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A protein chip membrane-capture assay for botulinum neurotoxin activity

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ABSTRACT

Botulinum neurotoxins A and B (BoNT/A and B) are neuromuscular blocking agents which inhibit neurotransmission by cleaving the intra-cellular presynaptic SNARE proteins SNAP-25 and VAMP2, localized respectively in plasma membrane and synaptic vesicles. These neurotoxins are both dangerous pathogens and powerful therapeutic agents with numerous clinical and cosmetic applications. Consequently there is a need for *in vitro* assays of their biological activity to screen for potential inhibitors and to replace the widely used *in vivo* mouse assay. Surface plasmon resonance (SPR) was used to measure membrane vesicle capture by antibodies against SNAP-25 and VAMP2. Substrate cleavage by BoNTs modified capture providing a method to assay toxin activity. Firstly using synaptic vesicles as a substrate, a comparison of the EC_{50}s for BoNT/B obtained by SPR, ELISA or flow cytometry indicated similar sensitivity although SPR assays were more rapid. Sonication of brain or neuronal cultures generated plasma membrane fragments with accessible intra-cellular epitopes adapted to measurement of BoNT/A activity. SPR responses were proportional to antigen concentration permitting detection of as little as 4 pM SNAP-25 in crude lysates. BoNT/A activity was assayed using monoclonal antibodies that specifically recognize a SNAP-25 epitope generated by the proteolytic action of the toxin. Incubation of intact primary cultured neurons with BoNT/A yielded an EC_{50} of 0.5 pM. The SPR biosensor method was sensitive enough to monitor BoNT/A and B activity in cells cultured in a 96-well format providing an alternative to experimental animals for toxicological assays.

Introduction

Botulism is a flaccid paralysis often contracted by food poisoning. The clinical symptoms of botulism result from defective acetylcholine release at the neuromuscular junction, which in the absence of medical assistance can lead to death. The causative agents are microbial toxins, synthesized by bacteria of the genus *Clostridium*, of which serotypes A, B, E, and more rarely F, are typically associated with human botulism. Botulinum neurotoxins (BoNTs) are two chain proteins. The heavy chain vectors the neurotoxin to motoneuron nerve terminals via recognition of specific receptors. Synaptic vesicle protein 2 (SV2) and synaptotagmins I or II, in association with gangliosides, have been identified as BoNT/A and B receptors respectively. Receptor binding is followed by intra-cellular penetration of the light chain. The light chain is a zinc metalloprotease which interferes with neuromuscular transmission by cleaving essential protein components (SNARE proteins) implicated in acetylcholine release. BoNT/A and E cleave SNAP-25, which is anchored by palmitoylation in the inner leaflet of the plasma membrane, while BoNT/B and F clip VAMP2 (Vesicle Associated Membrane Protein 2 or synaptobrevin), a synaptic vesicle (SV) membrane protein (Verderio et al., 2006; Montecucco and Molgo, 2005).

BoNTs are remarkably potent molecules with a lethal intravenous dose in humans between 1 and 5 ng/kg. As they can be easily produced, they constitute a potential threat as biological weapons. There is currently no antidote against botulism and post-exposure serotherapy is ineffective because of the rapid onset of neurotoxin action. Despite their toxicity, BoNT/A and B are also established therapeutic and cosmetic agents. Local injections of minute doses can produce long-lasting but reversible paralysis of specific muscles. Thus, these neurotoxins have become a treatment of choice for peripheral syndromes caused by hyperactivity of cholinergic nerve terminals and recently the list of indications has expanded to include pain, migraine, cerebral palsy and hyperhydrosis (Verderio et al., 2006; Montecucco and Molgo, 2005).

Abbreviations: BoNT, Botulinum neurotoxin; SPR, surface plasmon resonance; VAMP2, vesicle associated membrane protein 2; SV, synaptic vesicle; MFI, mean fluorescence intensity; RU, resonance unit.

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Sensitive and accurate assays are essential for BoNT quality control in the preparation of therapeutic formulations and this is currently achieved using the mouse lethality assay. The mouse seroneutralization assay is also the most common diagnostic test for botulism (Lindstrom and Korkeala, 2006). These bioassays are sensitive, but lengthy (several days) and large scale use may be unethical. Therefore, there is a crucial need to develop *in vitro* methods to detect the biological activity of BoNTs. Existing *in vitro* tests are based on measuring cleavage of peptides or recombinant proteins (Ekong et al., 1997; Schmidt and Stafford, 2003). They detect the endopeptidase activity of BoNT light chains at low concentrations permitting large scale inhibitor screening. However these tests are performed in optimized conditions and use for neurotoxin detection in complex media requires neurotoxin preconcentration to eliminate interfering substances (Kalb et al., 2006; Wictome et al., 1999; Bagramyan et al., 2008).

Cell-based assays constitute an alternative approach to mouse bioassays as they provide information on the activity of both the heavy and light chains including the capacity of the heavy chain to confer binding and internalization. Thus they are more informative when screening for BoNT inhibitors, as they can monitor effects that block BoNT action at any of the steps between cell surface binding and proteolysis of SNAREs (Boldt et al., 2006). Moreover cell-based assays enable very sensitive detection of serum antibodies against BoNT in some patients receiving repeated neurotoxin treatment (Pellett et al., 2007). However western blotting methods traditionally used to measure toxin activity with living cells (Pellett et al., 2007; Foran et al., 2003; O'Sullivan et al., 1999; Stahl et al., 2007) are generally too slow and quantification for routine use is laborious.

Protein chips are an emerging technology in medical and biochemical biology for detection and quantification of proteins providing methods that can be automated, multiplexed and miniaturized (Cherif et al., 2006; Fitzgerald et al., 2005). Among the different protein chip methodologies, SPR allows direct label-free detection of molecular interactions between an interactant immobilized on a sensor chip and analytes injected over the surface. We have previously reported that anti-VAMP antibodies coupled to an SPR chip capture intact SVs and capture is inhibited when epitopes are clipped by BoNT/B (Ferracci et al., 2005). We have now extended these studies and adapted them to assay the more widely used BoNT/A. Firstly we have assessed how SPR detection performs compared to more conventional approaches (flow cytometry and ELISA). Secondly we have established methods for SPR analysis of native membrane-anchored SNAP-25 to provide rapid readout of BoNT/A action and in particular in an intact cell culture assay that provides an alternative to the mouse lethality assay.

Materials and methods

Reagents. Monoclonal antibody (mAb) 4F3 against a SNAP-25 epitope produced by BoNT/A action was obtained from Research & Diagnostic Antibodies. MAbs against VAMP2 (Cl69.1) and SNAP-25 (Cl71.2) were obtained from Synaptic Systems. Monoclonal antibodies against synaptophysin (171B5), SNAP-25 N-terminal region (BR05), syntaxin1 (10H5), synaptotagmin1 (1D12), and SV2 and polyclonal antibodies against VAMP2 N-terminal region and cysteine string protein (CSP) have been described previously (Ferracci et al., 2004). MAb9A7 is a pan-specific monoclonal anti-Na^+/K^+-ATPase recognizing a cytoplasmic epitope (Choi et al., 1997). Rabbit polyclonal anti-mouse Fcγ antibodies, sensor chips (CM3 and C1) and amine-coupling kits were from GE Healthcare. *Clostridium botulinum* holotoxins serotypes A (1×10^8 LD_{50}/ml) and B (1×10^8 LD_{50}/ml) were produced and purified as described previously (Sakaguchi, 1982). cDNAs coding for His-tagged recombinant light chains of *C. botulinum* type A, B and E neurotoxins (BoNT/A-Lc, BoNT/B-Lc and BoNT/E-Lc) were generously provided by the late Dr. H. Niemann. Proteins were expressed in *Escherichia coli* and purified by affinity chromatography on nickel beads using 100 mM imidazole as eluting buffer. Proteins were dialyzed overnight against 20 mM Hepes/NaOH pH 7.4, 0.15 M NaCl and frozen. The specific activity of BoNT/B-Lc *in vitro* is 25 times lower than that of BoNT/B.

Membrane preparations. The rat brain fraction SnH was obtained in a single step by homogenizing a rat brain with a Teflon/glass homogenizer in 10 ml of cold 0.32 M sucrose buffered with 10 mM Hepes/NaOH, pH 7.4 and containing 5 mM DTT. Homogenates were centrifuged at 10 000 ×*g* for 15 min at 4 °C. Supernatants were filtered (Millex-GP 0.22 μm, Millipore), aliquoted and stored frozen at −20 °C. SnH contains about 40% of brain SV and is depleted of large plasma membrane fragments that are removed by 10 000 ×*g* centrifugation (Ferracci et al. 2004).

SnS was prepared by cutting a rat brain into pieces of about 60 mg. Each fragment was sonicated twice for 30 s, with a 1 min pause between each sonication, at 40 W using an ultrasonic processor (Vibra Cell 75022, Bioblock Scientific) in 0.6 ml of homogenization buffer (10 mM Hepes–NaOH pH 7.5, 0.32 M sucrose, 5 mM dithiothreitol), centrifuged at 10 000 ×*g* for 15 min at 4 °C and pooled supernatants were filtered through 0.22 μm membranes, aliquoted and stored frozen at −20 °C. Total protein content in SnS and SnH (~7 and 3 mg protein/ml respectively) was measured using Coomassie blue protein determination kit (Pierce). His-SNAP-25 was produced, purified and used as a standard for native SNAP-25 quantification in SnS by western blotting. His-SNAP-25, BoNT/A-Lc, BoNT/B-Lc and BoNT/E-Lc concentrations were determined by amino-acid analysis.

Assay for proteolytic activity. SnH (100 μl) diluted 30-fold in cleavage buffer (50 mM Hepes/NaOH pH 7.4, 20 μM $ZnCl_2$, 0.2 mg/ml BSA, 0.16 mM DTT) was incubated for 3 h in the presence or absence of recombinant BoNT/B-Lc. Cleavage was stopped by addition of NaCl (final concentration 0.4 M) and by placing samples on ice. SnS was diluted (0.23 mg protein/ml) into BoNT/A buffer (20 mM Hepes/NaOH, pH 7.4, 0.1 M NaCl, 20 μM $ZnCl_2$, 1 mM dithiothreitol) and incubated with recombinant BoNT/A-Lc for 3 h at 37 °C.

SPR analysis. SPR detection is based on an optical phenomenon that reports changes in bound mass at the surface of a sensor chip functionalized with antibodies. SPR measurements were performed at 25 °C on a Biacore 3000 or Biacore×instrument (GE Healthcare) generally with CM3 sensor chips (exceptions are indicated) using 50 mM Tris/HCl pH 7.4, 0.15 M NaCl as running buffer. Monoclonal antibodies were captured indirectly using anti-mouse IgG antibodies. Sensor chips were chemically activated by the injection of 35 μl of a 1:1 mixture of 50 mM *N*-hydroxysuccimide (NHS) and 200 mM *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodi-imide (EDC) at 5 μl/min. The reaction enables carboxymethylated groups to bond covalently to the free amino groups of rabbit polyclonal anti-mouse Fcγ antibodies subsequently injected at pH 5. The remaining active sites were then blocked with 35 μl 1 M ethanolamine. About 4000–5000 RU of anti-mouse IgG antibodies were routinely coupled in flow cells. Monoclonal antibodies (50 μg/ml) were immunocaptured to obtain between 400–800 RU. Non-immune IgG or anti-SNAP-25 C-ter rabbit polyclonal antibodies were directly coupled to the chip using the same procedure. Non-immune IgG coated in flow cell 1 was systematically used to assess non-specific binding which was always less than 10%. Data are the signals obtained with specific antibodies minus the non-specific signal with non-immune IgG (except Fig. 2B). SPR assays were performed by serially injecting samples from an autosample rack at 4 °C.

For BoNT/B assays, cleavage of VAMP2 was detected by measuring SV capture on functionalized chips with antibodies against VAMP2, synaptophysin and SV2 (Ferracci et al. 2005). Samples (30 μl) were diluted to 1 μg/ml in 50 mM Tris/HCl pH7.4,

0.4 M NaCl and injected at 5 µl/min into flow cells. Regeneration was performed after a cycle of injections using 100 mM octylglucoside diluted in running buffer. For BoNT/A assays, diluted SnS or cell lysates (25–30 µl) were serially injected at 5 µl/min over mAb4F3 and reference antibodies directed against syntaxin 1, synaptophysin or anti-SNAP-25 N-ter. The ratio of specific membrane binding to mAb4F3 *versus* reference antibodies was measured in the presence or absence of BoNT/A. Rundown in membrane binding to antibodies was measured by injecting reference samples at regular intervals during analysis.

ELISA. To immunoisolate SVs, anti-synaptotagmin1 antibody (10 µg/ml) was coated overnight at 4 °C. Wells were washed and blocked with 2.5% casein in PBS. After rinsing with buffer A (0.25% casein in PBS), 50 µl samples (10 µg protein/ml in buffer A) were added to each well and incubated 1 h at 37 °C. After 3 washes, 50 µl primary antibody (10 µg/ml anti-VAMP2 N-ter, anti-CSP or non-immune IgG were added for 30 min at 37 °C. After washing, 50 µl of alkaline phosphatase-linked goat anti-rabbit antibody (1/10 000 in buffer A) were incubated for 30 min at 37 °C. The reaction was revealed with TMB (3,3′,5,5′-tetramethylbenzidine, Sigma) reagent and read at 450 nm. Samples were run in duplicate, coefficients of variation between duplicates were less than 5%. Direct coating experiments were performed as described above except that 50 µl of SnH or SnS (6 and 14 µg of protein/ml respectively) were directly adsorbed to wells overnight at 4 °C.

Flow cytometry. Aldehyde/sulfate 4 µm diameter latex beads (Invitrogen) covalently coupled to anti-mouse antibodies were functionalized with anti-synaptotagmin1 antibodies, in 0.1% BSA/PBS. 25 µl beads (100 000 beads per point coated with 0.3 µg anti-synaptotagmin1) were added to 200 µl SnH (10 µg protein/ml) and agitated 1 h at 37 °C. Washed vesicle-coated beads were incubated with 100 µl detection antibodies (anti-VAMP2 N-ter, anti-CSP or non-immune IgG) at 10 µg/ml. After washing, beads were agitated with 100 µl FITC-anti-rabbit antibodies (10 µg/ml) for 30 min at 37 °C. For each measurement, 10 000 events were analyzed on a FACSCanto flow cytometer using FACSDiva software. The mean fluorescence intensity (MFI) of the negative control was subtracted in each condition.

Cell culture. Cell cultures were prepared essentially as described (Foran et al., 2003) at 400 000 cells/cm^2. After 10–12 days *in vitro*, neurotoxins were added for 24 h. Neurons were washed 3 times with PBS and frozen without buffer. Lysis buffer (5 mM Hepes–NaOH pH 7.4) was added (0.3 ml/well for 24-well plate or 0.1 ml for 96-well plate) and cell lysates were sonicated 10 s (24-well plate) or 5 s (96-well plate) and centrifuged at 10 000 ×g for 15 min at 4 °C. Supernatants were collected, diluted in running buffer before injection over the chip surface.

Results

Our experimental rationale is based on the use of membrane fractions which contain anchored VAMP2 and SNAP-25, the intracellular substrates for the protease activity of BoNT/B and BoNT/A respectively. Membrane fractions from rat brain are treated with BoNT light chains *in vitro*. Alternatively membrane fractions are prepared from cultured neurons previously incubated with whole BoNT. In both cases antibodies directed against the appropriate substrate are then used to capture membranes. Antibodies may be directed against substrate epitopes that are clipped off by toxin action. In this case BoNT activity is assayed as a reduction in membrane capture. Other antibodies bind to epitopes that are absent in the native substrate, but are specifically produced by the proteolytic action of BoNTs (e.g. a new carboxyl terminus). Here BoNTs are assayed from the increase in membrane capture.

Comparative BoNT/B assays using synaptic vesicles

BoNT/B activity was measured using SVs in a 10 000 ×*g* supernatant of rat brain homogenate (SnH), which constitutes a convenient substrate (Ferracci et al., 2005). In order to compare different detection methods, SnH was incubated with a range of concentrations of recombinant BoNT/B light chain (BoNT/B-Lc) and the extent of VAMP2 cleavage was determined by SPR, flow cytometry and ELISA.

Proteolysis of rat brain SVs by BoNT/B removes an N-terminal 1-76 fragment of VAMP2. SPR assays were carried out using a monoclonal anti-VAMP2 antibody against a N-terminal peptide (1–18), which thus recognize an epitope clipped off by BoNT/B action. This antibody was immobilized in a flow cell and BoNT/B-Lc-treated or control SnH samples were injected. Anti-SV2 antibodies and non-immune IgG were also used in parallel flow cells to assess cleavage specificity and non-specific binding respectively. Samples from a range of BoNT/B-Lc concentrations were injected sequentially without antibody regeneration as each injection saturated a maximum of 2–5% of antibody capacity. SV capture was measured on a chip functionalized with anti-VAMP2 and anti-SV2 antibodies. The ratio of the anti-VAMP2/anti-SV2 SPR signals was thus set at 100% in the absence of BoNT/B-Lc. Increasing BoNT/B-Lc concentrations inhibited SV capture by anti-VAMP2 antibodies, but did not affect capture on anti-SV2 antibodies. Toxin activity is thus represented as the SPR signal ratio (anti-VAMP2/anti-SV2 × 100%). Hence the anti-SV2 signal normalizes any variability in the quantity of substrate injected. Dose–response curves from 3 independent experiments yielded an EC_{50} of 1.1 ± 0.1 pM (Fig. 1A).

A flow cytometry method was developed to measure VAMP2 immunoreactivity. As SVs are too small to be resolved by flow cytometry, they were first captured on calibrated 4 µm beads. SnH treated with BoNT/B-Lc was incubated with latex beads functionalized with monoclonal antibodies against the SV protein synaptotagmin1. Organelle-coated beads were mixed with a saturating concentration of rabbit anti-VAMP2 N-ter antibodies, anti-CSP antibodies or irrelevant IgG. The beads were then incubated with FITC-conjugated anti-rabbit antibodies and analyzed in a flow cytometer. The dot plot representation of forward and side scatter reveals a population of singlet beads which were gated for fluorescence analysis. Flow cytometry analysis demonstrated a high MFI value for VAMP2 and a weaker value for CSP (not shown) consistent with their relative expression in SVs (Takamori et al., 2006). Pre-treatment of SnH with BoNT/B-Lc resulted in a concentration-dependent decrease in the VAMP2 signal whereas CSP fluorescence, used as internal control, remained constant (result not shown). Three independent experiments gave an EC_{50} of 1.3 ± 0.2 pM (Fig. 1B).

Immunodetection by ELISA was carried out by two methods. The first involved an antibody sandwich format with SVs immobilized in wells coated with anti-synaptotagmin1 antibodies. After a blocking step and washing, captured SVs were incubated with anti-VAMP2 or anti-CSP polyclonal antibodies and bound immune complexes revealed using a colorimetric reaction. In the second method, SnH was directly coated on plastic wells and SV antigens detected as above. In both assays, VAMP2 immunoreactivity was dependent on BoNT/B-Lc concentration while CSP signals were not affected (not shown). Figs. 1C and D illustrate dose–response curves obtained by both approaches yielding EC_{50} values respectively of 1.9 ± 0.1 pM and 1.1 ± 0.1 pM. Thus SPR, flow cytometry and ELISA produced quasi-identical results.

SPR quantification of plasma membrane antigens

We next investigated measurement of BoNT/A activity using SPR to detect native SNAP-25. SNAP-25 is a palmitoylated protein, mainly localized on the cytoplasmic face of the plasma membrane, although a minor population is associated with secretory vesicles. SNAP-25 was present in the SnH fractions used for BoNT/B assays, probably mainly associated with synaptic vesicles. Western blotting experiments were

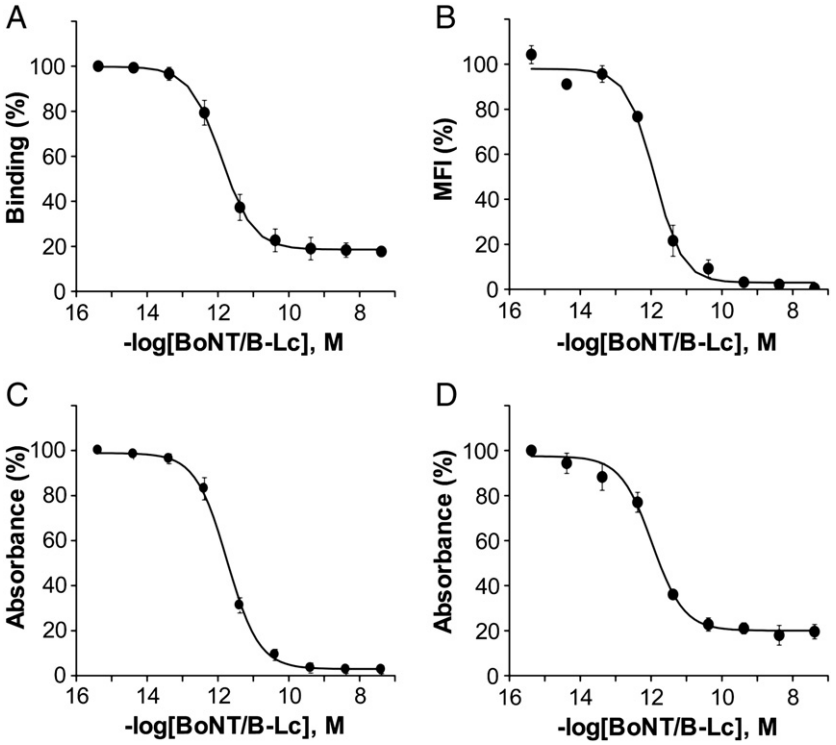

Fig. 1. Measurement of BoNT/B light chain activity by SPR, flow cytometry and ELISA. SnH (100 μg protein/ml) was incubated with increasing concentrations of BoNT/B-Lc, then analyzed. (A) SPR: SV capture was measured on a chip functionalized with anti-VAMP2 and anti-SV2 antibodies. The ratio of the SPR signal (anti-VAMP2/anti-SV2 × 100%) measured in the presence or absence of BoNT/B-Lc yields Binding (%) on the ordinate. (B) Flow cytometry: SVs were immunocaptured on beads functionalized with anti-synaptotagmin1 antibodies and VAMP2 was quantified. (C, D) ELISA: SVs were immunoisolated (C) or SnH membranes were directly coated (D). Immunoreactivity with anti-VAMP2 antibody was measured. All plots are means ± SD of three independent experiments.

performed to assess whether the SnH fraction provides a good substrate for BoNT/A which clips off the nine C-terminal amino acids (residues 198–206) of SNAP-25 (Blasi et al., 1993). SnH was incubated in presence of a high concentration of BoNT/A-Lc and immunoblots (Fig. 2A) stained with a polyclonal anti-SNAP-25 C-ter antibody or with a monoclonal antibody (mAb4F3) that specifically recognizes the

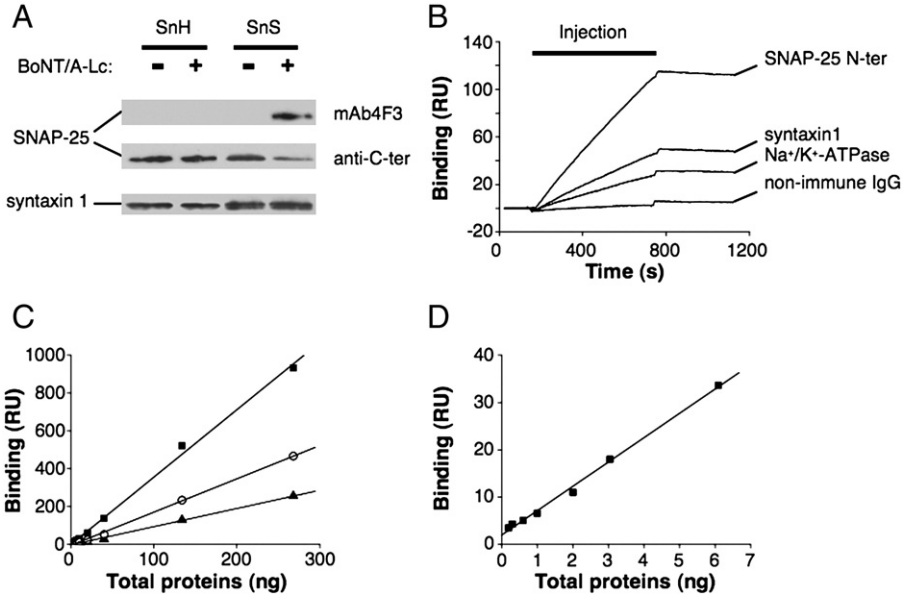

Fig. 2. Detection and assay of plasma membrane proteins. (A) Cleavage of SNAP-25 by BoNT/A. Proteins from SnH (100 μg protein/ml) and SnS (200 μg protein/ml) were incubated in the presence or absence of 10 nM BoNT/A-Lc. Proteins (40 μl SnH and 20 μl SnS) were resolved on 12% SDS-PAGE. Immuno-blotting was performed using anti-SNAP-25 antibodies recognizing either only the BoNT/A-cleaved form (mAb4F3) or clipped C-terminal domain (anti-C-ter) that is clipped off. Specificity for proteolysis of SNAP-25 was confirmed with anti-syntaxin 1 antibody. (B) SPR detection of plasma membrane proteins. SnS diluted in running buffer (12 μg/ml) was injected (30 μl at 3 μl/min) on a CM3 sensor chip coated with anti-SNAP-25 N-ter, anti-syntaxin1, anti-Na^+/K^+ ATPase and non-immune IgG. (C, D) SPR dose–response curve for plasma membrane proteins: (C) SnS (2–270 ng protein in 30 μl) were injected onto anti-SNAP-25 N-ter (■), syntaxin1 (○) and Na^+/K^+-ATPase (▲) antibodies immobilized on a CM3 sensor chip ($R^2 > 0.99$). (D) Dilution of SnS (0.2–6 ng of total proteins in 30 μl) was injected onto anti-SNAP-25 N-ter (■) coated on a C1 sensor chip ($R^2 > 0.99$). All data represent specific binding obtained after subtraction of background on a flow cell coated with non-immune IgG. RU values were recorded 25 s after the injection of SnS dilution.

BoNT/A-cleaved form of SNAP-25. Results indicate that SNAP-25 in SnH was apparently insensitive to attack by BoNT/A. When SNAP-25 is associated with VAMP2 and syntaxin1 to form SNARE complexes, it is protected from BoNT/A action (Hayashi et al., 1994). This is the case in SV membranes in which SNAP-25 is probably totally incorporated into complexes by the molar excess of VAMP2 and syntaxin 1 (Otto et al., 1997). Hence SNAP-25 in SnH is not an appropriate substrate for BoNT/A assay. Membrane fractions containing SNAP-25 anchored in synaptic plasma membranes could potentially provide a better substrate. To produce small plasma membrane vesicles we directly sonicated rat brain fragments, and prepared a 10 000 ×*g* supernatant which was then filtered (0.22 μm). This fraction, designated SnS (supernatant of sonicated brain), contains SNAP-25 which was cleaved by BoNT/A-Lc (Fig. 2A). In the same conditions, the level of syntaxin1, used as an internal control protein, was not changed. A minor fraction of toxin-resistant SNAP-25 was also observed in all experiments.

To determine whether SnS can be used to detect plasma membrane markers and provide a BoNT/A substrate for SPR, membranes were injected over SPR chips carrying antibodies against Na^+/K^+-ATPase, syntaxin1 and SNAP-25. Injection of diluted SnS produced a linear increase in the SPR response during the association phase, terminating in a plateau when injection stopped (Fig. 2B). The rate of membrane capture differed with each antibody, presumably depending on the relative density of exposed epitopes. When SnS was injected at a high concentration, the SPR signal saturated at a plateau of about 3000 RU, with each antibody (not shown). As illustrated in Fig. 2B, the non-specific signal obtained with non-immune IgG was in all cases less than 10% of the signal obtained with specific antibodies. Interactions were specific as preincubation of SnS with the antibody in solution before injection blocked subsequent interaction with the same antibody immobilized on the SPR chip. Furthermore specific interactions involved membrane particles, as centrifugation of SnS at 100 000 ×*g* before injection completely abolished the SPR signal (not shown). Sensorgram analysis of the post-injection phase indicates that plasma membrane binding to the sensor chip was relatively stable. It was regenerated by injection of concentrated octylglucoside which solubilizes membranes and dissociates antibody/antigen interactions (Ferracci et al., 2004, 2005). Multiple injections yielding signals from 5–20 RU can be carried out sequentially which associated with cyclic regeneration, allows several hundred injections using the same antibody chip over a one week period (not shown, Ferracci et al., 2005; Dillon et al., 2005). These results indicate that synaptic plasma membranes can be specifically captured on SPR chips via antibodies against cytoplasmic epitopes.

As shown in Fig. 2C, a linear dose–response curve was established by measuring the increase in mass subsequent to injections of SnS ranging from 2 to 270 ng protein over CM3 sensor chips. A higher sensitivity was obtained with dextran-free C1 chips corresponding to an absolute detection limit of 2.7 pg SNAP-25 (based on SnS SNAP-25 content determined by quantitative western blotting) from only 200 pg of total protein (Fig. 2D). Thus SPR can be used to directly quantify native plasma membrane-anchored antigens over an extended linear dynamic range. This high sensitivity (5–10 fold higher than for ELISA in our hands) is in agreement with previous findings with synaptic vesicle antigens (Ferracci et al., 2005) and is due to amplification of SPR signals by the high molecular masses of the analytes.

Detection of BoNT/A-Lc endoprotease activity by SPR

The effects of BoNT/A-Lc on membrane capture from SnS was evaluated using three different anti-SNAP-25 antibodies immobilized on the chip. A polyclonal anti-C-terminal (anti-SNAP-25 C-ter) antibody chemically coupled to the chip or two monoclonal antibodies captured indirectly: one against an N-terminal domain of SNAP-25 (anti-SNAP-25 N-ter) and mAb4F3 which specifically recognizes the new SNAP-25 C-terminal domain produced by BoNT/A cleavage. Upon injection of SnS, a strong SPR signal was generated with anti-SNAP-25 N-ter while responses with SNAP-25 C-ter and mAb4F3 were weaker (Fig. 3A upper). Following BoNT/A-Lc treatment (Fig. 3A lower), membrane capture by anti-SNAP-25 C-ter was abolished, while it

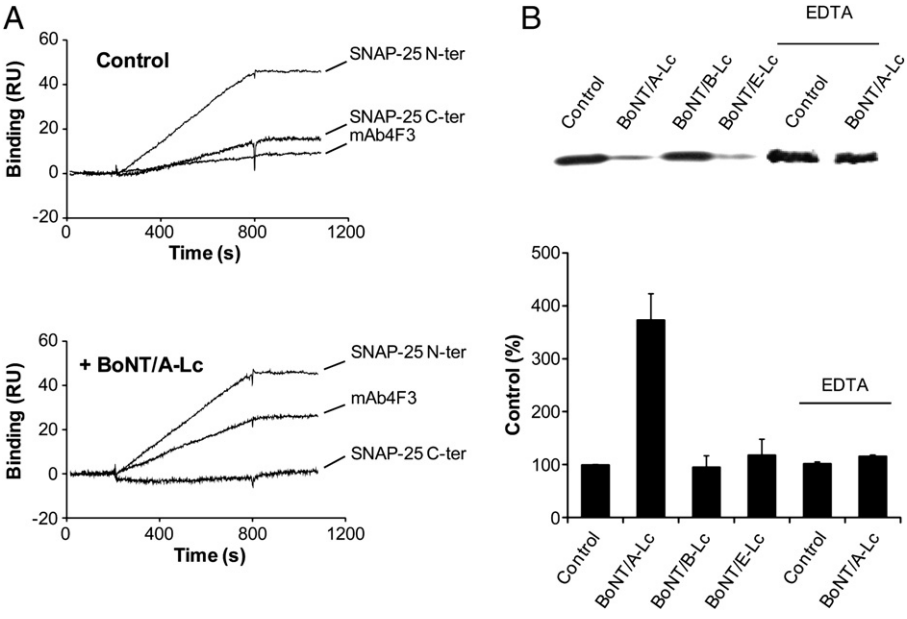

Fig. 3. Detection of BoNT/A light chain activity by SPR. (A) SPR detection of membrane-associated SNAP-25 using anti-SNAP-25 antibodies: CM3 sensor chip was functionalized with mAb anti-SNAP-25 N-ter, mAb4F3 and anti-SNAP-25 C-ter polyclonal antibodies. SnS (220 μg/ml) was incubated for 3 h at 37 °C in the absence (upper) or presence (lower) of 10 nM BoNT/A-Lc. SnS was diluted 100 fold (SNAP-25 N-ter and mAb4F3) or 10 fold (SNAP-25 C-ter) before injection, then sensorgrams were superimposed. (NB polyclonal anti-SNAP-25 C-ter antibodies are an IgG fraction with a lower binding capacity than monoclonal anti-SNAP-25 N-ter antibodies). (B) SnS (220 μg/ml) was incubated for 3 h at 37 °C with 10 nM recombinant BoNT/A, B or E-Lc in BoNT/A buffer. Parallel incubations were performed in the presence of 20 mM EDTA. Samples (20 μl) were denatured and SNAP-25 detected by western blotting with anti-SNAP-25 C-ter antibody (upper). The same samples were analyzed using SPR (lower). Specific membrane capture was measured on a sensor chip functionalized with mAb4F3 and anti-SNAP-25 N-ter (mAb71.2) antibodies. The ratio of SPR signals (mAb4F3/anti-SNAP-25 N-ter) measured in absence of BoNT yields the control value. Data are representative of two independent experiments (SPR results are the mean±SD of triplicates).

increased with mAb4F3, but was unchanged with the anti-SNAP-25 N-ter. These data illustrate that cleavage of membrane-anchored SNAP-25 by BoNT/A can be detected by SPR analysis of epitopes that are either clipped off or generated by toxin action. Monitoring an unchanged epitope in parallel provides internal calibration of SNAP-25 content that can be used for normalization.

In subsequent experiments mAb4F3 was chosen to allow maximum use of sensor chips by indirect immobilization. The specificity of membrane capture by mAb4F3 for BoNT/A-treated samples was also verified. SnS was incubated with BoNT/A-Lc, B and E (10 nM). Western blotting analysis (Fig. 3B upper) with anti-SNAP-25 C-ter antibody confirmed that SNAP-25 was cleaved by BoNT/A-Lc and E (BoNT/E removes a 26 amino acid C-terminal peptide (Binz et al., 1994)), but not by BoNT/B-Lc. In these conditions VAMP2 was cleaved by BoNT/B (not shown). Analysis of the same samples by SPR demonstrated that only BoNT/A-Lc induced membrane capture by mAb4F3. However capture was abolished when incubations were performed in the presence of EDTA (20 mM) which blocks the metalloprotease activity of BoNT/A-Lc by chelating Zn^{2+} (Fig. 3B lower). Taken together these experiments demonstrate that cleavage of native SNAP-25 by BoNT/A-Lc can be detected by direct injection of a crude fraction over an SPR chip that carries appropriate antibodies.

SPR assays measuring BoNT/A activity

SnS was treated with a range of recombinant BoNT/A-Lc concentrations then analyzed by SPR using mAb4F3. Results display an increment in capture by mAb4F3 with increasing BoNT/A-Lc concentration. Typical dose–response curves are illustrated in Fig. 4 using either a synaptic vesicle protein or a plasma membrane antigen as an internal control. The EC_{50} from 3 independent experiments was 34 ± 3 pM (Fig. 4A). Similar results were obtained by western blotting (Fig. 4B) or ELISA (not shown) using loss of labelling by anti-SNAP-25 C-ter antibody to evaluate BoNT/A-Lc activity. Capture of BoNT/A-Lc-treated membranes on mAb4F3 (Fig. 4A) reached a plateau at a comparable concentration to that producing a maximum loss of C-terminal epitopes (Fig. 4B). Comparable EC_{50}s were obtained using

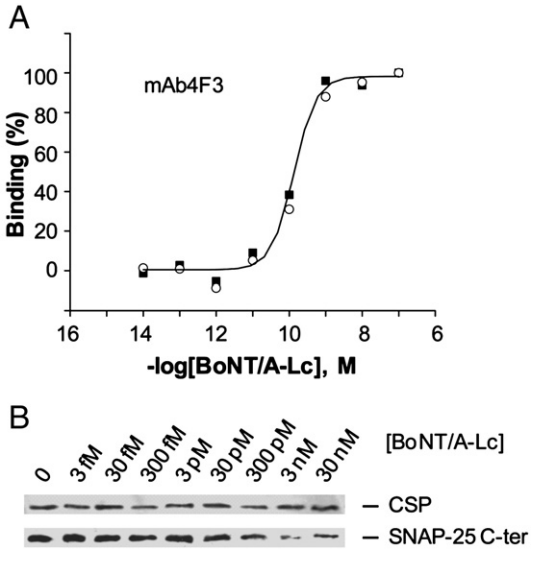

Fig. 4. SPR method to assay BoNT/A light chain activity. SnS (220 μg protein/ml) was incubated with the indicated concentrations of BoNT/A-Lc. (A) Specific membrane capture was measured on a chip functionalized with mAb4F3, anti-syntaxin1 and anti-synaptophysin antibodies. Signals obtained on mAb4F3 were normalized to anti-syntaxin1 (○) and anti-synaptophysin (■) antibodies. The signal obtained in the absence of BoNT/A-Lc was taken as 0%. Plots are representative data from one of three independent experiments. (B) Samples (20 μl) were processed on 12% SDS-PAGE before immunoblotting with anti-SNAP-25 C-ter or anti-CSP antibodies.

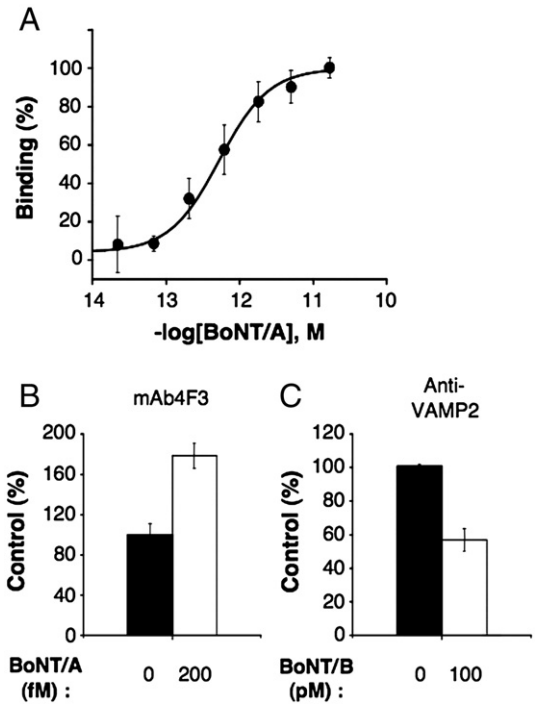

Fig. 5. Measurement of BoNT/A holotoxin activity with cultured neurons. (A) Cerebellar granule neurons cultured in 24-well plates were incubated with the indicated concentrations of BoNT/A. Diluted cell lysates were injected over chips functionalized with anti-syntaxin1 and mAb4F3 antibodies and specific signals measured. The signal obtained with lysates from untreated cells was taken as 0%. The result shown is the mean ± SD of 4 independent experiments. (B, C) Cerebellar granule neurons cultured in 96-well plates were incubated with either 200 fM BoNT/A (B) or 100 pM BoNT/B (C). Diluted cell lysates were injected over anti-VAMP2, mAb4F3 and anti-synaptophysin antibodies and specific signals measured. The ratios of SPR signals (mAb4F3/anti-synaptophysin for BoNT/A assay and anti-VAMP2/anti-synaptophysin for BoNT/B assay) measured in the absence of BoNT yield the control values. Results shown are the mean ± SD of 3 independent experiments.

purified reduced BoNT/A instead of the recombinant light chain or soluble his-SNAP25 rather than the native protein (measured by western blotting, not shown).

We next asked whether SPR methods could be used to measure proteolytic cleavage of SNAP-25 in a cell culture model. Cerebellar granule neurones, plated in 24-well dishes were incubated with a range of concentrations of BoNT/A for 24 h. Cultures were sonicated and fractions equivalent to SnS analyzed by SPR using mAb4F3 (Fig. 5A). This yielded an EC_{50} of 571 ± 192 fM in accordance with EC_{50} measured by immunoblot using the same cell model (Foran et al., 2003).

Chip-based analysis of BoNT activity with cell cultures is expected to be applicable to inhibitor screening. Automated analysis of lysates from cells intoxicated with BoNTs was then carried out using cerebellar granule neurones cultured in 96-well plates. After incubation with a fixed concentration of BoNT/A or B near to their respective EC_{50}s (Foran et al., 2003) an increase in signal was observed for BoNT/A with mAb4F3 (178 ± 12% Fig. 5B) and a decrease for BoNT/B with anti-VAMP2 N-ter (−43 ± 7%, Fig. 5C) in comparison to control conditions without neurotoxins. SNARE cleavage in intact cells requires BoNT heavy chain binding to cell surface receptors followed by internalization and translocation of proteolytic light chains into the cytoplasm. Thus this method provides a sensitive assay for the global biological activity of BoNTs.

Discussion

This study shows that protein chip methods constitute a reliable and accurate method to measure SNARE cleavage produced by BoNT/A

and B in crude fractions or cell cultures. Conventional immunological quantification of membrane proteins requires several steps including specific or non-specific solid-phase adsorption (nitrocellulose, bead, plastic well,…) of the antigen followed by incubation with primary and secondary labelled antibodies. Assays are based upon membrane protein analysis by SPR, in which antigens are directly titrated by injecting membranes over specific antibodies immobilized at high density upon sensor chips. SPR detects molecular interactions in real time rather than in terms of end point analysis. Assay of soluble components by SPR has been reported (Karlsson et al., 1993) and we have previously shown that membrane-anchored synaptic vesicle proteins are also amenable to this method (Ferracci et al., 2005). We have now extended this observation to plasma membrane antigens using crude preparations. The performance of SPR detection for BoNT/B assay, was compared to ELISA and cytometry, both well-established methods for membrane proteins (Clayton et al., 2001; Lamparski et al., 2002). EC_{50}s for BoNT/B-Lc measured by SPR were practically identical to those obtained by ELISA and flow cytometry. However SPR confers several advantages in terms of speed and reagent economy over conventional methods.

Plasma membrane antigens were detected at less than 100 pg/ml comparable to synaptic vesicle antigens (Ferracci et al., 2005). Absolute quantification can also be achieved if the preparation is calibrated for example by quantitative western blotting. The high sensitivity found in SPR is inherent to the small flow cell volume allowing antibody concentrations considerably higher than 10× K_D and the high molecular mass of the analytes which amplifies the SPR signals. The sensitivity of SPR, combined with parallel measurements on a single sample of both non-specific binding and internal reference antigens for normalization, facilitate the quantification of BoNTs activity in cultured cells correcting well-to-well variability in cell seeding and extraction.

Attempts to cleave membrane-anchored SNAP-25 by BoNT/A in sub-cellular fractions in absence of added proteins or detergent have not been previously reported. Our data suggest that SNAP-25 is resistant to BoNT/A and E in synaptic vesicles whereas VAMP2 is accessible to proteolytic attack by BoNT/B, BoNT/F and TeNT (Ferracci et al., 2005). It confirms the fact that vesicular SNAP-25 mostly assembles into *cis* SNARE complexes, which protect it from endoproteolytic activity (Otto et al., 1997). *Cis* SNARE complexes also contain VAMP2, which is presumably equally resistant to neurotoxin action. However vesicles contain about 30 times more VAMP2 than SNAP-25. Thus the majority of vesicular VAMP2 is available as a substrate for BoNT/B, F, and TeNT. We have established a simple sonication method which generates fragments of brain plasma membrane suitable for SPR analysis on chips carrying antibodies against intra-cellular domains of plasma membrane proteins. This fraction containing plasma membrane-anchored SNAP-25 provides a convenient native substrate to detect and assay BoNT/A activity. Like the BoNT/B assay, the EC_{50} for BoNT/A obtained by SPR is similar to that found by western blotting or ELISA.

There are currently no effective small molecule inhibitors of BoNTs. Our method has several advantages as a potential screening assay. Firstly the native membrane-anchored proteins constitute a closer approximation to the relevant physiological substrate than the widely used peptide substrates. Secondly the low cost of reagents, the abundance of the substrate (1 rat brain yields enough substrate for 100 000 points) and the "one-step" detection method has the potential to provide an economical screening assay using multiplexed SPR analysers.

Native membrane VAMP2 is more efficiently cleaved by BoNT/B than synthetic substrates (Ferracci et al., 2005). Furthermore localization of SNAP-25 to the plasma membrane is critical for its efficient cleavage by BoNT/A in transfected cells (Dong et al. 2004). Our results indicate that BoNT/A sensitivity is identical using membrane-anchored SNAP-25 or cognate recombinant protein. Thus in our experimental conditions cleavage of SNAP-25 is not promoted by a native environment. The EC_{50} (34 pM) is in the range of published data (3 pM to 1 nM) using recombinant SNAP-25 (O'Sullivan et al., 1999; Ibanez et al., 2004; Chaddock et al., 2002; Dong et al., 2004), although published reports claim a significantly higher sensitivity in particular using peptide mass spectrometry (Ekong et al., 1997; Boyer et al., 2005).

Cell-based assays provide the most appropriate biological model for the assessment of BoNTs since they closely recapitulate the mechanism by which the neurotoxins act *in vivo*. Previously published cell-based assays for BoNTs involved biochemical measurement of neurotransmitter release or quantification of SNARE cleavage by western blotting (Blasi et al., 1993; Foran et al., 2003). FRET signals generated by transfected SNAREs also constitute an elegant method for the detection of BoNT/A and B activity (Dong et al., 2004). However this approach using clonal neuroendocrine cell lines requires high BoNT concentrations as these cells are less sensitive to toxin action than neurons. The SPR method reported here was successfully used to measure SNARE cleavage in cultured neurons and was sensitive enough to detect the activity of BoNTs in cells cultured in a 96-well format. It can provide a routine assay for quality control during commercial BoNT production, and constitute a new assay for inhibitor screening or evaluation of the presence of neutralizing serum antibodies in patients undergoing treatment.

BoNT/A assays with cultured neurones were about 60 times more sensitive than with plasma membrane fragments. Presumably this may in part be due to the capacity of neurones to concentrate toxin light chain in their cytoplasm, via heavy chain binding to a membrane receptor (the intraluminal domain of SV2 associated with a ganglioside), endocytosis and translocation. In contrast in cell free assays BoNT/A-Lc could be rapidly inactivated (Ibanez et al., 2004).

In conclusion, immunocapture of membrane-associated SNARE proteins monitored by SPR constitutes a highly sensitive method to analyze SNARE proteolysis. It provides a rapid and label-free one-step method for routine assay of BoNT/A and B activity.

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