Functional characterisation of tetanus and botulinum neurotoxins binding domains

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SUMMARY

Tetanus and botulinum neurotoxins constitute a family of bacterial protein toxins responsible for two deadly syndromes in humans (tetanus and botulism, respectively). They bind with high affinity to neurons wherein they cause a complete inhibition of evoked neurotransmitter release. Here we report on the cloning, expression and use of the recombinant fragments of the heavy chains of tetanus neurotoxin and botulinum neurotoxin serotypes A, B and E as tools to study the neurospecific binding of the holotoxins. We found that the recombinant 50 kDa carboxy-terminal domains of tetanus and botulinum neurotoxins alone are responsible for the specific binding and internalisation into spinal cord cells in culture. Moreover, we provide evidence that the recombinant fragments block the internalization of the parental holotoxins in a dose-dependent manner, as determined by following the neurotoxin-dependent cleavage of their targets VAMP/synaptobrevin and SNAP-25. In addition, the recombinant binding fragments cause a significant delay in the paralysis induced by the corresponding holotoxin on the mouse phrenic nerve-hemidiaphragm preparation. Taken together, these results show that the carboxy-terminal domain of tetanus and botulinum neurotoxins is necessary and sufficient for the binding and internalisation of these proteins in neurons and open the possibility to use them as tools for the functional characterisation of the intracellular transport of clostridial neurotoxins.

Key words: Tetanus toxin, Botulinum neurotoxin, Spinal cord neuron, SNARE protein

INTRODUCTION

The clostridial neurotoxin (CNT) family is composed of tetanus neurotoxin (TeNT) and seven different serotypes of botulinum neurotoxins (BoNT) (Minton, 1995). The active holotoxin is comprised of two fragments, termed heavy (H, 100 kDa) and light (L, 50 kDa) chains (Fig. 1A) and blocks neurotransmitter release in vitro and in vivo (Schiavo and Montecucco, 1997). The L chain is responsible for the intracellular activity of CNTs and contains the catalytic domain of the neurotoxins. CNTs are zinc-endoproteases which specifically cleave a family of proteins, named SNAREs (soluble NSF attachment protein receptor) (Söllner et al., 1993). These proteins, whose integrity is necessary to sustain regulated secretion, are involved in multiple steps leading to the docking and fusion of small synaptic vesicles (SSVs) with the presynaptic plasma membrane and in a variety of other intracellular trafficking events (Hanson et al., 1997; Hay and Scheller, 1997; Robinson and Martin, 1998).

Vesicle-associated membrane protein (VAMP)/synaptobrevin is localised on SSVs and other vesicular compartments and is cleaved by TeNT and four serotypes of BoNT (B, D, F and G) (Schiavo et al., 1992, 1993a,c, 1994; Yamasaki et al., 1994a,b,c). BoNT/A and E instead cleave synaptosomal-associated protein of 25 kDa (SNAP-25) (Blasi et al., 1993a; Schiavo et al., 1993a,b), whilst BoNT/C has a dual-specificity for SNAP-25 and syntaxin 1 (Blasi et al., 1993b; Schiavo et al., 1995; Foran et al., 1996; Osen Sand et al., 1996; Williamson et al., 1996; Vaidyanathan et al., 1999). Both syntaxin and SNAP-25 are mainly localised on the plasma membrane (Garcia et al., 1995). They are therefore defined as target SNAREs or t-SNAREs, whilst VAMP/synaptobrevin is a vesicular SNARE or v-SNARE. v- and t-SNAREs form a very stable complex, possibly promoting a strict apposition of the vesicular and target lipid bilayers which is likely to drive membrane fusion (Sutton et al., 1998; Weber et al., 1998). CNTs alter the formation and assembly of this complex by reducing its stability (Hayashi et al., 1994, 1995) and in the case of syntaxin and VAMP/synaptobrevin, by detaching the cytoplasmic portion from their membrane anchors (Schiavo and Montecucco, 1997).

Despite the widespread distribution of the cleavable isoforms of VAMP, SNAP-25 and syntaxin, the action of CNTs in vivo is absolutely neurospecific. All the clinical symptoms of botulism can be ascribed to the inhibition of acetylcholine
release at the neuromuscular junction (NMJ), whilst the spastic paralysis of tetanus is due to TeNT targeting to the inhibitory interneurons of the spinal cord (Halpern and Neale, 1995; Schiavo and Montecucco, 1997). The neurospecificity and the differential trafficking of BoNTs and TeNT appear to be due to structural elements present at the level of their H chains. These contain two functional domains: a carboxy-terminal portion (Hc) that is likely to play a role in the interaction with the receptor(s), and an amino-terminal part (Hd) of 50 kDa involved in the translocation of the L chain in the cytosol (Fig. 1A) (Montecucco and Schiavo, 1993). The recently reported crystal structure of TeNT Hc domain (Umland et al., 1997; Knapp et al., 1998) and of BoNT/A (Lacy et al., 1998) show that the Hc domains of CNTs are highly related and consist of two sub-domains with folds related to those of lectin sugar-binding proteins and the trypsin inhibitor family, respectively.

The presence of two putative binding elements in the Hc fragment supports a dual receptor model for the binding of CNTs to neuronal membranes (Montecucco, 1986). Low (Kd in the nMolar range) and high (Kd, sub-nM) affinity receptors for CNTs have been described and experimental evidence indicates that both lipid and protein receptors are essential for high affinity binding (Halpern and Neale, 1995). Whilst the contribution of certain polysialoangangliosides of the G1b series to CNTs binding is well documented (Habermann and Dreyer, 1986; Halpern and Neale, 1995; Schiavo and Montecucco, 1997), the characterisation of their protein receptors at the peripheral and central level has proved elusive. Synaptotagmins, integral proteins of SSVs, have been suggested as the neuronal receptors for BoNT/B, E and A (Li and Singh, 1998; Nishiki et al., 1996a,b), thus supporting a model envisaging BoNTs entry at the NMJ via SSV recycling (Matteoli et al., 1996).

Here we report on the expression and functional characterisation of the Hc fragments of TeNT and of BoNT/A, B and E which are the three botulinum neurotoxins more frequently involved in human botulism. We describe the purification to homogeneity of Hc fragments that are able to bind to the surface of murine spinal cord neurons, a model system which contains physiological CNT receptors. These fragments antagonise the binding and cellular trafficking of the system which contains physiological CNT receptors. These bind to the surface of murine spinal cord neurons, a model of interneurons of the spinal cord (Halpern and Neale, 1995; Nishiki et al., 1996a,b), thus supporting a model envisaging BoNTs entry at the NMJ via SSV recycling (Matteoli et al., 1996).

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Cultures were grown for two weeks in a humidified 10% CO₂ incubator at 37°C. Culture medium was MEM (minimum essential medium with Earle’s salts, with sodium bicarbonate 3.7 g/l, glucose 6 g/l, pH 7.3-7.4), containing B27 supplement (Gibco-BRL), heat-inactivated horse serum (5% v/v) and L-glutamine (2 mM). Five days after plating, 35 µg/ml of uridine and 15 µg/ml of 5’-fluoro-2’-deoxyuridine (both from Sigma) were added to the medium for 96 hours to block cell proliferation. Medium was changed every 3-4 days.

CNT binding and internalisation

Spinal cord cultures were cooled to 4°C, washed twice with 0.1% bovine serum albumin (BSA) in Hanks’ solution (20 mM Hepes-Na, pH 7.4, 0.44 mM KH₂PO₄, 0.42 mM Na₂HPO₄, 5.36 mM KCl, 136 mM NaCl, 0.81 mM MgSO₄, 1.26 mM CaCl₂, 6.1 mM glucose) and incubated with recombinant TeNT Hc, native TeNT, BoNT/B Hc (80 nM), BoNT/A Hc or BoNT/E Hc (200 nM) for 1 hour at 4°C. In selected samples, native TeNT (20 µM) was pre-incubated at 4°C for 15 minutes before addition of the recombinant Hc. For internalisation studies, cells were incubated for 1 hour at 37°C with the recombinant TeNT Hc (80 nM) and BoNT/E Hc (100 nM) domains diluted in culture medium. After washing, cells were transferred to room temperature and fixed in 4% paraformaldehyde, 20% sucrose in phosphate-buffered saline (PBS) without calcium and magnesium for 15 minutes. After rinsing twice with PBS, cells were incubated for 20 minutes with 50 mM NH₄Cl in PBS, washed and then blocked with 2% BSA, 0.25% porcine skin gelatin, 0.2% glycine, 15% foetal calf serum in PBS for 1 hour. Coverslips were then incubated for 30-60 minutes with mouse anti-α-VSV-G (1:100) (Soldati and Perràrd, 1991), mouse anti-HA (1:50) (Niman et al., 1983) or polyclonal anti-TeNT (1:1:500) antibodies diluted in PBS containing 1% BSA, 0.25% porcine skin gelatin. After rinsing, cells were incubated for 25 minutes with Alexa 488 goat anti-mouse IgG or Alexa 488 goat anti-rabbit IgG (1:200) (Molecular Probes). To monitor internalisation, cell were treated with the same solutions with the addition of 0.1% Triton X-100. Coverslips were mounted on slides with Mowiol 4-88 (Harco) and stored at 4°C.

Hc binding to purified polysialogangliosides was monitored by dot-blot assay (Thomas et al., 1999) by using 0.5 µg of phosphatidylcholine (PC) (Avanti) or purified gangliosides G₃M₁, GD₁b, GT₁b and GQ₁b (Sigma) adsorbed on nitrocellulose. After blocking with 5% dried skimmed milk in PBS, Hc fragments (80 nM) were diluted in 20 mM Tris-acetate, pH 6.0, 5% milk and incubated for 2 hours at room temperature. Binding was detected with anti-α-VSV-G (1:1000) and anti-HA (1:200) antibodies, followed by an anti-mouse peroxidase-conjugated IgG and Enhanced Chemi-Luminescence method (ECL, Amersham Pharmacia Biotech, UK).

CNT treatment and SNARE analysis in spinal cord cells

Spinal cord cultures were rinsed twice with MEM and incubated for 20 hours at 37°C in serum-free culture medium with different CNTs (TeNT and BoNT/A 100 pM, BoNT/E 500 pM, BoNT/B 2.5 nM) purified as previously described (Schiavo and Montecucco, 1995). Some samples were pre-incubated with the recombinant Hc (1 nM, 10 nM, 100 nM and 1 µM, unless otherwise stated) for 1 hour at 37°C before adding the corresponding CNT. For immunocytochemistry, cells were fixed and treated as described with the addition of 0.1% Triton X-100 in all solutions. SNARE detection was carried out with a polyclonal antibody recognising the carboxy terminus of SNAP 25 (1:200) (Osen Sand et al., 1993) or with a monoclonal anti-VAMP-2 (1:100) (Edelmann et al., 1995). For western blot analysis, spinal cord cells were washed twice with PBS and then scraped in the same buffer. Proteins were recovered by precipitation with 6.5% trichloroacetic acid (TCA) and analysed by SDS-PAGE containing urea (Süllner et al., 1993). Western blotting was performed by using anti-VAMP-2 antibody (1:200) (Edelmann et al., 1995), followed by ECL detection.

For quantitation, protein recovery was normalised using syntaxin 1 immunoreactivity as internal standard.

Mouse phrenic nerve-hemidiaphragm recording

Mouse phrenic nerve-hemidiaphragms were dissected from animals weighing about 20 g, mounted in 10 ml of oxygenated (95% CO₂ - 5% O₂) Krebs-Ringer solution containing 11 mM glucose, pH 7.4, and kept at 37°C. The phrenic nerve was stimulated via two ring platinum electrodes by supramaximal stimuli of 3-6 V amplitude and 0.1 millisecond pulse duration with a frequency of 0.1 Hz. Isometric muscle contraction was monitored via a displacement force transducer connected to a recorder. In control experiments (without any added toxin), the amplitude of muscle contraction under stimulation was constant for at least 8 hours. CNTs were added to the bath at a concentration of 10 nM for TeNT and 0.2 nM for BoNTs under conditions that allow binding to go to completion (Simpson, 1980). For this purpose, tissues were incubated with toxin in physiological medium at 25°C for 60 minutes without nerve stimulation. At the end of incubation, tissues were washed and transferred to 37°C in a bath without CNTs. Nerve stimulation was applied until a reduction of 90% of the initial muscle twitch was observed. In the competition assays, mouse phrenic nerve-hemidiaphragms were incubated with each Hc fragment (Hc:CNT ratio 100:1 for TeNTand 1:300:1 for BoNTs) for 15 minutes at 25°C followed by a co-incubation with the parental CNT at the same concentration of the control for 60 minutes at 25°C without nerve stimulation. After incubation, all tissues were washed and paralysis times were monitored. Results were expressed as the time necessary to obtain a 50% reduction of the initial muscle response following nerve stimulation. Data are the average of n=3 experiments.

RESULTS

Expression and characterisation of TeNT and BoNTs Hc fragments

Experimental evidence suggests that the Hc fragment is mainly responsible for the specific binding of CNTs to their acceptor(s) in neurons. However, with the exception of TeNT, the molecular analysis of this process has been hampered by the lack of suitable protocols for the expression of this domain in a recombinant form. Here we used variants of the expression vector pGEX, encoding glutathione S-transferase, for the purification of fusion proteins containing the Hc fragments of TeNT and three BoNT serotypes (A, B and E) (Fig. 1A). These new vectors contain either the VSV-G (pGEX-4T3-VSV-G) or the HA (pGEX-4T3-HA) epitopes after the thrombin cleavage site. We adopted a PCR approach using as a template crude genomic DNA from selected toxigenic C. botulinum strains. Whilst the amplification with BoNT/A and E specific primers generated fragments corresponding to the published sequences, the same procedure also allowed the isolation of a new BoNT/B variant with 91% identity to M81186 and Y13630 (strain Danish, ATCC 43757) and 88% identity to X71343 (strain Eklund, ATCC25765, non-proteolytic) at the amino acid level. The neurotoxin corresponding to this new variant is fully toxic and is immunologically indistinguishable from the classical B serotype.

The length of the sequences corresponding to the different Hcs was chosen on the basis of sequence alignments and the crystal structures (Umland et al., 1997; Knapp et al., 1998; Lacy et al., 1998), which highlight the similar organisation of CNT Hc fragments. This structure predicts that both the folding and the binding activity of the Hc domains are
independent of the remainder of the neurotoxin molecule. To test this hypothesis, we expressed and purified GST-fusion proteins containing the different HC fragments in *E. coli* (Guan and Dixon, 1991). In the case of TeNT, BoNT/A and E, this procedure allowed the isolation of recombinant proteins with an apparent molecular mass ranging from 44 to 47 kDa (Fig. 1B, left panel) depending on the serotype, which are specifically recognised by either anti-VSV-G (Fig. 1B, central panel) or anti-HA antibodies (Fig. 1B, right panel). In contrast, the same procedure applied to BoNT/B was not successful and allowed the expression of only a very small amount of HC within inclusion bodies. When another *E. coli* strain (TOPP3, Stratagene) was used for expression, a soluble fusion protein was obtained which, after thrombin cleavage, gave a 48 kDa homogeneous band recognised by an anti-HA antibody (Fig. 1B, left and right panels).

**Binding and internalisation of recombinant HC fragments**

CNTs are known to bind to polysialogangliosides in vitro and this interaction is mediated by the HC domain (reviewed by Habermann and Dreyer, 1986; Halpern and Neale, 1995). We tested the competence of our recombinant fragments to bind polysialogangliosides by using a dot-blot assay (Thomas et al., 1999). As shown in Fig. 1C, TeNT HC interacts with G*_{T1b}* and to a less extent to G*_{D1b}, whereas BoNT/B binds G*_{T1b}* and G*_{O1b}. No interaction was observed with PC or the monosialilated ganglioside G*_{M1}, thus confirming the preference of CNTs for a subset of polysialogangliosides (Habermann and Dreyer, 1986; Schengrund et al., 1991; Halpern and Neale, 1995).

Whilst BoNT/E HC mirrors the binding behaviour of BoNT/B, the HC of BoNT/A presents a much lower interaction with these glycolipids (not shown).

In order to test the HC fragments in a more physiological context, their binding was assayed on the most appropriate cellular system presently available to study CNT activity, a mixed population of foetal spinal cord cells containing motoneurons, dorsal root ganglia, glial and stromal cells (Ransom et al., 1977). The neuronal components of this culture display evoked neurotransmitter release which can be blocked by physiological concentrations of CNTs (Williamson et al., 1996), thus indicating the presence of functional CNT binding sites on their surface.

As shown in Fig. 2, native TeNT and its recombinant HC fragment bind to mouse spinal cord cells in culture with similar distribution. At 4°C, the immunoreactivities of both the VSV-G tagged HC fragment (Fig. 2A) and TeNT (Fig. 2B), present in the incubation medium at a final concentration of 80 nM, revealed a punctate surface distribution. Membrane staining is not homogenous and is distributed all along the neurites and on the cell soma. In some cases, labelling has the morphological appearance of neuronal varicosities, areas where neurotransmitter release and active endocytosis are suggested to take place (Bennett et al., 1997; Brain et al., 1997; Bennett, 1998). The staining of HC fragment is specific as demonstrated by competition with excess of native TeNT (Fig. 2C). The absence of staining of the homogenous layer of non-neuronal supporting cells, further demonstrates that TeNT and HC bind selectively to neurons.
CNT binding fragments interact with neurons

B and E is very similar to that observed with TeNT, as shown in Fig. 2D-F. Staining is concentrated in distinct patches on the plasma membrane of neurites and the cell soma. Qualitatively, the extent of binding with the three BoNT HCs is lower than the one observed with TeNT. This may reflect a reduced number of membrane acceptors, which in turn could explain the higher concentration of native BoNTs required to observe a physiological intracellular effect (see below). Alternatively, the reduced staining seen with BoNT HCs could be due to an overall lower binding affinity of these fragments.

The same cellular system was then used to monitor the ability of the HCs to be internalised following surface binding. After incubation at 37°C, confocal microscopic analysis revealed that Hc immunostaining was concentrated in bright intracellular structures with very few patches still present on the cell surface (Fig. 3). These endocytic vesicles and their intracellular distribution appear to be identical for BoNT and TeNT Hc fragments. Future biochemical experiments are necessary to assess the precise nature of these vesicles and to test the possibility that TeNT and BoNTs use, at least in part, the same internalisation pathway.

Recombinant Hc fragments block the binding and intracellular activity of the native CNTs in spinal cord cell cultures

In order to establish that the Hc is able to bind to the physiological receptor of the parental CNT, and therefore represents the bona fide binding domain, we tested the different Hc fragments for their ability to inhibit the intracellular activity of the corresponding holotoxin in spinal cord cultures. As shown in Fig. 4A, the neuronal components of this preparation express the SSV protein VAMP/synaptobrevin, which localises in bright clusters corresponding to synaptic contacts and to points of accumulation of SSVs. Treatment of the culture for 20 hours at 37°C with 100 pM native TeNT caused the complete disappearance of VAMP immunostaining (Fig. 4B), following its specific proteolysis by this neurotoxin. As previously reported (Osen Sand et al., 1996; Williamson et al., 1996), TeNT-dependent VAMP ablation did not alter neuronal morphology nor affect cell survival.

Pre-treatment of the cells with the Hc fragment of TeNT potently inhibited the proteolytic action of the neurotoxin in a dose dependent manner (Fig. 4C-F). Complete protection of VAMP immunostaining was achieved by pre-treatment of the culture with 100 nM of TeNT Hc. This result was confirmed by western blotting of the treated cells and analysis of the VAMP immunoreactivity (Fig. 4, inset).

The effect of the Hc fragment of BoNT/A, B and E on the intoxication mediated by the native neurotoxin was similarly tested. In this case, spinal cord cells were probed with an antibody against the substrate of the botulinum neurotoxin (VAMP-2 for BoNT/B and SNAP-25 for BoNT/A and E), whose staining disappears upon toxin cleavage (Blasi et al., 1993a; Schiavo et al., 1993a,b). As shown in Fig. 5A and G, SNAP-25 localises along the neurite membrane without a clear concentration at the synapses or at varicosities (Garcia et al., 1995). Cell treatment for 20 hours at 37°C with 100 pM of BoNT/A (Fig. 5B) and 500 pM BoNT/E (Fig. 5H) abolished SNAP-25 immunostaining. SNAP-25 immunoreactivity was preserved when the cells were pre-incubated with 1 μM of the corresponding Hc fragment (Fig. 5C,I). BoNT/B was less potent than the other CNTs on these cells and a concentration of 2.5 nM of BoNT/B was necessary to achieve complete loss of VAMP immunostaining (Fig. 5E). Under such conditions, only partial protection was elicited by
pre-incubation of the spinal cord cells with BoNT/B H\textsubscript{C} (500 nM, Fig. 5F). This is consistent with a lower ratio between H\textsubscript{C} and native neurotoxin present in the medium, although other explanations are also possible (i.e. the presence of different conformational isoforms in the preparation of BoNT/B H\textsubscript{C}, only a fraction of which are competent for binding). The immunofluorescence protection experiments were confirmed by western blot using an anti-VAMP-2 antibody and, for SNAP-25, an antibody recognising both the intact protein and the cleaved fragment (not shown).

**Fig. 3.** Internalisation of H\textsubscript{C} fragments in mouse spinal cord cells. Spinal cord cells were incubated with the H\textsubscript{C} fragment of TeNT (A,B) or BoNT/E (C,D) for 1 hour at 37\degree C before fixing and permeabilisation. Binding was detected using anti-VSV-G (A,B) or anti-HA (C,D) antibodies as described in Materials and Methods and images were collected with a confocal laser scanning microscope. (A and C) Two z-sections obtained at 1.5 \textmu m from the substrate, whereas B and D display the merged images of the z-sections (14\times0.4 \textmu m) corresponding to the entire cell. Bars, 5 \textmu m.

**Fig. 4.** The recombinant H\textsubscript{C} fragment of TeNT blocks the binding and intracellular activity of the native neurotoxin. Mouse spinal cord cells were incubated for 20 hours at 37\degree C in the absence (A) or in the presence (B-F) of TeNT holotoxin. Before TeNT addition, samples C-F were treated with increasing amounts of the recombinant TeNT H\textsubscript{C} (C: 1 nM; D: 10 nM; E: 100 nM; F: 1 \mu M). Intact VAMP was then immunodetected with an anti-VAMP-2 antibody. In parallel samples, cells were recovered and levels of VAMP-2 analysed by western blot (upper right panel) as described in Materials and Methods. Bar, 5 \textmu m.
Recombinant Hc fragments antagonise CNT-dependent inhibition of neuromuscular transmission

BoNTs elicit an irreversible paralysis of the well-established system of the mouse phrenic nerve-hemidiaphragm preparation. Its onset of paralysis (which is commonly expressed as 50% of paralysis time) is dose- and temperature-dependent and ranges from 23 to 37 minutes (Table 1). Due to its site of action, TeNT is less potent on peripheral synapses with a longer (110 minutes) 50% of paralysis time (Schmitt et al., 1981; Simpson, 1984a,b). We tested the protective activity of the recombinant Hc fragments on the CNT-dependent inhibition of neuromuscular transmission by adding them to the electrophysiological bath. All the recombinant Hc fragments antagonised the action of the parental CNT which is seen as a delay in the onset of paralysis (Table 1). The increase in the 50% of paralysis time ranged from 75% for BoNT/A and TeNT to more than 150% in the case of BoNT/B and E. This protective effect of Hc is strictly serotype-dependent. In fact, the Hc fragment of BoNT/B did not interfere with the paralysis induced by BoNT/E (not shown), as expected from serotypes not competing for the same cellular acceptors (Evans et al., 1986; Habermann and Dreyer, 1986).

Table 1. Effect of recombinant Hc fragments on the 50% time of paralysis in mouse phrenic nerve-hemidiaphragm preparations intoxicated with TeNT and BoNTs

<table>
<thead>
<tr>
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<th>Control CNT*</th>
<th>CNT + Hc*</th>
<th>(+%)‡</th>
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<tr>
<td>BoNT/A</td>
<td>37±6</td>
<td>65±8</td>
<td>(+75)†</td>
</tr>
<tr>
<td>BoNT/B</td>
<td>23±5</td>
<td>60±9</td>
<td>(+160)†</td>
</tr>
<tr>
<td>BoNT/E</td>
<td>24±3</td>
<td>61±7</td>
<td>(+154)†</td>
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<tr>
<td>TeNT</td>
<td>111±11</td>
<td>195±20</td>
<td>(+75)†</td>
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*The values given are the 50% paralysis time (in minutes) of tissues (n=3) exposed to CNTs alone or in the presence of their Hc fragment. Each value represents the mean ± s.d.

‡Increase in the 50% of paralysis time following pre-treatment with Hc (percentage of the control).
analysis of the TeNT Hc (Halpern and Loftus, 1993), no further data on the precise definition of the neurotoxin binding domain is currently available for the different BoNT serotypes. In particular, the purification of these domains from bacteria has been hampered by low expression levels and relatively high insolubility. Here we demonstrate the suitability of distinct E. coli expression systems for the production of functional binding domains of CNTs which are able to antagonise the activity of the native neurotoxins in different experimental systems.

Despite the relatively low level of sequence homology, the structures of the Hc domain of TeNT and BoNT/A are very similar (Umland et al., 1997; Knapp et al., 1998; Lacy et al., 1998), suggesting an analogous function and folding for the uncharacterised BoNT/B and E. In fact, the Hc comprises two similarly sized sub-domains which have virtually no or very limited surface interactions with the other half of the H chain. The tertiary structure suggests that this part of the molecule folds independently and therefore constitutes a good candidate for functional expression in an heterologous system.

Using variants of the expression vector pGEX, the Hc domain of TeNT, BoNT/A and E were expressed as a soluble fusion protein in E. coli. In contrast, no expression of soluble BoNT/B Hc was observed in E. coli strains based on the K-12 genotype. The reason for this failure is unclear. Expression levels are known to be lowered by the insertion of tandem rare codons into homologous genes (Makoff et al., 1989). On inspection, the number and type of rare codons (Sharp and Li, 1986) in BoNT/B Hc are similar to those present in the other expressed Hcs. However, the Hc of BoNT/B does contain multiple triplets of consecutive rare codons concentrated in the first third of the sequence, a fact that may explain its poor expression. To overcome this problem, three non K-12 bacterial strains were tested and a homogeneous BoNT/B Hc of the expected molecular mass was obtained in the TOPP3 strain.

Recombinant Hc fragments bind immobilised polysialogangliosides and their biological activity was further tested in various functional assays. The first consisted of the binding of these domains to mouse embryonic spinal cord cells. This mixed culture contains the cell types responsible for the uptake of CNTs and the target of their final physiological action in vivo. Recombinant Hc fragments of TeNT and BoNT/A, B and E bind efficiently to spinal cord neurons and reveal a punctate staining pattern with areas of high toxin concentration, suggesting specialised arrangements of CNT cellular receptors. This binding is functional, as demonstrated by the ability of the spinal cord neurons to internalise the different Hcs in intracellular vesicular structures upon transfer to 37°C. In this regard, these fragments and their mutants will play a central role for the future dissection of the endocytic pathway of TeNT and BoNTs in isolated neurons.

Recent findings indicate the involvement of synaptogamins in the cellular recognition of BoNT/A, B and E (Nishiki et al., 1994, 1996a,b; Li and Singh, 1998). The domain acting as BoNTs receptor is the amino-terminal domain of synaptogamin, which is localised in the lumen of the SSV (Schiavo et al., 1998), but becomes accessible to the extracellular medium following the fusion of SSV with the presynaptic membrane (Matteoli et al., 1992). CNTs might exploit the SSV exo-endocytic cycle to gain access to the SSV lumen and then enter the neuronal cytoplasm (Matteoli et al., 1996). Although the distribution of the neurotoxin binding sites observed here is compatible with areas of SSV exocytosis, our experimental work does not provide direct evidence in support of this model nor for the role of synaptotagmins as BoNTs receptors.

We also showed that the Hc domains of CNTs inhibit the intracellular proteolytic activity of the native neurotoxins, which was conveniently followed by immunofluorescence and immunoblotting. This represents a very sensitive approach to test the binding, entry and translocation of native CNTs at a concentration as low as picomolar in different cellular systems (Williamson et al., 1996; Schiavo and Montecucco, 1997). All four Hc fragments effectively inhibited the proteolysis of the parental native neurotoxin in a concentration dependent manner, indicating that the Hc fragments bind to functional acceptor(s), which mediate the “productive” entry of the neurotoxin into the cell. This result is in contrast to the conclusions of a recent study showing no competition between the binding of native BoNT/A and its H chain, prepared by chemical dissociation, at the murine NMJ (Daniels-Holgate and Dolly, 1996). This preparation, which preserves the integrity of a fully-developed neuromuscular junction, is widely used to study the effects of various neurotoxins, including CNTs (Wohlfarth et al., 1997). In our experimental conditions, all four Hc fragments were found to be potent antagonists of the corresponding CNT, causing a significant delay in the onset of paralysis (75-150%). The discrepancy between the two results could be due to loss of biological function of the entire H chain during isolation or alternatively, the isolated fragments may have different conformations or aggregation states which would result in different biological activities.

Although the final concentrations necessary to delay the onset of paralysis are similar for TeNT and BoNT Hcs, the ratio between Hc and the holotoxin is higher for BoNTs than for TeNT (1,300:1 and 100:1, respectively). This reflects the well-documented fact that higher doses of TeNT compared to BoNTs are needed to observe a paralytic effect at the NMJ (10 nM vs 0.2 nM) (Dreyer and Schmitt, 1981; Schmitt et al., 1981; Simpson 1984a,b, 1985). Due to the large volume of the electrophysiological bath, a ratio between Hc and holotoxin higher than 100:1 was possible only for BoNTs. Experiments performed with lower excess of BoNT Hcs revealed a reduced level of protection, possibly due to the sequestration of Hc by non-specific low affinity binding sites present on the tissue. These low affinity sites would release bound Hc molecules only slowly, resulting in an apparently lower protecting effect of BoNT Hcs in this assay.

The specificity of the antagonism of the Hc on the paralysis caused by the native neurotoxin is further demonstrated by its strict serotype-dependency. We observed a complete lack of competition with the Hc fragment of BoNT/B on the action of BoNT/E. This result is particularly relevant because it is consistent with the well-documented lack of competition between BoNT/B and BoNT/E, which do not appear to share the same cellular receptor (Habermann and Dreyer, 1986). This last result was recently challenged by a series of reports suggesting that BoNT/A, B and E bind to the SSV proteins synaptotagmin I and II (Li and Singh, 1998; Nishiki et al., 1994, 1996a,b). The physiological relevance of the interaction
between synaptotagmin and CNTs remains controversial (Bakry et al., 1997). The recombinant Hc fragments will provide a valuable tool to verify these conclusions in vitro and in vivo, allowing further investigation into the nature and distribution of their still uncharacterised neuronal receptors. In addition, they will provide an experimental system for the precise mapping of the Hc-receptor protein-protein interaction and the definition of the minimal effector domain which is essential for their possible use as neurospecific protein carrier.

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