Synaptic vesicle chips to assay botulinum neurotoxins

Géraldine FERRACCI*, Raymond MIQUELIS*†, Shunji KOZAKI‡, Michael SEAGAR§ and Christian LÉVÊQUE*§¹

*Unité de Méthodologie des Interactions Moléculaires, Institut Jean Roche, Faculté de Médecine secteur Nord, 13916 Marseille, France, †CNRS/Université de la Méditerranée FRE 2738, Institut Jean Roche, Faculté de Médecine secteur Nord, 13916 Marseille, France, ‡Department of Veterinary Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai-shi, Osaka 599-8531, Japan, and §INSERM/Université de la Méditerranée UMR 641, Institut Jean Roche, Faculté de Médecine secteur Nord, 13916 Marseille, France

BoNTs (botulinum neurotoxins), considered to be the most toxic of all biological substances, inhibit neurotransmission through proteolytic cleavage of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins [VAMP (vesicle-associated membrane protein, or synaptobrevin), SNAP-25 (25 kDa synaptosome-associated protein) or syntaxin]. Expansion in the use of BoNTs as therapeutic and cosmetic agents, and the potential threat they constitute as biological weapons, underlines the need for rapid and sensitive *in vitro* assays. Here, we present new automatized bioassays to detect VAMP cleavage by BoNT/B and F. Western blotting and SPR (surface plasmon resonance) methods revealed that BoNT/B and F totally cleave their substrate on immunisolated SVs (synaptic vesicles). Real-time monitoring of the immunocapture of native SVs from crude lysates on SPR sensor chips enabled the detection of picogram

amounts of different SV proteins. Pre-incubation of a membrane fraction containing SVs with BoNT specifically inhibited capture by anti-VAMP antibodies, and amounts as low as 0.1 pg of BoNT/B were detected. This automated SPR assay is approx. 200 times more sensitive, and 25 times more rapid, than the *in vivo* BoNT/B test currently used. Moreover, the method can be performed using a few thousand cultured neurons and constitutes a new screening assay for inhibitors. Our data indicate that native VAMP is an optimal substrate for *in vitro* BoNT assays that can be monitored by SPR.

Key words: botulinum neurotoxin, surface plasmon resonance, synaptic vesicle, synaptobrevin, VAMP (vesicle-associated membrane protein).

INTRODUCTION

Clostridium botulinum produces seven distinct types of botulinum neurotoxin (BoNT/A–G). BoNT/A, B, E and F are the most potent in humans [lethal dose 0.05–0.1 µg by inhalation or a few µg *per os* (by mouth)], and produce clinical botulism by blocking neurotransmitter release at peripheral synapses. BoNTs are structurally similar, consisting of a heavy (H; 100 kDa) chain and a light (L; 50 kDa) chain linked through a disulphide bond and non-covalent interactions. H-chains mediate binding to specific neuronal receptors, leading to internalization of the L-chains and their translocation into the cytoplasm of nerve terminals. The L-chains are zinc endopeptidases which cleave the presynaptic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins VAMP (vesicle-associated membrane protein/synaptobrevin; cleaved by BoNT/B, D, F and G), located in SVs (synaptic vesicles), and SNAP-25 (25 kDa synaptosome-associated protein; cleaved by BoNT/A, C and E) and syntaxin (cleaved by BoNT/C), expressed mainly in the plasma membrane. Assembly of these three proteins into a ternary SNARE complex is an essential step in SV fusion with the plasma membrane, and the correlation between SNARE protein cleavage by BoNTs and block of neurotransmission is well established [1,2].

The need to detect and quantify BoNTs can arise in different contexts. In addition to the three naturally occurring forms of human botulism (food-borne, wound and intestinal), the military or terrorist use of BoNTs as biological weapons constitutes a menace. Furthermore, the use of BoNTs to treat human disease is expanding. Local injection of minute amounts of BoNT (mainly BoNT/A or B) relieves muscle hyperactivity underlying contractures, spasms and focal dystonia [3]. BoNT/A is also

increasingly used cosmetically to reduce facial wrinkles. Assays for BoNTs must therefore fulfil a variety of needs, including detection in the environment (surveillance), precise measurement of their specific activity (quality control), and measurement of inhibitor potency (development of drugs and antibodies to treat botulism). Only assays that monitor the biological activity of BoNTs can meet these requirements. Toxin activity can be assayed by measuring toxicity *in vivo* or by evaluating SNARE cleavage *in vitro*. The mouse bioassay is currently the most widely used method. Introduced several decades ago, it is highly sensitive but lacks specificity, can take up to 4 days to complete and requires numerous animals, thus raising ethical issues.

In vitro assays based on VAMP cleavage typically rely upon separating the substrate from the resulting cleavage fragments, which are then quantified. Alternatively, BoNT endopeptidase activity has been measured using fluorescence-based techniques [4,5]. However, assays with synthetic peptide substrates are generally insufficiently sensitive to detect small quantities of toxin, probably because, unlike native proteins, they do not assume the optimal conformation for toxin cleavage [6,7].

Native SNARE cleavage can be detected using a variety of neuronal preparations, including subcellular fractions such as SVs, plasma membrane, synaptosomes and intact cells, either in culture or in brain slices [8–10]. Cleavage in intact cells requires BoNT binding, internalization and translocation into the cytoplasm, and thus provides a more complete assay for both H- and L-chain activity. However, a major obstacle in using native substrates to measure BoNT activity is the lack of a sensitive routine assay for SNARE cleavage [11]. Detection by Western blotting is time-consuming, quantification is laborious and most reports only provide qualitative results. Thus new assays for BoNT activity must

Abbreviations used: BoNT, botulinum neurotoxin; DTT, dithiothreitol; GST, glutathione S-transferase; RU, resonance unit; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; Sn10, the 10 000-*g* supernatant fraction; SPR, surface plasmon resonance; SV, synaptic vesicle; SYT, synaptotagmin; VAMP, vesicle-associated membrane protein/synaptobrevin.

¹ To whom correspondence should be addressed (email leveque.c@jean-roche.univ-mrs.fr).

be introduced, particularly automated tests with high sensitivity to replace mouse LD₅₀ bioassay, but also to rapidly quantify BoNT activity in cell cultures.

Biosensors use biological molecules to monitor biochemical interactions. SPR (surface plasmon resonance) spectroscopy allows real-time measurement of interactions between immobilized ligands such as antibodies and analytes in solution. Labelling of molecules is not required, and automatic analysis of small samples can be performed. SPR has been used previously [12] to characterize SVs, a homogeneous population of small (≈ 50 nm) presynaptic organelles. Here, we report an SPR method to quantify native membrane proteins in intact SVs. It provides a means to assay BoNT/B and BoNT/F activity, and constitutes the most sensitive *in vitro* assay described to date. Moreover, the method is particularly suitable for rapid assay of BoNT action on cultured neurons.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibodies against VAMP 2 (Cl69.1) and synaptogyrin (Cl80.1) were obtained from Synaptic Systems (Göttingen, Germany). A monoclonal anti-SV2A antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA, U.S.A.), and non-immune mouse IgG from Interchim (Montluçon, France). Monoclonal anti-synaptophysin (171B5), SYT 1 (synaptotagmin-1; 1D12), SNAP-25 (BRO5) and VAMP (1H7 and 3D10), and polyclonal anti-(VAMP 2 N-ter), anti-(VAMP 60–88) and CSP antibodies have been described previously [12,13]. Rabbit polyclonal anti-mouse Fc γ antibodies, sensor chips (CM5, CM3 and C1) and amine-coupling kits were from Biacore International SA (Neuchâtel, Switzerland). Nitrocellulose membranes (0.2 μ m pore size) were from Schleicher and Schuell (Dessau, Germany). Octyl glucoside (*n*-octyl β -D-glucopyranoside) and fatty-acid-free BSA were purchased from Sigma. BoNT/B, F and E (respectively, 0.5×10^8 , 0.5×10^7 and 0.5×10^7 LD₅₀/mg of protein) were produced, and purified as described previously [14].

Subcellular fractionation

Rat brain fraction Sn10 (the 10000-*g* supernatant fraction) containing SVs was obtained in a single step, essentially as described previously [12]. Briefly, fresh rat cerebral cortex (1 g) was homogenized with a Teflon homogenizer in 10 ml of cold 10 mM Hepes/NaOH, pH 7.5, containing 0.32 M sucrose with or without 5 mM DTT (dithiothreitol). Homogenates were centrifuged at 10 000 *g* for 15 min at 4°C, and the supernatants (≈ 3 mg of protein/ml) were filtered through 0.22 μ m membranes.

SVs were immunoprecipitated from Sn10 using monoclonal antibodies against SYT 1 (1D12), VAMP 2 (Cl 69.1) or control IgG [15]. Immune complexes were incubated for 3 h at 4°C under agitation with pre-washed Protein A–Sepharose. Beads were collected by centrifugation and washed three times with TBS buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl] containing 0.1% BSA, once in TBS and then denatured in SDS/PAGE sample buffer.

Primary cultured hippocampal neurons (40 000 per dish) were prepared from embryonic day-18 rats [16]. Cells were washed three times with PBS, followed by a 3 min incubation with 0.5 ml ice-cold lysis buffer [5 mM Hepes/NaOH (pH 7.4)/1 mM EDTA/1 mM PMSF] under agitation. Well contents were collected and centrifuged in a Microfuge at 10 000 *g* for 10 min at 4°C to remove cell debris.

Assays for proteolytic activity

For experiments using Sn10, purified BoNTs were reduced for 45 min at 37°C in 10 mM Hepes/NaOH, pH 7.5/150 mM NaCl solution, containing 10 mM DTT. Inactivated toxins were obtained by heating for 5 min at 90°C. Sn10 was diluted in PCR tubes (3 μ g of protein/ml for SPR analysis, or 600 μ g of protein/ml for immunoblotting) in cleavage buffer [50 mM Hepes/NaOH (pH 7.5)/20 μ M ZnCl₂/0.2 mg/ml BSA/0.16 mM DTT] and treated for 3 h at 37°C or 4°C with reduced or inactivated BoNTs. Cleavage was stopped by placing samples on ice, followed by addition of NaCl to a final concentration of 0.4 M. For experiments on neuronal cultures, holo-BoNTs (H + L-chains) were diluted in cell-culture medium (1 ml per dish) and incubated 24 h at 37°C.

SPR analysis

SPR measurements were performed with a Biacore 3000 apparatus (Biacore International SA) at 25°C with TBS as the running buffer. Anti-SV antibodies were captured as described previously [12] on rabbit polyclonal anti-mouse Fc γ antibodies using the amine-coupling kit, as recommended by Biacore. All sensorgrams show data in which the non-specific signal obtained from the control surface coated with non-immune IgG has been subtracted from the signal obtained with anti-SV antibodies.

To detect VAMP cleavage after SV capture on chips, Sn10 was incubated in the presence or absence of BoNTs and injected over anti-SYT 1 (0.6 ng) and control IgG (0.6 ng) at 5 μ l/min to immobilize 1200–1900 RU (resonance units) [12]. Flow was increased to 10 μ l/min, and sensor chips were pre-saturated with non-immune IgG (50 μ g/ml). Anti-SV antibodies (30 μ g/ml) were then injected over the sensor surface in alternation with non-immune IgG.

To assay SV proteins, anti-SV antibodies (Cl69.1, 171B5 or 1D12) and non-immune IgG were bound at equivalent densities (0.8 ng for CM3 chips) to separate flow cells. Before analysis, sensor surfaces were conditioned by several injections of buffer containing 0.4 M NaCl and 0.2 mg/ml BSA. The buffer signal was then subtracted from test signals. All samples were adjusted to 0.4 M NaCl and 0.2 mg/ml BSA, and injected (30 μ l) at 3 μ l/min into flow cells. For each sample injection, interactions were monitored simultaneously in different flow cells. Regeneration was performed after each injection with octyl glucoside, as described previously [12].

For BoNT assays, samples (diluted Sn10 or cell lysates) were placed in the sample rack at 4°C and injected serially over anti-SV or control antibodies. The ratio of specific SV binding to anti-VAMP 2 antibodies compared with specific SV binding to control anti-SV antibodies (1D12, 171B5 or Cl80.1) was measured in the presence or absence of BoNTs. Typically, injection of control samples (with inactivated toxin) yielded 20–30 RU. A slight linear rundown in vesicle binding to antibodies was measured by injecting control samples at regular intervals during analysis, and this was used to correct data. Chips were regenerated before reaching a total of 200 RU of captured vesicles. The total capacity of a CM3 chip was approx. 2000 RU.

Other methods

Protein concentrations were measured using the BCA (bicinchoninic acid) assay (Pierce, Rockford, IL, U.S.A.), following the manufacturer's instructions with a BSA standard. GST (glutathione S-transferase)–VAMP 2_{1–96} and BoNT concentrations were also determined by amino acid analysis. SDS/PAGE was performed using 12% mini-gels. Proteins were transferred to

Figure 1 Western blot analysis of BoNT/B and F activity

(A) A rat brain was homogenized in sucrose buffer in the absence (Control) or presence (+ DTT) of 5 mM DTT. Sn10 (3 mg/ml) was diluted 30-fold in cleavage buffer and incubated for 3 h at 4°C or 37°C. Samples (30 µl) were denatured and processed by SDS/PAGE before probing with anti-SYT 1 or anti-VAMP 2 antibodies. (B) Diluted Sn10 (500 µg/ml) was incubated for 3 h at 37°C in the absence (Control) or presence of 100 nM BoNT/B or F in cleavage buffer. Aliquots of Sn10 fractions (200 µl) were cooled to 4°C, adjusted to 0.15 M NaCl, and diluted 2-fold with TBS/0.1% BSA before immunoprecipitation of SVs with 10–15 µg of anti-SYT 1, anti-VAMP 2 or non-immune IgG. Sn10 (3 µg) or immunoprecipitated fractions were denatured, resolved by SDS/PAGE and analysed by Western blotting with anti-VAMP 2 and anti-SYT 1 antibodies.

nitrocellulose, and stained with Ponceau S. Membranes were blocked with 5% milk in TBS, and incubated with 0.5–5 µg/ml primary IgG for 1 h at room temperature. Antigens were detected using chemiluminescent horseradish-peroxidase substrate (Super Signal, West Pico Chemiluminescent Substrate, Pierce) coupled with anti-mouse IgG antibody (Jackson Immunoresearch Laboratories Inc., Baltimore, MD, U.S.A.).

The concentration of VAMP 2 in Sn10 was determined using GST–VAMP 2 as a standard [13]. Purity was monitored by SDS/PAGE and Coomassie Blue staining. Aliquots of GST–VAMP 2 or Sn10 were run on the same gel. Quantitative Western blotting indicated the presence of 200 pmol of VAMP 2/mg of Sn10 protein. Using molar proportions of VAMP 2/synaptophysin/SYT 1 of 1/0.33/0.22 [17], we calculated a synaptophysin and SYT 1 content in Sn10 of 66 and 44 pmol/mg of protein respectively. This is in accordance with calculations based on reported synaptophysin content [18,19].

RESULTS

Cleavage of VAMP 2 in intact SVs by BoNT/B and F

BoNT/B and F specifically cleave the integral SV protein VAMP 2 at two distinct sites located in the cytoplasmic region of the protein [1]. Experiments were performed to assess whether VAMP 2 in intact SVs from rat brain is a useful substrate for BoNT assays. Since BoNT assays are performed at 37°C, we initially asked whether endogenous proteases might interfere by non-specifically cleaving SV proteins [20]. Sn10 from rat brain homogenate, which contains approx. 40% of the total SVs [19], was incubated for 3 h at 4°C or 37°C, and then analysed by Western blotting. The data indicated a significant loss of both SYT 1 and VAMP 2 (Figure 1A, left panel). We therefore attempted to identify inhibitors that block endogenous proteases, but do not interfere with BoNT activity. This led to the finding that homogenization in the presence of 5 mM DTT followed by incubation in 0.16 mM DTT prevented degradation of both SV proteins during incubation at 37°C

(Figure 1A, right panel). Subsequently, Sn10 was prepared and incubated with BoNT/B or BoNT/F in the presence of DTT. Treatment with both BoNTs led to a strong reduction in immunoreactive VAMP 2 (Figure 1B). However, in spite of relatively high concentrations of BoNTs, a small fraction of VAMP 2 in Sn10 was resistant to cleavage. This could represent VAMP 2 that is engaged in SNARE complexes and thus protected from toxin action [1], or is associated with other unidentified proteins. To determine whether this resistant fraction resides on SVs, intact vesicles were immunoprecipitated from Sn10 using anti-SV antibodies immobilized on agarose beads. In the absence of treatment with BoNTs, SVs contained both SYT 1 and VAMP 2, irrespective of whether they were captured by anti-SYT 1 or anti-VAMP 2 antibodies (Figure 1B), whereas SV proteins were not detected when the immunoprecipitation procedure was carried out with non-immune IgG. When BoNT-treated vesicles were pulled down with anti-SYT 1 antibodies, no staining was detectable using an antibody directed against an N-terminal region of VAMP 2, although the level of SYT 1 was unaffected. Furthermore, no signal was revealed by an antibody that recognizes both VAMP 1 and VAMP 2 (results not shown). These results indicate that VAMP 1 was undetectable in this preparation [17], and that VAMP cleavage by BoNT/B and F in intact SVs is apparently complete. These results are consistent with observations by Hu et al. [21] using freshly purified SVs. Thus the BoNT-resistant VAMP detected in Sn10 is probably located in a compartment other than SVs. When immunocapture of toxin-treated vesicles was performed with anti-VAMP 2 antibodies, neither SYT 1 nor VAMP 2 was detected (Figure 1B). Incubation with BoNT/B or F removes the epitope for the anti-VAMP 2 antibody, and therefore vesicle capture can no longer occur. These observations provide the basis for the BoNTs assay that we will describe below.

SPR with intact SVs reveals differential cleavage by BoNT/B and F

BoNT/B and F cleave VAMP at peptide bonds Gln⁷⁶–Phe⁷⁷ and Gln⁵⁸–Lys⁵⁹ respectively (Figure 2, upper diagram) generating fragments that have proven to be extremely difficult to detect by Western blotting [22]. Thus the activity of toxins is generally evaluated from the decrease in the amount of full-length VAMP, a criterion which does not distinguish BoNT/B from BoNT/F. A panel of antibodies directed against VAMP (Figure 2, upper diagram) was therefore used to determine whether the distinct action of these two BoNTs could be resolved with intact vesicles. SVs were analysed by SPR using methods we have recently described [12]. Briefly, vesicles are immunocaptured on the sensor chip of a Biacore 3000 apparatus via anti-SV antibodies. Immobilization is extremely stable with time, thus the accessibility of various epitopes can be probed by injecting a panel of antibodies online and monitoring their binding by SPR. Sn10 was incubated for 3 h at 37°C in the presence or absence of BoNTs. Aliquots were then injected over a sensor chip coated with an anti-SYT 1 antibody to obtain specific capture of approx. 1500 RU of SVs. Specific capture was calculated by subtraction of non-specific adsorption measured on a parallel-flow cell coated with an irrelevant antibody. Four different anti-VAMP antibodies were injected consecutively, resulting in four distinct stepwise increments in the SPR signal with control samples (Figure 2A). After incubation with BoNT/F, interactions with anti-(VAMP 60–88) antibodies were identical with the control, whereas binding of anti-(VAMP N-ter), 3D10 and 1H7 antibodies was abolished. This is consistent with the fact that the first antibody is directed against the 60–88 domain, which remains anchored in the vesicle membrane, whereas the epitopes for the other three antibodies are contained within residues 1–58, corresponding to the N-terminal fragment

Figure 2 SPR analysis of SVs after treatment with BoNT/B and F

VAMP domains recognized by anti-VAMP antibodies (anti-VAMP 2 N-ter, 1H7, 3D10 and anti-VAMP 60–88) are shown schematically at the top of the Figure. TMR indicates the transmembrane region, and black arrowheads show the cleavage sites for BoNT/B and F. Sn10 was pre-incubated for 3 h at 37 °C in the absence (Control) or presence of BoNT/F (**A** and **C**) or BoNT/B (**B**). SVs from Sn10 were captured by injection over anti-SYT 1 antibodies. Sensorgrams in (**A**) and (**B**) illustrate the specific binding of four different anti-VAMP antibodies to immobilized SV. Superposed sensorgrams in (**C**) show the absence of effect of BoNT/F treatment on other SV proteins. White arrowheads indicate injection of non-immune mouse IgG (50 µg/ml) between each anti-SV antibody.

clipped off by BoNT/F. Treatment with BoNT/B abolished binding of all four antibodies (Figure 2B). The fact that BoNT/B totally eliminated binding of antibody anti-(VAMP 60–88) is not fully consistent with predictions based upon protein sequence alone. Region 60–88 overlaps the BoNT/B cleavage site (Gln⁷⁶–Phe⁷⁷), and certain specificities in this polyclonal antibody recognize residues 77–88 [13], which are C-terminal to the cleavage site and remain associated with the vesicle. However, this membrane-proximal domain dips tangentially into the vesicle phospholipid bilayer, and is not accessible for antibody binding

[13,23]. Control experiments indicated that epitopes associated with a variety of SV proteins were unaffected by BoNT/F (Figure 2C), BoNT/B or BoNT/E (results not shown).

These data confirm that VAMP in intact SVs is fully accessible to BoNT/B and F, and that SPR reveals differential cleavage of VAMP by these two toxins.

SPR assays for SV proteins

Quantitative analysis of cleavage of VAMP by BoNTs using the SPR method outlined above (Figure 2) has certain disadvantages for routine experiments. It necessitates several independent vesicle-capture steps (i.e. in the absence of BoNT and at each BoNT concentration), and injection of saturating concentrations of antibodies, followed by regeneration. We have therefore established an alternative procedure which is quicker, more sensitive and can be used to assay VAMP 2 or other vesicle membrane components.

In the condition of mass transport, we asked whether analysis of the capture of SVs by antibody-coated SPR sensor chips could provide a rapid means of assaying the component recognized by the antibody. In Figure 3(A), diluted Sn10 (3 µg of protein/ml) was injected at a low flow rate over an anti-synaptophysin antibody, yielding a linear signal terminating in a plateau. Similar patterns were obtained with anti-SYT 1 and anti-VAMP 2 antibodies. Vesicle binding was systematically performed in 0.4 M NaCl, which strongly promoted capture by an anti-N-terminal VAMP 2 monoclonal antibody but did not affect recognition by anti-SYT 1 or synaptophysin antibodies. Non-specific adsorption to a control antibody was < 5% of the total signal. Similar results were obtained with the 10000-g supernatant of a hypotonic lysate from primary cultured neurons (Figure 3A). Under these conditions, the capacity of the immobilized antibody is far from saturation (0.5–3% occupation), and many injections could be performed in the same flow cell without regeneration. Figure 3(B) illustrates the fact that consecutive injection of a diluted Sn10 solution gives reproducible results (4.4 ± 0.2 RU; $n = 5$), even for small SPR signals.

The signal amplitude depends on the flow rate, the injection time, the surface density of antibody and the antigen concentration, which is maintained constant during injection. In order to establish a standard SPR curve for VAMP 2 using this method, the VAMP 2 content of Sn10 was first calibrated by quantitative Western blotting. The signals obtained by successive injections of decreasing amounts of Sn10 (i.e. decreasing amounts of VAMP 2) over anti-VAMP 2 antibodies are shown in Figure 3(C). The scale does not allow illustration of the lowest dilutions, which were significantly different from background. Similar profiles were obtained with anti-SYT 1 and synaptophysin antibodies. Plateau phases indicating a lack of dissociation were obtained with all three antibodies, although these antibodies display very different binding kinetics (results not shown), when they are injected over a sensor chip coated with SVs (as in Figure 2). Thus irreversible binding is probably due to Velcro-like capture of a single SV by several antibody molecules.

The injection of Sn10 over the three antibodies, each in a distinct channel, allows assay of the three antigens in a single step. Signals measured at the plateau were plotted (Figures 3D and 3E), and were linear over three orders of magnitude (from picogram to nanogram levels). The sensitivity of the assay depends on the type of sensor chip used, because the SPR signal decreases exponentially as a function of the square of the distance from the gold surface film. Sensitivity was increased (results not shown) when antibodies were coupled with 40 nm dextran spacer CM3 chips compared with 100 nm dextran CM5 chips, and the best results (Figure 3F) were obtained using dextran-free C1 chips

Figure 3 An SPR method to assay SV proteins

(A) Typical sensorgrams obtained by injecting Sn10 (3 μ g/ml) or hippocampal cell lysate (50 μ g/ml) over anti-synaptophysin antibodies coated on a CM3 sensor chip. (B) Superimposed traces of five consecutive injections of Sn10 (60 ng/ml) over anti-VAMP 2 coated on a CM3 sensor chip. (C) Specific SPR responses obtained with a serial dilution of Sn10 containing the indicated quantities of VAMP, injected over anti-VAMP 2. (D) Plots of the specific plateau response obtained after injection of different concentrations of Sn10 over anti-synaptophysin (●), anti-VAMP 2 (○) and anti-SYT 1 (△) antibodies immobilized on a CM3 chip ($r > 0.98$). (E) As in (D), with expanded scales. (F) Calibration curves obtained from specific plateau SPR responses with injections of diluted fractions of Sn10 over anti-VAMP 2 coated on a C1 surface ($r = 0.99$). All data represent specific binding obtained after subtraction of background ($< 5\%$) on a flow cell coated with non-immune IgG.

($\times 4$ compared with CM3 chips). Under this condition and using an injection of only 570 pg of total Sn10 protein, the detection threshold for VAMP 2 was 1.5 pg (120 amol), which was 40-fold more sensitive than a carefully optimized Western blotting procedure [24]. The detection threshold for synaptophysin was 1.2 pg (35 amol), and this sensitivity is 60 times higher than an ELISA assay of detergent-solubilized synaptophysin [25]. Furthermore, our SPR method allows the assay of VAMP 2 in hypotonic lysates from cultured neurons. Experiments using serial dilutions of the Sn10 from a lysate of 40 000 hippocampal neurons yielded a specific signal with an aliquot equivalent to the SV content of approx. 10 neurons (results not shown). Therefore the rapid quantification of vesicular antigens can be achieved in extracts containing only minute amounts of neuronal proteins that are not readily accessible to traditional biochemical methods. High sensitivity is conferred by the irreversibility of binding and the fact that the amplitude of the SPR signal is directly proportional to the molecular mass of the analyte bound to the sensor chip. As maximal sensitivity was not required for detection of VAMP 2 in the BoNT assay, and because the regeneration procedure had been optimized for CM3 chips [12], they were used in all the following experiments.

Assays for BoNT/B and F activity with a brain fraction containing SVs

We then investigated the potential use of this method to measure VAMP 2 cleavage by BoNT/B and F. Sn10 was diluted to 1 μ g of protein/ml (0.2 nM VAMP) in a low-ionic-strength buffer containing Zn^{2+} and BSA [6]. BoNTs were reduced with DTT, and serial dilutions were incubated with Sn10 for 3 h at 37°C. The reaction was stopped by cooling to 4°C and addition of NaCl to give 0.4 M final concentration [6]. Samples were then injected over three flow cells coated with anti-VAMP 2, anti-SYT 1 and control antibodies. Figure 4(A) indicates that the SPR signal resulting from vesicle capture on the anti-VAMP 2 antibody

Figure 4 An SPR method to assay BoNT/B light chain activity

Sn10 was pre-incubated with the indicated concentrations of BoNT/B, and then analysed. Typical sensorgrams representing binding to anti-VAMP 2 (A) or anti-SYT 1 (B) are illustrated after subtraction of binding to non-immune IgG.

decreases as BoNT/B concentration is increased. In contrast, capture on anti-SYT 1 antibodies remained relatively constant (Figure 4B), and was used to correct for the minor signal fluctuations resulting from variations in vesicle concentration. When

Figure 5 Concentration-dependence of BoNT/B and F light-chain activities

(A) Sn10 was incubated with increasing concentrations of BoNT/B (○), BoNT/F (■) or BoNT/E (△) for 3 h. Specific SV capture was measured on a sensor chip functionalized with anti-VAMP 2 or other anti-SV antibodies. Plots are representative data from one of at least two independent experiments. (B) Sn10 fractions were incubated at 37°C or 4°C in the presence or absence of 3 pg/ml BoNT/B for 3 h, and specific SV binding was measured over anti-VAMP 2 and anti-SYT 1 antibodies. The signal obtained with each antibody in the absence of BoNT/B was taken as 100 %.

200 RU of vesicles had been captured, the antibodies were regenerated by injecting a concentrated solution of octyl glucoside. Thus the BoNT assay is based on a decrease of SV binding to anti-VAMP 2 antibodies using SV binding via other anti-SV antibodies as an internal control.

Using this method we have generated concentration-dependence curves for reduced BoNT/B, F and E (Figure 5A). The EC_{50} for BoNT/B was 4.9 ± 1.2 pg/ml, which is more sensitive than the mouse bioassay (LD_{50} 40 pg/ml). The absolute threshold is 0.1 pg (sample volume 50 μ l) compared with 20 pg (per 0.5 ml injected) for the mouse bioassay. The sensitivity should in theory be increased by using a smaller injection volume and longer contact time (flow rate of 1 μ l/min), but the duration of the analysis would be increased. Thus, in terms of threshold, this assay is at least 200 times more sensitive and 25 times more rapid than the mouse bioassay (4 h versus 4 days), and significantly more sensitive than reported *in vitro* assays [4,5]. In principle, it can be transposed to tetanus toxin and to the other BoNT serotypes that cleave VAMP 2. BoNT/F was approximately three orders of magnitude less active, yielding an EC_{50} of 2.1 ± 0.4 ng/ml. This result contrasts with mouse toxicity assays in which BoNT/F was only 10 times less active than BoNT/B. The discrepancy may be due to more effective bioavailability, targeting and/or internalization of BoNT/F *in vivo*. Alternatively, it may result from the fact that our *in vitro* assay conditions have been carefully

Figure 6 Measurement of BoNT/B and F holotoxin activity in cultured neurons

Hippocampal neurons were incubated for 24 h with increasing concentrations of BoNT/B (○) or BoNT/F (■) and hypotonically lysed. Cell lysates were rapidly centrifuged, diluted 5-fold in running buffer and adjusted to 0.2 mg/ml BSA and 0.4 M NaCl. Diluted supernatants were injected over anti-VAMP 2 and anti-synaptogyrin antibodies and specific SV capture was measured. The ratio of SPR signals (anti-VAMP 2/anti-synaptogyrin) measured in the presence or absence of BoNTs yields the percentage control values. The results shown are representative of one out of three independent experiments.

optimized for BoNT/B, but may not be ideal for BoNT/F. BoNT/E, which does not cleave VAMP, was inactive.

Inhibition of VAMP capture did not occur when Sn10 was incubated with 3 pg/ml BoNT/B for 3 h at 4°C (Figure 5B) or when the BoNT/B reduction step was omitted, or when BoNT was heat-inactivated (results not shown). Thus inhibition of capture monitors specific enzymatic activity.

Assays for BoNT/B and F activity with intact cultured neurons

Primary cultured hippocampal neurons were incubated in the presence of a range of concentrations of BoNT/B or F. Cells were then lysed hypotonically, and the supernatants were centrifuged, diluted and processed as described for Sn10. As shown in Figure 6, the capture of SVs from cultured neurons was strongly inhibited by both BoNT/B (EC_{50} 1.5 ± 1.2 ng/ml) and F (EC_{50} 2.7 ± 1.3 ng/ml), with an almost identical concentration-dependence. BoNT/F thus displayed similar potency to that measured *in vitro* with isolated SVs (EC_{50} 2.1 ng/ml in Figure 5), whereas BoNT/B was approximately three orders of magnitude less potent in hippocampal neurons than *in vitro*. This may be due to relatively poor penetration of BoNT/B into hippocampal neurons. The membrane receptor for BoNT/F has not yet been identified. The receptor for BoNT/B is SYT 1 or 2 in association with gangliosides, although the toxin binds with significantly higher affinity to SYT 2 [26,27]. In the hippocampus, SYT 2 mRNA is only detected in occasional cells, whereas SYT 1 transcripts are much more widely expressed [16,28]. In contrast, in cerebellar granule neurons, BoNT/B inhibits transmitter release and cleaves VAMP with an EC_{50} that is 10-fold lower than that for BoNT/F [22]. This correlates with a relatively strong expression of SYT 2 mRNA in the cerebellum, and particularly in granule cells [28].

DISCUSSION

We have established SPR assays to monitor VAMP 2 cleavage by BoNT/B and F using intact SVs from rat brain as substrate. Initial studies indicated that, in the presence of DTT, SV proteins

were not degraded at 37 °C, even in fairly crude extracts. This is consistent with our previous findings indicating that SVs captured on sensor chips were stable for several days at 25 °C [12]. Immunodetection of VAMP on captured vesicles provided a means to monitor BoNT/B and F activity, and identified cytoplasmic epitopes located in the Lys⁵⁹–Gln⁷⁶ fragment that were spared by BoNT/F, but clipped off by BoNT/B. Thus SPR analysis on SV chips provides a differential assay for BoNT/F compared with BoNT/B (or potentially BoNT/D, G and tetanus toxin) activity by revealing fragments that have, until now, resisted detection by Western blotting [22].

Real-time SPR analysis of the capture of minute quantities of SVs on chips coated with specific antibodies provides an assay for vesicle antigens from different biological models, without membrane solubilization. SPR-based assays have many advantages compared with Western blotting, including sensitivity, rapidity, easy quantification and detection in a complex medium.

The high sensitivity is partly due to the size of the analyte. SPR signals are directly proportional to the mass of the analyte for globular proteins. Thus a general drawback of SPR assays is the lack of sensitivity for low-molecular-mass analytes. Indirect competitive displacement assays have been used to circumvent this limitation. Alternatively, the molecular mass of the analyte can be increased by coupling to latex beads or colloidal gold, by incorporation into liposomes or by using peroxidase-catalysed precipitation [29]. The sensitivity of the assay reported here can be attributed in part to the use of native 50 nm organelles as analytes, which have considerably higher mass than a single VAMP molecule. A single SV has a protein mass of approx. $(6-9) \times 10^6$ Da [associated with lipids at a lipid:protein ratio of 1.44 (w/w)] compared with a VAMP molecule of 12 kDa. However, the amplification factor cannot be directly calculated, since a single vesicle probably binds to multiple antibody molecules, and furthermore because vesicles do not behave like globular proteins.

This assay is significantly quicker than Western blotting. The total assay time for neurons in culture including sample preparation and analysis is under 1 h. The SPR measurement itself takes 5–10 min per point, with the limitation that analysis must be performed point by point. Furthermore, modification of a given SV membrane protein can be readily quantified with respect to the parallel analysis of several different proteins in the same organelle. Finally, the SPR method is compatible with measurements in complex solutions (e.g. sera, food samples and solutions with high concentrations of carrier proteins to stabilize toxins) that are difficult to analyse by Western blotting. The SV protein assay also constitutes a novel means to monitor BoNT/B and F activity in a variety of biological model systems (SVs, cultured neurons, synaptosomes, brain slices, etc.) that provide a more physiological context than assays using recombinant proteins or synthetic peptides. It constitutes the first method described to date to rapidly quantify BoNT activity in cell culture. Cell-based assays can be particularly useful to detect neutralizing anti-toxin antibodies in patients treated with BoNTs and to screen for potential inhibitors, and can also provide an *in vitro* model in which to study long-term activity of BoNTs.

Direct incubation of SVs with BoNT/B provides the first *in vitro* assay that is significantly more sensitive than the mouse bioassay, which is the current reference. It is also the most sensitive *in vitro* assay for BoNT/F activity reported to date, approaching that of the mouse bioassay. Use of the native substrate with an optimal conformation determined by lipid environment and protein partners is probably a crucial factor. The subcellular environment of SNARE proteins is important for proteolysis by BoNTs. Syntaxin is not cleaved by BoNT/C unless it is inserted into an acidic phospholipid bilayer [30]. Similarly, SNAP-25 *in situ*,

but not the bacterially expressed protein, is a substrate for BoNT/C [31]. Heterologously expressed SNAP-25 and VAMP 2 are only cleaved by BoNT/A and B respectively if their subcellular distribution is correct [32]. All these findings suggest that native SNAREs inserted in a specific membrane environment are ideal substrates for BoNT assays. In the case of VAMP 2, interactions with both lipids and proteins are likely to contribute. VAMP 2 cleavage by tetanus toxin and BoNTs is enhanced by acidic liposomes, probably because toxin binding to lipids increases local toxin concentration [33]. When recombinant VAMP 2 is reconstituted into liposomes, the membrane-proximal domain of the protein binds *cis* to negatively charged lipids at the vesicle surface, which modifies its conformation [13,23]. *cis*-Lipid binding is thought to prevent SNARE complex assembly and vesicle fusion [21,23,34]. The N-terminal domain of VAMP 2 binds to synaptophysin in the plane of the SV membrane, and this appears to promote cleavage by tetanus toxin, as does VAMP homodimerization [35].

In conclusion, the sensitivity of this *in vitro* BoNT assay results from the fact that SVs are both ideal substrates for a specific metalloprotease activity and appropriately sized analytes for SPR spectroscopy. SPR assay requires considerably fewer animals than the *in vivo* mouse assay, as a single rat brain provides sufficient vesicles for more than 100 000 assay points. It would be interesting to evaluate whether recombinant VAMP 2 incorporated into liposomes could provide a suitable alternative substrate, and thus completely obviate the use of animals. Future improvements could include extension of these methods to other BoNTs, and the use of antibodies directed against sequences that precisely flank the different cleavage sites. In this case, SPR analysis of vesicle capture would provide a direct indication of which BoNT serotype is present in a sample.

We thank M. Takahashi (Kitasato School of Medicine, Kanagawa, Japan) and S. Fujita (Mitsubishi Kasei Institute) for the gift of antibodies and B. Dargent (INSERM UMR641, France) for providing cultured neurons.

REFERENCES

- Schiavo, G., Matteoli, M. and Montecucco, C. (2000) Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* **80**, 717–733
- Humeau, Y., Dousseau, F., Grant, N. J. and Poulain, B. (2000) How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie* **82**, 427–446
- Cornella, C. L. and Pullman, S. L. (2004) Botulinum toxins in neurological disease. *Muscle Nerve* **29**, 628–644
- Anne, C., Cornille, F., Lenoir, C. and Roques, B. P. (2001) High-throughput fluorogenic assay for determination of botulinum type B neurotoxin protease activity. *Anal. Biochem.* **291**, 253–261
- Schmidt, J. J., Stafford, R. G. and Millard, C. B. (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D and F. *Anal. Biochem.* **296**, 130–137
- Foran, P., Shone, C. C. and Dolly, J. O. (1994) Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry* **33**, 15365–15374
- Shone, C. C. and Roberts, A. K. (1994) Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neurotoxin. *Eur. J. Biochem.* **225**, 263–270
- Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., Dasgupta, B. R. and Montecucco, C. (1992) Tetanus and Botulinum B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature (London)* **359**, 832–835
- Khoda, T., Kamada, Y. and Kozaki, S. (2000) Endocytosis of Clostridium botulinum type B neurotoxin into rat brain synaptosomes. *J. Vet. Med. Sci.* **62**, 1133–1138
- Welch, M. J., Purkiss, J. R. and Foster, K. A. (2000) Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. *Toxicon* **38**, 245–258
- Szule, J. A. and Coorsen, J. R. (2003) Revisiting the role of SNAREs in exocytosis and membrane fusion. *Biochim. Biophys. Acta* **1641**, 121–135

- 12 Ferracci, G., Seagar, M., Courageot, J., Miquelis, R. and Lévêque, C. (2004) Real time analysis of intact organelles using surface plasmon resonance. *Anal. Biochem.* **334**, 367–375
- 13 De Haro, L., Ferracci, G., Opi, S., Iborra, C., Quetglas, S., Miquelis, R., Lévêque, C. and Seagar, M. (2004) Ca²⁺/calmodulin transfers the membrane-proximal lipid-binding domain of the v-SNARE synaptobrevin from cis to trans bilayers. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1578–1583
- 14 Sakaguchi, G. (1983) Clostridium botulinum toxins. *Pharmacol. Ther.* **19**, 165–194
- 15 Lévêque, C., Boudier, J. A., Takahashi, M. and Seagar, M. (2000) Calcium-dependent dissociation of synaptotagmin from synaptic SNARE complexes. *J. Neurochem.* **74**, 367–373
- 16 Berton, F., Cornet, V., Iborra, C., Garrido, J., Dargent, B., Fukuda, M., Seagar, M. and Marqueze, B. (2000) Synaptotagmin I and IV define distinct populations of neuronal transport vesicles. *Eur. J. Neurosci.* **12**, 1294–1302
- 17 Walsh-Solimena, C., Blasi, J., Edelmann, L., Chapman, E. R., Von Mollard, G. F. and Jahn, R. (1995) The t-SNARE syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell. Biol.* **128**, 637–645
- 18 Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P. and De Camilli, P. (1986) Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* **103**, 2511–2527
- 19 Jahn, R., Schiebler, W., Ouimet, C. and Greengard, P. (1985) A 38000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4137–4141
- 20 De Paiva, A., Ashton, A. C., Foran, P., Schiavo, G., Montecucco, C. and Dolly, J. O. (1993) Botulinum A like type B and tetanus toxins fulfils criteria for being a zinc-dependent protease. *J. Neurochem.* **61**, 2338–2341
- 21 Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A. and Davletov, B. (2002) Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. *Nature (London)* **415**, 646–650
- 22 Foran, P. G., Mohammed, N., Lisk, G. O., Nagwaney, S., Lawrence, G. W., Johnson, E., Smith, L., Aoki, K. R. and Dolly, J. O. (2003) Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. *J. Biol. Chem.* **278**, 1363–1371
- 23 Kweon, D. H., Kim, C. S. and Shin, Y. K. (2003) Insertion of the membrane-proximal region of the neuronal SNARE coiled coil into the membrane. *J. Biol. Chem.* **278**, 12367–12373
- 24 Coorssen, J. R., Blank, P. S., Albertorio, F., Bezrukov, L., Kolosova, I., Backlund, P. S. and Zimmerberg, J. (2002) Quantitative femto-to-attomole immunodetection of regulated secretory vesicle proteins critical to exocytosis. *Anal. Biochem.* **307**, 54–62
- 25 Schlaf, G., Salje, C., Poethke, R., Felgenhauer, K. and Mader, M. (1996) A novel enzyme-linked immunosorbent assay for determination of synaptophysin as compared with other quantification procedures. *J. Neuroimmunol.* **67**, 59–65
- 26 Nishiki, T., Kamada, Y., Nemoto, Y., Omori, A., Ito, T., Takahashi, M. and Kozaki, S. (1994) Identification of protein receptor for Clostridium Botulinum B neurotoxin in rat brain synaptosomes. *J. Biol. Chem.* **269**, 10498–10503
- 27 Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A. and Chapman, E. R. (2003) Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol.* **162**, 1293–1303
- 28 Marqueze, B., Boudier, J. A., Mizuta, M., Inagaki, N., Seino, S. and Seagar, M. (1995) Cellular localization of synaptotagmin I, II and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *J. Neurosci.* **15**, 4906–4917
- 29 Kim, M. G., Shin, Y. B., Jung, J. M., Ro, H. S. and Chung, B. H. (2005) Enhanced sensitivity of surface plasmon resonance (SPR) immunoassays using a peroxidase-catalyzed precipitation reaction and its application to a protein microarray. *J. Immunol. Methods* **297**, 125–132
- 30 Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H. and Montecucco, C. (1995) Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* **270**, 10566–10570
- 31 Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E. and Neale, E. A. (1996) Clostridial neurotoxins and substrates proteolysis in intact neurons. *J. Biol. Chem.* **271**, 7694–7699
- 32 Dong, M., Tepp, W. H., Johnson, E. A. and Chapman, E. R. (2004) Using fluorescent sensors to detect botulinum neurotoxin activity *in vitro* and in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14701–14706
- 33 Caccin, P., Rossetto, O., Rigoni, M., Johnson, E., Schiavo, G. and Montecucco, C. (2003) VAMP/synaptobrevin cleavage by tetanus and botulinum neurotoxins is strongly enhanced by acidic liposomes. *FEBS Lett.* **542**, 132–136
- 34 Quetglas, S., Iborra, C., Sasakawa, N., De Haro, L., Kumakura, K., Sato, K., Lévêque, C. and Seagar, M. (2002) Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis. *EMBO J.* **21**, 3970–3979
- 35 Reisinger, C., Yelamanchili, S. V., Hinz, B., Mitter, D., Becher, A., Bigalke, H. and Aert-Hilger, G. A. (2004) The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis. *J. Neurochem.* **90**, 1–8

Received 26 May 2005/12 July 2005; accepted 13 July 2005

Published as BJ Immediate Publication 13 July 2005, doi:10.1042/BJ20050855