

Binding of *Clostridium botulinum* type B toxin to rat brain synaptosome

Moon Kook Park¹, Hyun Ho Jung² and Kyu Hwan Yang²

¹ Department of Biology Education, Chonbuk National University, Chonju, Korea

and ² Department of Biological Science and Engineering, Korea Advanced Institute of Science and Technology, Seoul, Korea

Received 4 June 1990

Accepted 18 June 1990

Key words: *Botulinum* neurotoxin; Synaptosome; Monoclonal antibody

1. SUMMARY

Purified toxin and its subunits from *Clostridium botulinum* type B were labeled with ¹²⁵Iodine and binding of them to rat brain synaptosomes was studied. Labeled toxin and heavy chain were shown to bind to synaptosomes and there was no significant difference in the molar quantity of bound toxin and heavy chain at several concentrations of synaptosomes, whereas labeled light chain did not bind to synaptosomes. The binding of labeled heavy chain to synaptosomes was inhibited by unlabeled toxin and heavy chain to a similar degree as that of labeled toxin. The binding of labeled toxin and heavy chain to synaptosomes were inhibited by a monoclonal antibody which is specific for the heavy chain.

2. INTRODUCTION

Clostridium botulinum neurotoxins have a common molecular structure composed of a heavy chain (*M_r* about 100 000) and a light chain (*M_r* about 50 000) linked by one or more disulfide

bond(s) [1]. These toxins inhibit the acetylcholine release from cholinergic nerve endings, causing neuromuscular paralysis [2]. They are thought to express toxic activity through the process of binding to presynaptic membrane, internalization, and blocking of transmitter release [2]. There exists indirect evidence to suggest that the heavy chain of the toxin molecule is responsible for binding the toxin to presynaptic membranes. This includes competition of heavy chain for binding sites on synaptosomes with whole toxin [3–5] and inhibition of binding of whole toxin to synaptosomes by polyclonal and monoclonal antibodies specific for the heavy chain [6,7]. However, there has been no report showing binding properties of heavy chain to presynaptic membranes. This paper reports the binding properties of radioiodinated heavy chain of botulinum type B toxin to rat brain synaptosomes.

3. MATERIALS AND METHODS

3.1. Preparation of type B toxin and its subunits

Clostridium botulinum type B strain Lamanna was grown in 6 liter's of toxin production medium [8] at 37°C for 5 days. Toxin purification was carried out by the method of DasGupta and Sugiyama [8]. Subunits of type B toxin were pre-

Correspondence to: M.K. Park, Department of Biology Education, Chonbuk National University, Chonju, 560-756, Korea.

pared by elution from SDS-polyacrylamide gels following the procedure of Hager and Burgess [9]. Protein concentration was determined by the method of Bradford [10] with ovalbumin as a standard. Toxicity was determined by the time to death method [11].

3.2. Preparation and characterization of monoclonal antibodies

Toxoid was prepared by dialyzing purified toxin against 0.4% formalin in 50 mM phosphate buffer, pH 6.0, at room temperature for 1 week. BALB/c mice were immunized with toxoid or heavy chain following the procedure of Hayakawa et al. [12]. Spleen cells were fused with myeloma cells (Sp2/0-Ag14) by 45% polyethylene glycol 4000 (Merck). Antibody-secreting hybridomas were screened by ELISA with alkaline phosphatase conjugated anti-mouse immunoglobulin (Sigma) and then cloned by limiting dilution. Cloned cells were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Sigma). The culture supernatant was fractionated with ammonium sulfate at 25 to 50% saturation and dialyzed against PBS at 4°C.

Immunodiffusion tests were carried out with goat antisera to mouse immunoglobulins (Sigma) to determine immunoglobulin (sub)classes of monoclonal antibodies. To determine the subunit of toxin to which each antibody binds, immunoblotting was carried out by the procedure of Winston et al. [13]. Neutralizing activity of each antibody was determined as described by Kamata et al. [14].

3.3. Radioiodination

150 μ Ci of ^{125} I (Amersham) was mixed with 20 μ g of toxin or subunit in 100 μ l of 100 mM phosphate buffer, pH 7.5. The reaction was initiated by adding 100 μ l of chloramine T solution (80 μ g/ml) and was stopped after 40 s at room temperature by adding 20 μ l of L-tyrosine solution (1 mg/ml). Labeled toxin or subunit was separated from the reaction mixture by gel filtration on a Sephadex G-25 column (1.0 $\times$ 10 cm), pre-equilibrated with 100 mM sodium phosphate buffer, pH 7.5, containing 0.1% bovine serum albumin, and then dialyzed against the same buffer for 1 day at 4°C.

3.4. Binding of type B toxin and its subunits to synaptosomes

Synaptosomes from rat brain were prepared by discontinuous sucrose-gradient centrifugation, as described by Whittaker [15]. Labeled toxin (300 ng), heavy chain (200 ng) or light chain (100 ng) was mixed with various amounts of synaptosomes (0–40 μ g protein) in a total volume of 100 μ l of incubation medium (120 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 4 mM MgCl_2 , 0.25% bovine serum albumin, 10 mM phosphate buffer, pH 7.2). After incubation at 37°C for 30 min, the reaction was stopped by dilution with 900 μ l of incubation medium and centrifugation (12000 $\times$ g for 15 min). 500 μ l of supernatant (a) and the remaining contents (b) of the vessel were measured for γ -radiation. Nonspecific binding (c) was determined by incubation in the presence of 500-fold excess of unlabeled toxin over the amounts of labeled toxin or subunits. The radioactivity bound (d) was calculated according to the formula $d = b - (a + c)$.

For testing the inhibition by unlabeled toxin and heavy chain, labeled toxin (30 ng) or heavy chain (20 ng) was mixed with various amounts of unlabeled toxin or heavy chain and then synaptosomes (10 μ g protein) were added. After incubation at 37°C for 30 min, the reaction was stopped by dilution and centrifugation.

For testing the effects by monoclonal antibodies, labeled toxin (30 ng) or heavy chain (20 ng) was preincubated with various amounts of monoclonal antibody for 15 min at 37°C and then synaptosomes (10 μ g protein) were added.

4. RESULTS AND DISCUSSION

When purified type B toxin was reduced with 5% β -mercaptoethanol, nearly all of the toxin was cleaved into a heavy and a light chain (Fig. 1A). The subunits of type B toxin were prepared by elution from preparative SDS-polyacrylamide gels instead of conventional QAE-Sephadex chromatography [16] or gel filtration [17]. The subunits were recovered with about 40% efficiency.

Four hybridoma cell lines were established and monoclonal antibodies produced by them were

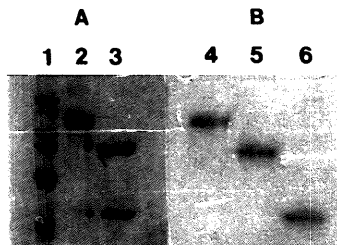


Fig. 1. SDS-PAGE (A) and autoradiography (B) of type B toxin and its subunits. Electrophoresis was performed with 7.5% running gel and 4% stacking gel. Lanes: 1, molecular mass markers: myosin (M_r 205000), β -galactosidase (M_r 116000), phosphorylase b (M_r 97000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000); 2, type B toxin under nonreducing condition; 3, type B toxin under reducing condition; 4, radioiodinated type B toxin under nonreducing condition; 5, radioiodinated heavy chain under reducing condition; 6, radioiodinated light chain under reducing condition.

characterized (Table 1). They all recognized the heavy chain of type B toxin. The neutralizing activity of antibody B3 was much higher than those of the others.

Specific radioactivities of the labeled toxin and subunits were 1.08–1.38 Ci/g protein and the toxicity of labeled toxin was about 84% that of unlabeled toxin. When the labeled toxin and subunits were examined by electrophoresis and autoradiography, they showed electrophoretic mobili-

TABLE 1

Some properties of monoclonal antibodies against type B toxin

Antibody	immunogen	(Sub)class	Epitope	Neutralization ^a (%)
B1	toxoid	IgM	H chain	69
B2	toxoid	IgG1	H chain	73
B3	toxoid	IgA	H chain	7
B4	heavy chain	IgG1	H chain	87

^a Type B toxin was diluted to 4×10^5 LD₅₀/ml with PBS and mixed with equal volume of each antibody preparation (2 mg/ml). After incubation at 37°C for 30 min, the remaining toxicity was determined. The data express remaining toxicity.

ties identical to those of unlabeled toxin and subunits (Fig. 13).

Labeled toxin and its subunits were examined for binding properties to synaptosomes. When labeled toxin or subunits were incubated with various amounts of synaptosomes, binding of light chain was not observed. In contrast, the specific binding of toxin and heavy chain was proportional to the amounts of synaptosomes up to 5 μ g of synaptosomal protein (Fig. 2) and there was no significant difference in the moles of bound toxin and heavy chain at the tested concentrations of synaptosomes (Table 2). In addition, there was no significant difference in the inhibitory effects of unlabeled toxin and heavy chain (Fig. 3). The concentrations of unlabeled toxin that induced half-inhibition of the binding of labeled toxin and heavy chain were 1.8 nM and 2.0 nM, respectively. The half-inhibition concentrations of unlabeled

TABLE 2

Amounts of toxin and heavy chain bound to synaptosome

Synaptosome concentration (μ g protein)	Amounts bound to synaptosome					
	Toxin			Heavy chain		
	cpm (10^4)	ng	mol ^a (10^{-14})	cpm (10^4)	ng	mol (10^{-14})
1.25	1.6	9.4	6.6	1.1	6.3	6.4
2.5	3.4	20.0	14.1	2.6	14.9	15.2
5.0	7.8	45.9	32.3	5.0	28.6	29.2
10.0	9.9	58.2	41.0	8.0	45.7	46.6
15.0	10.4	61.2	43.1	8.4	48.0	49.0
20.0	11.7	68.8	48.4	9.2	52.6	53.6

^a The molecular masses of toxin and heavy chain were assumed to be 142000 and 98000, respectively.

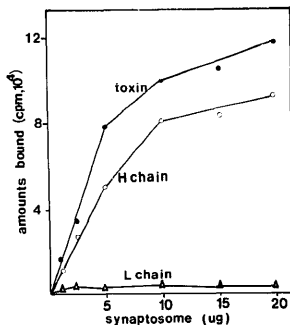


Fig. 2. Binding of labeled toxin and subunits to synaptosome. Labeled toxin (510000 cpm, 300 ng), heavy chain (350000 cpm, 200 ng) or light chain (166000 cpm, 100 ng) was incubated with various amounts of synaptosomes at 37°C for 30 min. Values are the means of two determinations.

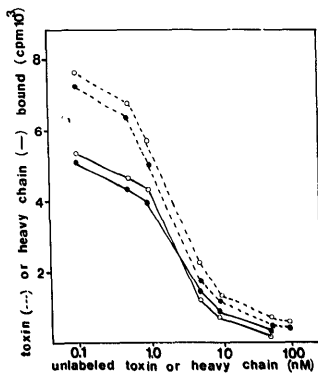


Fig. 3. Inhibition of binding of labeled toxin and heavy chain to synaptosome by unlabeled toxin and heavy chain. Labeled toxin (51000 cpm, 30 ng) or heavy chain (35000 cpm, 20 ng) was incubated with synaptosomes (10 µg protein) in the presence of unlabeled toxin (●) or heavy chain (○). Values are the means of two determinations.

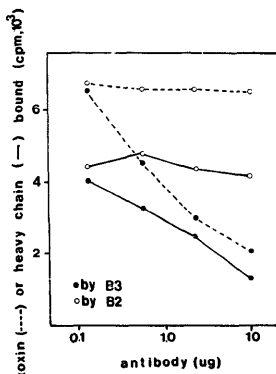


Fig. 4. Effects of monoclonal antibodies on binding of labeled toxin and heavy chain to synaptosome. Labeled toxin (51000 cpm, 30 ng) or heavy chain (35000 cpm, 20 ng) was preincubated with various amounts of each monoclonal antibody preparation at 37°C for 20 min and then synaptosomes (10 µg protein) was added and the mixture was incubated further at 37°C for 30 min. Only the results with antibodies B2 and B3 are shown and antibodies B1 and B4 showed similar effects as B2. Values are the means of two determinations.

heavy chain for the binding of labeled toxin and heavy chain were 2.1 nM and 1.8 nM, respectively. All the results indicate that the heavy chain of botulinum type B toxin has a similar binding ability to synaptosomes as the whole toxin. When compared to previous reports utilizing only labeled toxin [3-7], the results obtained in this study provide the first direct evidence for the involvement of heavy chain in binding of botulinum neurotoxin to presynaptic membranes.

When monoclonal antibodies, specific for the heavy chain, were tested for the effects on binding of toxin and heavy chain to synaptosomes, similar effects were observed in both whole toxin and heavy chain. Antibody B3 inhibited the binding of both toxin and heavy chain effectively, and the others showed little inhibitory effects (Fig. 4).

REFERENCES

- [1] DasGupta, B. R. and Sugiyama, H. (1972) *Biochem. Biophys. Res. Commun.* 48, 108–112.
- [2] Simpson, L.L. (1986) *Annu. Rev. Pharmacol. Toxicol.* 26, 427–453.
- [3] Kozaki, S. (1979) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 308, 67–70.
- [4] Kozaki, S., Miki, A., Kamata, Y., Ogasawara, J. and Sakaguchi, G. (1989) *Infect. Immun.* 57, 2634–2639.
- [5] Murayama, S., Syuto, B., Oguma, K., Iida, H. and Kubo, S. (1984) *Eur. J. Biochem.* 142, 487–492.
- [6] Agui, T., Syuto, B., Oguma, K., Iida, H. and Kubo, S. (1983) *J. Biochem.* 94, 521–527.
- [7] Kozaki, S., Kamata, Y., Nagai, T., Ogasawara, J. and Sakaguchi, G. (1986) *Infect. Immun.* 52, 786–791.
- [8] DasGupta, B.R. and Sugiyama, H. (1976) *Infect. Immun.* 14, 680–686.
- [9] Hager, D.A. and Burgess, R.R. (1980) *Anal. Biochem.* 109, 76–86.
- [10] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [11] Boroff, D.A. and Fleck, U. (1966) *J. Bacteriol.* 92, 1580–1581.
- [12] Hayakawa, S., Uchida, T., Mekada, E., Moynihan, M.R. and Okada, Y. (1983) *J. Biol. Chem.* 258, 4311–4317.
- [13] Winston, S.E., Fuller, S.A. and Hurrell, J.G.R. (1987) in *Current Protocols in Molecular Biology* (Ausbel, F.M., ed.), 10.8, Greene Publishing Associates and Wiley-Interscience, New York.
- [14] Kamata, Y., Kozaki, S., Nagai, T. and Sakaguchi, G. (1985) *FEMS Microbiol. Lett.* 26, 305–309.
- [15] Whittaker, V.P. (1959) *Biochem. J.* 72, 694–706.
- [16] Sathyamoorthy, V. and DasGupta, B.R. (1985) *J. Biol. Chem.* 260, 10461–10466.
- [17] Kozaki, S. and Sakaguchi, G. (1975) *Infect. Immun.* 11, 932–936.