Botulinum type F neurotoxin

Large-scale purification and characterization of its binding to rat cerebrocortical synaptosomes

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1. A large-scale purification procedure has been developed for *Clostridium botulinum* type F neurotoxin. Commencing with 160 litres of bacterial culture, 101 mg of purified type F neurotoxin with a specific toxicity of 2×10^7 mouse LD₅₀ (median lethal dose) · mg⁻¹ were obtained. 2. Purified type F neurotoxin was labelled to high specific radioactivity (900–1360 Ci/mmol) without loss of biological activity using a chloramine- τ procedure. Of the two neurotoxin subunits, the heavy chain was preferentially radiolabelled. 3. Radiolabelled type F neurotoxin displayed specific saturable binding to rat synaptosomes. At least two pools of acceptors were evident: a low content of high-affinity acceptors sites [$K_D \sim 0.15$ nM; B_{max} (maximal binding) 20 fmol/mg] and a larger pool of lower-affinity sites ($K_D > 20$ nM; $B_{max} > 700$ fmol/mg). Both pools of acceptors were sensitive to trypsin and neuraminidase treatment, which suggests that protein and sialic acid residues are components of the synaptosomal acceptors. 4. Experiments investigating competition among botulinum neurotoxin types A, B, E and F for acceptors on rat brain synaptosomes showed that type F neurotoxin binds to acceptor molecules which are completely distinct from those of the other three neurotoxins.

INTRODUCTION

Botulinum type F neurotoxin is one of seven antigenically different neurotoxins (types A–G) produced by various strains of the bacterium *Clostridium botulinum*. These neurotoxins represent a group of extremely potent neuromuscular agents which act presynaptically in the peripheral nervous system, blocking the release of the neurotransmitter acetylcholine, and are responsible for the syndrome botulism, a frequently fatal disease affecting both man and animals (Shone, 1987). Of the seven neurotoxin types, just four types (A, B, E and F) have been found to be clearly associated with the human disease. Structurally the botulinum neurotoxins are very similar. Each is a protein of approx. 150 kDa, which in its most active form, consists of a heavy subunit (~100 kDa) linked by at least one disulphide bridge to a light subunit (~50 kDa). The neurotoxins most extensively studied (types A, B and E) appear to exert their neuromuscular effect through at least three stages: a binding stage, in which the neurotoxin binds to acceptor molecules on the presynaptic nerve surface, an energy-dependent internalization stage and, finally, an inhibition stage(s) in which the acetylcholine-release mechanism is disabled (Simpson, 1981).

Despite much study, the nature of the acceptor molecules to which these neurotoxins bind on the presynaptic nerve surface is not clearly understood. Binding studies using rat brain synaptosomes as a model suggest the botulinum neurotoxins bind in a complex manner, recognizing at least two pools of acceptor types. Neuraminidase treatment of synaptosomal membranes destroys toxin-binding activity, implicating a role for sialic acid residues in the acceptor(s) of types A (Williams *et al.*, 1983) and B (Evans *et al.*, 1986) neurotoxins. The demonstrated sensitivity of the acceptors to various proteinases also suggested the presence of a protein component. Despite the general similarities of the toxin acceptors, different neurotoxin types recognize different acceptors: types A and B neurotoxins appear to bind to different acceptors both on rat cerebrocortical synaptosomes and at the

murine neuromuscular junction (Evans *et al.*, 1986; Black & Dolly, 1986). The acceptors for botulinum type E neurotoxin are also different from that of type B toxin and appear to be similar to the type A acceptors (Kozaki, 1979).

Strains of *Clostridium botulinum* type F are generally poor producers of neurotoxin compared with those of types A and B, making purification of this neurotoxin difficult. A small-scale purification procedure developed by Yang & Sugiyama (1975) required five column-chromatography steps to a yield a small quantity of the purified neurotoxin. As a consequence very little is presently known about the structure and mode of action of botulinum type F neurotoxin (hereafter referred to as BoNTF). In the present paper a large-scale procedure for the purification of BoNTF is described. It is further demonstrated that BoNTF recognizes acceptors on cerebrocortical synaptosomes that are distinct from those recognized by neurotoxin types A, B and E.

MATERIALS AND METHODS

Purification of botulinum type F neurotoxin

All chromatography was carried out at room temperature using a BioPilot system (Pharmacia) and all dialysis steps were performed at 4 °C. All chemicals used for the preparation of buffers were reagent grade.

Clostridium botulinum type F (Langland strain), grown for 72 h on supplemented brain/heart infusion medium (Holdeman *et al.*, 1977) in 20-litre batches, was acidified with 2 M-H₂SO₄ to pH 3.0 and the resulting precipitate collected by continuous-flow centrifugation at 30000 g. Acid-precipitated toxin from 160 litres of culture was resuspended, using a stomacher homogenizer (Seward Medical, London, U.K.), in 1400 ml of sodium phosphate buffer (0.2 M, pH 6.0). The pH of the mixture was adjusted to 6.0 with 2 M-NaOH and the suspension stirred for 1 h at room temperature, then centrifuged at 25000 g for 40 min. The supernatant fluid was retained and the sediment

Abbreviations used: LD₅₀, amount of toxin which kills half the animals within 4 days; K_D , dissociation constant; B_{max} , maximal binding; BoNTF, botulinum type F neurotoxin; IC₅₀, concentration causing 50% inhibition.

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extracted with a further 1400 ml of the phosphate buffer. The supernatant fluids from both extractions were combined and treated with RNAase (Sigma) (100 µg/ml for 1 h at 37 °C) and then precipitated with solid (NH₄)₂SO₄ at 60% saturation. The precipitated toxin, collected by centrifugation at 25000 g for 30 min, was resuspended in 600 ml of Bistris/HCl buffer (0.05 M, pH 5.8) and dialysed for 48 h against 2 × 10 litres of the same buffer. The dialysed toxin was centrifuged at 25000 g for 60 min, then loaded on to a column (11.2 cm diameter × 7 cm long) of Sepharose Q Fast Flow (Pharmacia) equilibrated with the Bistris/HCl buffer. After loading, the column was washed with 500 ml of the Bistris/HCl buffer, and the toxin was eluted with Bistris/HCl buffer (0.05 M, pH 5.8) containing 0.15 M-NaCl, at 50 ml/min. The eluted neurotoxin was then dialysed for 16 h against triethanolamine buffer (0.05 M, pH 8.5) containing 0.05 M-NaCl (2 × 10 litres). After dialysis the toxin was loaded on to a second column (5 cm × 8 cm) of Sepharose Q Fast Flow equilibrated in the pH 8.5 buffer, and then eluted with triethanolamine buffer (0.05 M, pH 8.5) containing 0.15 M-NaCl. The neurotoxin was dialysed for 16 h against 10 litres of succinate buffer (0.05 M, pH 5.5) and loaded on to a column (1.6 cm × 5 cm) of Sepharose S equilibrated in the same buffer. The column was washed with 100 ml of the succinate buffer, and the purified type F neurotoxin was eluted with succinate buffer (0.05 M, pH 8.5) containing 0.15 M-NaCl. The neurotoxin was dialysed against Tris/HCl buffer (0.1 M, pH 8.0) and stored frozen at -25 °C.

Purification of the heavy subunit of BoNTF

The heavy subunit of BoNTF was purified in the manner previously described for the purification of the heavy chain of botulinum type A neurotoxin (Shone *et al.*, 1985). Neurotoxin (2.5 mg/ml) was incubated with 1 M-NaCl/2.5 M-urea and 100 mM-dithiothreitol in Tris/HCl buffer (0.1 M, pH 8.0) for 16–20 h at 22 °C. The precipitated light chain of BoNTF was removed by centrifugation (15000 g, 2 min) and the supernatant fluid, containing the purified heavy chain, was dialysed overnight against Krebs bicarbonate buffer, pH 7.4 (Dolly *et al.*, 1987).

Purification and radiolabelling of the botulinum neurotoxins

Clostridium botulinum types A, B and E neurotoxins were purified as described previously (Shone *et al.*, 1985; Evans *et al.*, 1986; Schmidt & Siegel, 1986).

Botulinum types A, B, E and F neurotoxins were iodinated with ¹²⁵I by the chloramine-T method, as described previously for type A neurotoxin (Williams *et al.*, 1983). After the removal of portions necessary for the determination of protein content and specific radioactivity, ¹²⁵I-neurotoxin preparations were stored at 4 °C in 0.25% (w/v)-gelatin/phosphate buffer. The free ¹²⁵I content of the preparation was assessed by precipitation of the labelled toxin using ice-cold trichloroacetic acid (10%, w/v) with BSA (0.75 mg/ml) as a carrier protein. ¹²⁵I-neurotoxins were compared with unlabelled neurotoxins by PAGE run in the presence or absence of 5% (w/v) 2-mercaptoethanol.

Saturable binding of ¹²⁵I-BoNTF to rat synaptosomal membranes

Routine centrifugation assays as described elsewhere (Williams *et al.*, 1983; Evans *et al.*, 1986) were performed using rat cerebrocortical synaptosome preparations (Dodd *et al.*, 1981). Membranes (5–6 mg/ml) were sedimented and resuspended in Krebs bicarbonate buffer, pH 7.4, containing BSA (1 mg/ml). Saturable binding was determined by incubating membrane portions with increasing concentrations of ¹²⁵I-BoNTF (0.01–20 nM). Non-specific binding at each concentration was determined by the inclusion of a 100-fold molar excess of unlabelled toxin. After incubation (1 h at 22 °C) samples were

centrifuged (9000 g for 2 min) and portions of the supernatant fluid removed for the determination of free ¹²⁵I-BoNTF. The pellets were then resuspended and further sedimented twice (2 × 1 ml of ice-cold buffer) before their radioactivity was determined by γ-radiation counting. The ability of other botulinum neurotoxin types to compete with ¹²⁵I-BoNTF for its acceptor-binding site(s) was assessed by incubation of synaptosomal membranes (1 h at 22 °C) with 1 nM-¹²⁵I-BoNTF in the presence of 0–1 µM test ligand. Binding was terminated by dilution and centrifugation as described above.

Characterization of BoNTF acceptors on rat brain synaptosomes

Synaptosomal membranes (5–6 mg of total protein/ml) were treated with trypsin [tosylphenylalanylchloromethane ('TPCK')-treated; Sigma] at 1 mg/ml for 40 min at 37 °C in the presence or absence of a 5-fold (w/w) excess of soybean trypsin inhibitor. Likewise, synaptosomal suspensions were incubated with neuraminidase (type X from *Clostridium perfringens*; Sigma) at a concentration of 1 unit/ml for 40 min at 37 °C with or without (20 mM of) the selective inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Sigma). After incubation, membranes were washed (as above) and resuspended in buffer containing the appropriate inhibitor. Samples (50–100 µg) of these membranes, or membranes heat-treated at 95 °C for 2 min, were incubated with 1 nM-¹²⁵I-BoNTF. Specific binding was quantified as described above and compared with that observed with non-treated membranes.

Other analytical determinations

Protein content was determined by the method of Bradford (1976), with BSA as a standard. Mouse toxicity tests were performed as described elsewhere (Maisey *et al.*, 1988).

RESULTS

Purification of BoNTF

BoNTF exists as a protein complex in association with one other protein which has no toxicity (Ohishi & Sakaguchi, 1974). After precipitation by (NH₄)₂SO₄, BoNTF, in its complexed format pH 5.8 in Bistris/HCl buffer, bound to the anion-exchange resin, Sepharose Q (Table 1). At pH 5.5 in succinate buffer (0.05 M) the bulk of the protein components in this mixture, including the toxin, were not retained by the cation-exchange resin, Sepharose S. The neurotoxin complex eluted from Sepharose Q column was dissociated by raising the pH to 8.5, and the neurotoxin was separated from the non-toxic protein of the complex by chromatography on a second column of Sepharose Q at pH 8.5. Finally, chromatography of the eluted neurotoxin on a column of Sepharose S at pH 5.5 yielded the purified neurotoxin. At this final column stage the BoNTF appears to be the only protein to bind to the Sepharose S (Table 1). Purified BoNTF had a specific toxicity of 2 × 10⁷ mouse LD₅₀ (median lethal dose) · mg⁻¹, and on SDS/PAGE appeared > 95% pure, running as a single band of molecular mass 155 kDa with only two trace impurities visible at lower molecular masses (Fig. 1). Under reducing conditions on SDS/PAGE the toxin dissociated into two subunits corresponding to molecular masses of 105 and 55 kDa (Fig. 2).

Properties of ¹²⁵I-BoNTF

Homogeneous preparations of BoNTF were [¹²⁵I]iodinated to high specific radioactivity (~ 900–1360 Ci/mmol) using the chloramine-T method, under conditions found to be optimal for botulinum neurotoxin types A and B (Williams *et al.*, 1983; Evans *et al.*, 1986). ¹²⁵I-labelled BoNTF retained an appreciable

Table 1. Purification of botulinum type F neurotoxin

Purification stage	Volume (ml)	[Protein] (mg/ml)	Toxicity (LD ₅₀)		Yield (%)	
			Total	Specific	Stage	Overall
Resuspended acid ppt.	2000	nd	4.6×10^9	nd*	100	100
Phosphate-buffer extract	2190	19.5	4.6×10^9	1.1×10^5	100	100
(NH ₄) ₂ SO ₄ resuspension	1300	20	3.6×10^9	1.5×10^5	85	85
Sephacrose Q (pH 5.8) column						
Wash	1500	5.5	$< 3 \times 10^7$		< 1	
Elution with 0.15 M-NaCl	700	15.5	3.5×10^9	3.2×10^5	90	76
Elution with 1.0 M-NaCl	700	9.5	7.0×10^7		1.8	
Sephacrose Q (pH 8.5) column						
Elution with 0.15 M-NaCl	400	14	2.5×10^9	4.3×10^5	71	54
Elution with 0.2 M-NaCl	300	3	3.0×10^7		< 1	
Sephacrose S (pH 5.5) column						
Wash	270	5.5	$< 2 \times 10^5$		< 1	
Neurotoxin peak (elution with 0.15 M-NaCl)	44	2.3	2.1×10^9	2.1×10^7	84	45

* Abbreviation: nd, not determined.

fraction of its original neurotoxicity (> 75%) and migrated identically with native BoNTF when analysed by SDS/PAGE; autoradiography of the dried gel showed that, although both constituent chains contained radioactivity, it was the larger polypeptide that was preferentially labelled (Fig. 2). Most (90%) of the radioactivity in ¹²⁵I-BoNTF samples was acid-precipitable. Storage of ¹²⁵I-labelled BoNTF in gelatin/phosphate buffer at 4 °C enabled preparations to be used for more than 1 week without appreciable loss of specific binding activity.

Demonstration of saturable binding of ¹²⁵I-BoNTF to rat brain synaptosomal membranes

Incubation of increasing concentrations of ¹²⁵I-BoNTF (0.01–20 nM) with rat cerebrocortical synaptosomal membranes revealed that the level of measurable binding reached a plateau at high concentrations (Fig. 3, inset). Inclusion of a 100-fold molar excess of unlabelled BoNTF over each concentration used showed that, at low concentrations, the non-specific contribution to total binding remained acceptably low; however, at high concentrations this proportion became predominant (Fig. 3, inset). Computer analysis of four sets of binding data using the

Fig. 1. SDS/PAGE of samples of BoNTF during purification

Electrophoresis was performed on gradient gels (4–30%; Pharmacia) as detailed previously (Shone *et al.*, 1985). Samples are: track 1, molecular-mass standards (94, 67, 43, 30 and 20.1 kDa); track 2, phosphate-buffer extract; track 3, resuspended (NH₄)₂SO₄ precipitate; track 4, 0.15 M-NaCl fraction from Sepharose Q (pH 5.8) column; track 5, 0.15 M-NaCl fraction from Sepharose Q (pH 8.5) column; track 6, purified BoNTF [0.15 M-NaCl fraction from Sepharose S (pH 5.5) column]. All samples were electrophoresed in the absence of a reducing agent.

Fig. 2. SDS/PAGE-autoradiography of ¹²⁵I-BoNTF

Purified BoNTF was radiolabelled with ¹²⁵I- to high specific radioactivity and subjected to electrophoresis on a 10% polyacrylamide gel run under non-reducing and reducing conditions. Under non-reducing conditions (track 1) the disulphide-linked di-chain form of the neurotoxin is observed, which, in the presence of 5% (w/v) 2-mercaptoethanol (track 2), dissociates into its constituent heavy (HC) and light (LC) chains. Notably the heavy chain is labelled predominantly, the light chain by comparison containing little radioactivity. The autoradiogram presented is representative of the pattern of labelling obtained with three different preparations of ¹²⁵I-labelled BoNTF.

Fig. 3. Saturable binding of ^{125}I -BoNTF to rat cerebrocortical synaptosomes

Purified synaptosomal membranes in Krebs bicarbonate buffer containing 1 mg of BSA/ml were incubated (1 h at 22 °C) with increasing concentrations of ^{125}I -BoNTF (0.01–30 nM). Binding was terminated by dilution with ice-cold buffer, followed by centrifugation; sedimented pellets were washed once and subjected to γ -radiation counting. Non-specific binding was determined by inclusion of a 100-fold molar excess of unlabelled BoNTF over each concentration used. Presentation of the specific binding as a Scatchard plot was non-linear, indicating heterogeneity in BoNTF-binding sites. This was reaffirmed by computer analysis of the data, which revealed at least two acceptor populations consisting of high-affinity sites ($K_D > 20$ nM; $B_{\max} > 700$ fmol/mg). Inset: Specific binding (●) was calculated by subtracting non-specific binding (○) from the total binding (▲). The findings shown in these representative Figures were reproduced with three different preparations of both ^{125}I -labelled BoNTF and synaptosomal membranes.

program LIGAND (Munson & Rodbard, 1980) indicated the presence of at least two acceptor populations: a low content of high-affinity acceptor sites with a K_d (dissociation constant) value between 0.07 and 0.15 nM [average K_d 0.11 nM; $B_{\max}$ (maximal binding) 18 fmol/mg], together with a much larger population of low-affinity sites ($K_D > 20$ nM; $B_{\max} > 700$ fmol/mg). Presentation of the specific binding data as a Scatchard plot (Fig. 3) showed the relationship to be non-linear; this reaffirmed heterogeneity in the neuronal acceptor sites for ^{125}I -BoNTF.

Characterization of the synaptosomal acceptors for BoNTF

The involvement of a protein component together with *N*-acetylneuraminic acid residues in the constitution of the central acceptors for botulinum neurotoxin types A and B has been demonstrated (Williams *et al.*, 1983; Evans *et al.*, 1986). In the present investigation purified rat cerebrocortical synaptosomal membranes were trypsin-, heat- and neuraminidase-treated and assessed for their ability to bind ^{125}I -BoNTF (1 nM). These studies suggest that a proteinaceous component was inherent to both high- and low-affinity sites, since measurable specific binding was completely destroyed by either heat or trypsin treatment (Table 2). The specificity of this trypsin-mediated proteolysis was shown by the ability of soybean trypsin inhibitor to prevent the inactivation. The involvement of *N*-acetylneuraminic acid resi-

dues in the constitution of synaptosomal acceptors for BoNTF was shown by the ability of neuraminidase to inhibit completely the measurable specific binding. This inactivation of acceptors resulted specifically from the action of neuraminidase and was not due to proteinase contamination of the enzyme preparation,

Table 2. Nature of the synaptosomal-membrane acceptors for BoNTF

Rat synaptosomal membranes in Krebs bicarbonate buffer were heat-, trypsin- or neuraminidase-treated as detailed in the Materials and methods section. After washing, centrifugation and resuspension in buffer containing the appropriate inhibitor, membrane samples were incubated with 1 nM- ^{125}I -BoNTF for 1 h at 22 °C in the presence or absence of a 100-fold molar excess of unlabelled BoNTF. Specific binding was quantified as described in Fig. 3 and expressed as a percentage of that for control samples (mean $\pm$ S.D., $n = 4$)

Membrane treatment	Relative specific binding (%)
None (total binding)	100 $\pm$ 3
Neuraminidase (1 unit/ml)	0
+Inhibitor	83 $\pm$ 12
Trypsin 1 mg/ml	0
+Inhibitor	86 $\pm$ 6
Heat treatment (95 °C for 2 min)	0

Fig. 4. ^{125}I -labelled BoNTF binding to synaptosomal membranes in the presence of other botulinum neurotoxin types

In a series of experiments, (a)–(d), 1 nM ^{125}I -botulinum types A, B, E and F neurotoxins were added to increasing concentrations of unlabelled neurotoxins. In each case binding was initiated by the addition of synaptosomal membranes ($\sim 100\text{ }\mu\text{g}$); after incubation for 1 h at 22°C , binding was terminated by dilution and centrifugation as detailed in the Materials and methods section. The amount of ^{125}I -neurotoxin binding was expressed as a percentage of that for control samples (in which no competing ligand was present) and plotted against $\log(\text{ligand concentration})$. (a), (b), (c) and (d) involve 1 nM ^{125}I -botulinum neurotoxin types F, A, B and E respectively; competing ligands are BoNTF (●), the heavy chain of BoNTF (■), and botulinum neurotoxins A (□), B (○) and E (▲). Data points represent means for four replicates; the range in all cases was $< 10\%$.

because the inclusion of 20 nM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (an inhibitor of neuraminidase) was shown to antagonize this effect. These data provide evidence that the acceptors recognized by BoNTF may contain sialoglycoproteins.

Effect of botulinum neurotoxin types A, B and E on the binding of BoNTF to rat brain synaptosomes

In view of the demonstrated structural similarities between the acceptors for BoNTF and the other botulinum neurotoxin types, evidence for toxin similarity was sought by testing the ability of types A, B and E botulinum neurotoxins to compete for the

binding components of ^{125}I -labelled BoNTF. As Fig. 4(a) shows, even high concentrations of these neurotoxins failed to produce any measurable antagonism of BoNTF (1 nM) binding to synaptosomal membranes. Control samples, in which unlabelled BoNTF was the competing ligand, showed, as expected, a dose-dependent antagonism of binding with an IC_{50} (concentration causing 50% inhibition) of $\sim 18\text{ nM}$. Furthermore, the constituent heavy chain of BoNTF was shown to mediate this acceptor recognition, since homogeneous preparations of this subunit antagonized ^{125}I -BoNTF binding to synaptosomes with the same efficacy as the native toxin.

In order to investigate the possibility of any competition between the neurotoxins being overlooked because of differences in binding affinities, BoNTF was tested for its ability to affect the synaptosomal binding of 1 nM ^{125}I -labelled botulinum neurotoxin types A, B and E (Figs. 4b, 4c and 4d respectively). These results showed that no significant similarity in the acceptor-binding sites exists between BoNTF and any of the other botulinum neurotoxin types tested. Indeed, in accordance with previous findings (Kozaki, 1979), only botulinum neurotoxins types A and E demonstrated partial similarity of their membrane acceptors (Fig. 4b and 4d).

DISCUSSION

All of the botulinum toxins so far studied are secreted from the bacterium in the form of protein complexes consisting of the neurotoxin moiety in association with at least one other non-toxin protein. These protein complexes are stable at low pH ($< \text{pH } 6$), but dissociate at higher pH values ($> \text{pH } 8$). This pH-dependent association of the complex proteins has been used to purify several of the botulinum neurotoxins. Botulinum neurotoxins A and B, whose complexes contain a haemagglutinin component, have been purified by affinity chromatography (Shone *et al.*, 1985; Evans *et al.*, 1986) and type E neurotoxin using h.p.l.c. (Schmidt *et al.*, 1986).

In the present paper a large-scale purification procedure for *Clostridium botulinum* type F neurotoxin, based on the dissociable nature of the toxin complex, is described. The procedure includes three column-chromatography stages, in each of which the column is eluted with a simple stepwise salt step. The final column stage yields BoNTF more than 95% pure with a specific toxicity of 2×10^7 mouse $\text{LD}_{50} \cdot \text{mg}^{-1}$, which agrees favourably with previously reported values for the purified neurotoxin (Yang & Sugiyama, 1975).

The characteristics of the interaction of BoNTF with rat brain synaptosomes were similar to those previously observed with botulinum type A and B neurotoxins. The neurotoxin appears to bind to at least two different acceptors on the synaptosomal membranes: a small pool of high-affinity acceptors and a larger pool recognized with lower affinity. The affinity with which BoNTF binds to the high-affinity acceptor on the brain synaptosomes is of a similar order of magnitude (K_D between 10^{-9} and 10^{-10} nM) to that previously observed for type A and B neurotoxins (Williams *et al.*, 1983; Evans *et al.*, 1986). BoNTF, in common with the other botulinum neurotoxins, appears to bind its synaptosomal acceptors by a region on the heavy chain, and the acceptors recognized by this active-site region appear to contain both protein and sialic acid residues, indicating a glycoprotein structure.

Despite these similarities, the acceptors on rat brain synaptosomes recognized by BoNTF were found to be clearly distinct from those recognized by either type A, B or E neurotoxins. Previous studies using rat brain synaptosomes as a model have also shown that botulinum types A and B neurotoxins recognize different acceptor molecules (Evans *et al.*, 1986), whereas botu-

linum type E neurotoxin appears to bind acceptors similar to those of type A (Kozaki, 1979). Thus the four botulinum neurotoxins most implicated in human botulism, namely A, B, E and F, appear to recognize a family of three distinct acceptor types on brain synaptosomes.

Although rat brain synaptosomes provide a convenient model with which to study the binding properties of the botulinum neurotoxins, there is some doubt as to whether or not the synaptosomal acceptors bear any resemblance to those at the neuromuscular junction. Indeed, in contrast with central acceptors at which the heavy chain apparently binds with equal affinity to the native toxin, recent evidence (Maisey *et al.*, 1988; Poulain *et al.*, 1989) has suggested that only the di-chain species of the neurotoxin is able to recognize efficiently those acceptors involved in the poisoning of peripheral motor neurons. Some similarities between the brain and peripheral acceptors are evident, since the lack of competition between the binding of type A and B neurotoxin observed with brain synaptosomes has also been demonstrated at the neuromuscular junction (Black & Dolly, 1986). Furthermore, in a previous study with type A neurotoxin, a very close correlation between a reduction in its binding to synaptosomes and a loss of its toxicity in the peripheral nervous system was observed (Shone *et al.*, 1985).

Hence, despite the fact that differences appear to exist between the acceptors at the brain and peripheral tissue, it seems unlikely that the binding-site regions present on the heavy subunits of the botulinum neurotoxins, which are capable of very-high-affinity binding to the central nervous system, have no significant role in the biological (peripheral) action of the toxins. In the present study it has been clearly demonstrated that, in the case of BoNTF, this binding-site region has properties which make it distinct from those present on botulinum types A, B and E neurotoxins.

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