusion chromatography. Unless stated otherwise, 30 μL of a liposome emulsion was subjected to size exclusion chromatography (SEC) using a microcentrifuge spin column system (Thermo Scientific, Rockford, IL, USA) with a gel bed volume of approximately 600 μL for separation from not-integrated and/or not-encapsulated compounds. A 50%-slurry of Sepharose 4B (Sigma-Aldrich, Buchs, Switzerland) in 12.5 mM HEPES pH 7.4 was used as gel matrix. Separation was enhanced by centrifugation in a table top centrifuge (Eppendorf AG, Hamburg, Germany) at 1200 rpm for 45 s at ambient temperature and the eluted sample fraction was collected. After the first elution, 30 μL of 12.5 mM HEPES pH 7.4 were gently added to the top of the Sepharose gel bed, followed by another centrifuge run with following collection of the eluted fraction. This elution step with HEPES buffer was repeated another 11 times to yield a total of 12 fractions.