The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin II associated with gangliosides G_{T1b}/G_{D1a}

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Abstract **^{125}I-labeled botulinum type B neurotoxin was shown to bind specifically to recombinant rat synaptotagmins I and II. Binding required reconstitution of the recombinant proteins with gangliosides G_{T1b}/G_{D1a}. Scatchard plot analyses revealed a single class of binding site with dissociation constants of 0.23 and 2.3 nM for synaptotagmin II and synaptotagmin I, respectively, values very similar to those of the high- (0.4 nM) and low-affinity (4.1 nM) binding sites in synaptosomes. The high-affinity binding of neurotoxin to synaptosomes was specifically inhibited by a monoclonal antibody recognizing with the amino-terminal region of synaptotagmin II. These results suggest that this region of synaptotagmin II participates in the formation of the high-affinity toxin binding site by associating with specific gangliosides.**

Key words: Botulinum toxin; Receptor; Synaptotagmin; Ganglioside

1. Introduction

Clostridium botulinum neurotoxin acts preferentially on nerve endings to inhibit neurotransmitter release [1]. The neurotoxin is classified into seven serotypes (A–G), each having a similar structure and mechanism of action [2,3]. They are cleaved by limited proteolysis to yield heavy and light chains held together by a disulfide bond. Toxin action has been proposed to involve a series of events, including binding to a type-specific receptor via the heavy chain, penetration through the membrane by receptor-mediated endocytosis, and the subsequent pH-induced translocation of the light chain into the neuronal cytosol [4]. Recent studies have demonstrated that the light chain is a zinc-dependent endopeptidase and specifically cleaves proteins involved in synaptic vesicle exocytosis [5,6]. Although the binding of the neurotoxin to the presynaptic membrane is an obligatory initial step, the receptor has not clearly identified.

Recently we have purified 58 kDa protein from rat brain synaptosomes, to which type B neurotoxin (BoNT/B) binds only in the presence of ganglioside G_{T1b} or G_{D1a} [7]. Partial amino acid sequences of the 58 kDa protein were identical to those of synaptotagmin [7], an integral membrane protein of synaptic vesicles [8,9]. These results suggest that synaptotagmin associated with gangliosides may be the receptor for BoNT/B. In the present study, we found that recombinant synaptotagmin II associated with gangliosides forms high-affinity toxin binding site located in an amino-terminal domain.

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Abbreviations: MEGA-9, nonanoyl-*N*-methylglucamide; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF; polyvinylidene difuluoride; mAb; mouse monoclonal antibody; ELISA; enzyme-linked immunosorbent assay; ABTS, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

2. Materials and methods

2.1. Materials

BoNT/B was purified as described [10] and iodinated with Na^{125}I (DuPont-NEN) by the chloramine-T method [11]. The specific activity of [^{125}I]BoNT/B was 3 to 4 mCi/mg of protein, and the residual toxicity was about 80% of that of the unlabeled toxin before labeling. The heavy and light chains of BoNT/B were separated as described [12]. Rat brain synaptosomes were prepared as described [7]. The 58-kDa protein receptor for BoNT/B was purified from the synaptosomes [7]. Rabbit polyclonal antibodies against a synthetic peptide corresponding to amino-terminal 20 amino acid residues of rat synaptotagmins I and II (PAbs Stg1N and Stg2N) were generated as described [13]. Gangliosides, G_{M1}, G_{M3}, G_{D3}, G_{D1a}, G_{D1b}, and G_{T1b} were purchased from Iatron Laboratories. A ganglioside mixture was prepared from mouse brains [14].

2.2. Preparation of recombinant synaptotagmin

Recombinant rat synaptotagmin expression plasmid was used to transform *E. coli* BL21(DE3)pLysS [7]. The recombinant proteins were prepared by solubilization with 20 mM MEGA-9 (Dojindo) from inclusion bodies after induction with isopropyl-1-thio-β-D-galactopyranoside. The amount of recombinant synaptotagmin I and II in the MEGA-9 extract was estimated by scanning and integrating after SDS-PAGE and Coomassie blue staining (~6% for synaptotagmin I and ~10% for synaptotagmin II).

2.3. BoNT/B binding experiments

The solubilized recombinant synaptotagmin was incorporated into phosphatidylcholine lipid vesicles by an acetone precipitation method as described previously [7,15]. The binding of [^{125}I]BoNT/B to the reconstituted lipid vesicles and synaptosomes was measured by a filtration assay as described [15]. Scatchard analysis of the binding data was performed by the computer program SP123 [16].

2.4. Dot-blot assay

Individual ganglioside (0.5 μg of NeuAc in 1 μl) dissolved in methanol was spotted on PVDF membrane according to the method of Chabraoui et al. [17]. The membranes were washed with HBS (3 mM HEPES-NaOH buffer, pH 7.0, containing 0.12 M NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) and blocked with HBS containing 5% skimmed milk. The membranes were then treated for 2 h with the recombinant synaptotagmin (10 μg of protein/ml) diluted in HBS containing 1% skimmed milk and 10 mM MEGA-9. After washing, the synaptotagmin which had bound to gangliosides was reacted with mAb and subsequently with goat anti-mouse IgG conjugated with alkaline phosphatase. The immunoreactive spots were visualized with the ProtoBlot Color Development System (Promega). In order to determine whether BoNT/B reacts directly to the complex of synaptotagmin and ganglioside, the membranes pretreated with synaptotagmin were incubated with [^{125}I]BoNT/B (1 nM) for 1 h. The membranes were washed and exposed to Kodak X-Omat film with an intensifying screen at −80°C.

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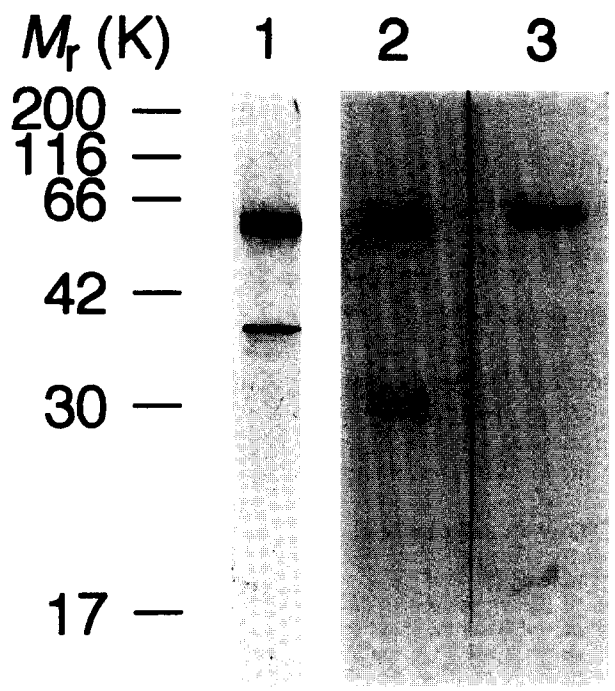

Fig. 1. Detection of synaptotagmins I and II in purified BoNT/B receptor. The purified BoNT/B receptor was subjected to SDS-PAGE (1 μg/lane, 12.5% gel) under reducing conditions followed by silver staining (lane 1) and by immunoblotting probed with antibodies against synaptotagmins I (PAb Stg1N, lane 2) and II (PAb Stg2N, lane 3). The positions of molecular mass standards are shown on the left.

2.5. Preparation of monoclonal antibodies

A synthetic peptide (MRNIFKRNQEPIVAPATTTA-(C)) corresponding to the N-terminus of rat synaptotagmin II was coupled to keyhole limpet hemocyanin and used for the immunization of BALB/c mice. Hybridomas were generated as described [18]. Antibody production by hybridomas was examined by ELISA with the synthetic peptide. A single hybridoma line (8G2b) was established.

A mAb, designated as ST209, against recombinant synaptotagmin II was prepared as before [7]. Prior to immunization, the recombinant synaptotagmin II was partially purified by immunoaffinity chromatography on CNBr-Sepharose 4B coupled with mAb 8G2b. Antibody production by hybridomas was examined by ELISA [19] with the synthetic peptide and immunoblotting.

The mAbs were purified from the ascitic fluid by affinity chromatography on Affi-Gel Protein A (Bio-Rad). The subclasses of the mAbs 8G2b and ST209 were G_1 and G_{2a}, respectively.

2.6. Epitope mapping with synthetic peptides

Overlapping octapeptides of synaptotagmin II were synthesized covalently bound to plastic pins with the Multipin Peptide Synthesis System (Chiron Mimotopes) according to the manufacturer's instructions. The solid phase-linked peptides were incubated with mAbs and the binding was detected by ELISA using rabbit anti-mouse IgG conjugated with horseradish peroxidase (Zymed) and ABTS as substrate.

2.7. Other methods

SDS-PAGE was performed by the method of Laemmli [20]. For immunoblotting, samples were transferred to PVDF membrane [21] and immunoreactive bands were detected as described [7]. Protein was measured by the method of Bradford [22] using bovine γ-globulin as a standard. The quantity of gangliosides was defined by the amount of NeuAc, determined by the method of Svennerholm [23].

3. Results

Immunoblotting of the purified BoNT/B receptor with isoform specific antibodies, indicated that the 58-kDa protein band contained not only synaptotagmin I but also synaptotagmin II (Fig. 1). In order to examine whether BoNT/B really interacts with synaptotagmin I and II, the binding assay was performed using the solubilized recombinant rat synaptotagmin I or II expressed in *E. coli*. Recombinant synaptotagmins showed toxin-binding activity only when ganglioside mixture was incorporated together into lipid vesicles (Fig. 2A). BoNT/B binding was also observed on the lipid vesicles in the presence of gangliosides G_{T1b} and G_{D1a}, but not the other gangliosides (G_{M1}, G_{M3}, G_{D3}, and G_{D1b}) (data not shown). [^{125}I]BoNT/B bound to recombinant synaptotagmin I and II incorporated into lipid vesicles in the presence of a ganglioside mixture in a concentration-dependent and saturable manner (Fig. 2A). No

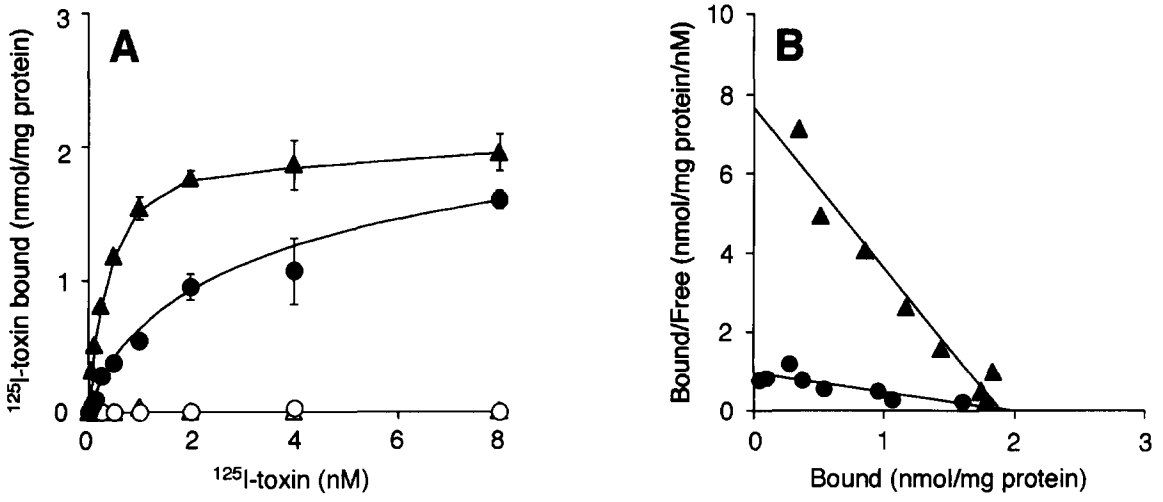

Fig. 2. Binding of [^{125}I]BoNT/B to recombinant rat synaptotagmin associated with gangliosides. (A) Dose dependence of [^{125}I]BoNT/B binding to synaptotagmin I (circles) and II (triangles) incorporated into lipid vesicles with (closed symbols) or without (open symbols) gangliosides. The solubilized recombinant synaptotagmin (60 ng protein) reconstituted into lipid vesicles with or without a ganglioside mixture (15 ng NeuAc) was incubated at 37°C for 1 h with increasing concentrations of [^{125}I]BoNT/B. Specific toxin binding per mg of synaptotagmin was plotted after correction for nonspecific binding measured in the presence of a 1,000-fold excess of unlabeled toxin. Values are the mean ± S.E. from three experiments. Error bars smaller than the symbols were omitted. (B) Scatchard plot of the binding data in the presence of gangliosides shown in A.

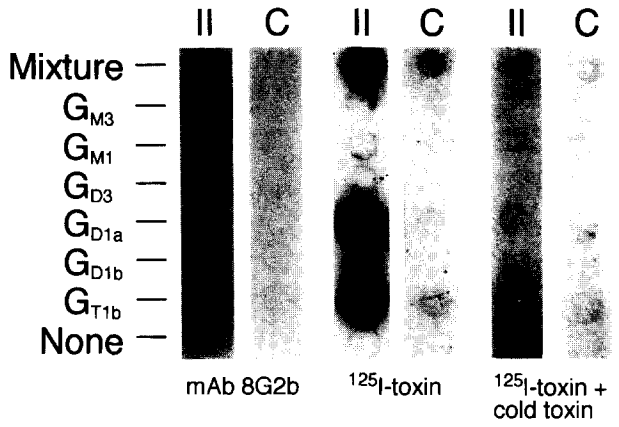

Fig. 3. Binding of [^{125}I]BoNT/B to the complex of synaptotagmin and gangliosides formed on PVDF membranes. After treatment of gangliosides (0.5 μg of NeuAc) spotted on the membranes with synaptotagmin II (panel II) for 1 h at room temperature, [^{125}I]BoNT/B (1 nM) was incubated for 1 h in the absence (middle) or presence (right) of excess of unlabeled toxin. The synaptotagmin which had bound to gangliosides was probed with mAb 8G2b (left). The MEGA-9 extract of *E. coli* transformed with vector (pET-3a) alone was used as a control (panel C).

binding was observed in lipid vesicles containing gangliosides without the recombinant protein (data not shown). Interestingly, Scatchard plot analyses indicated a single class of binding site with the dissociation constants (K_d) of 2.3 and 0.23 nM for synaptotagmins I and II, respectively (Fig. 2B). [^{125}I]BoNT/B binding to the reconstituted lipid vesicles was effectively inhibited by the heavy chain but not by the light chain of BoNT/B (data not shown). Previously, we have demonstrated that the 58-kDa protein fraction and brain synaptosomes contain two classes of binding site for BoNT/B [7,15]. These results suggest that synaptotagmins I and II contribute to the formation of the low- and high-affinity BoNT/B binding sites, respectively.

In order to confirm that the synaptotagmin/ganglioside complex could be recognized by BoNT/B without reconstitution into lipid vesicles, the direct interaction of the neurotoxin and synaptotagmins with gangliosides on PVDF membranes was examined (Fig. 3). Synaptotagmin II bound to all the gangliosides tested, suggesting that there was no restricted selectivity. However, [^{125}I]BoNT/B was found to react only with spots of the ganglioside mixture, G_{D1a}, and G_{T1b} which had been pretreated with synaptotagmin II. The radioactive spots were not observed in the presence of an excess of unlabeled toxin, suggesting that the interaction of [^{125}I]BoNT/B to the complex of synaptotagmin and ganglioside was specific.

The two mAbs (8G2b and ST209) obtained in this experiment specifically reacted with rat synaptotagmin II in immunoblots and with the synthetic amino-terminal 20 amino acid peptide in the ELISA (data not shown). The binding of [^{125}I]BoNT/B to synaptosomes was inhibited by mAb ST209 in a concentration dependent manner, whereas mAb 8G2b showed no inhibitory effect (Fig. 4). These results suggest that BoNT/B associates with the amino-terminal region of synaptotagmin II, probably within 20 amino acids from the amino-terminus. These results predict that mAb ST209 should inhibit [^{125}I]BoNT/B binding only to the high-affinity binding site in synaptosomes. Thus we conducted binding experiments with synaptosomes in the presence of the concentration (50 μg/ml) of mAb ST209 that produced 50% inhibition of the total binding. The capacity of the high affinity binding decreased to about 50% while the K_d was not modified. Neither of the capacity nor the K_d with the low affinity binding sites was affected by this treatment (Fig. 5). To delineate the respective epitopes of mAbs 8G2b and ST209, we designed 18 successive octapeptides each offset by one amino acid residue from Arg^2 to Pro^{26} and examined their immunoreactivities with mAbs 8G2b and ST209. As shown in Fig. 6, the reactivities of the two mAbs with their synthetic octapeptides overlapped partially. However, mAb ST209 strongly reacted with the peptides containing the sequence ^{9}QEPIVA^{14}, whereas mAb 8G2b bound preferentially to the peptides having the sequence ^{7}RNQE^{10}. The results indicate that the epitope recognizing mAb ST209 is located close to that of mAb 8G2b in the amino terminal region of synaptotagmin II.

4. Discussion

BoNT acts through a series of events, the first of which is the binding to cell surface receptors. The identity of these receptors and their role in neuronal function have not been clearly demonstrated. We have previously obtained evidence that the protein receptor for BoNT/B may be synaptotagmin I [7]. The present data indicate that not only synaptotagmin I but also synaptotagmin II interacts with BoNT/B in the presence of gangliosides, although they display different binding affinities. The dissociation constants of synaptotagmins I and II are comparable to those of the low- and high-affinity binding sites respectively in rat brain synaptosomes, and the 58-kDa protein fraction purified from synaptosomes [7,15,24]. In fact synaptotagmin II was also detected in the 58-kDa protein fraction by immunoblotting. Thus, it is probable that the low-and high-affinity sites in rat synaptosomes correspond to synaptotagmins I and II. Direct binding experiments with gangliosides spotted onto membranes revealed that BoNT/B only binds to

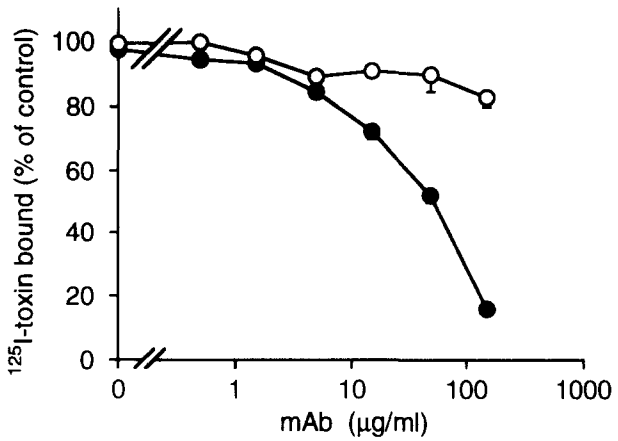

Fig. 4. Inhibition of [^{125}I]BoNT/B binding to synaptosomes by mAbs recognizing the amino-terminal region of synaptotagmin II. [^{125}I]BoNT/B (0.25 nM) was incubated at 37°C for 1 h with synaptosomes (10 μg of protein) in the presence of mAbs ST209 (●) and 8G2b (○). Specific toxin binding was determined by subtracting the nonspecific binding measured in the presence of excess unlabeled toxin. Each point represents the mean ± S.E. from three experiments. The [^{125}I]BoNT/B bound to synaptosomes in the absence of mAb was considered as 100%.

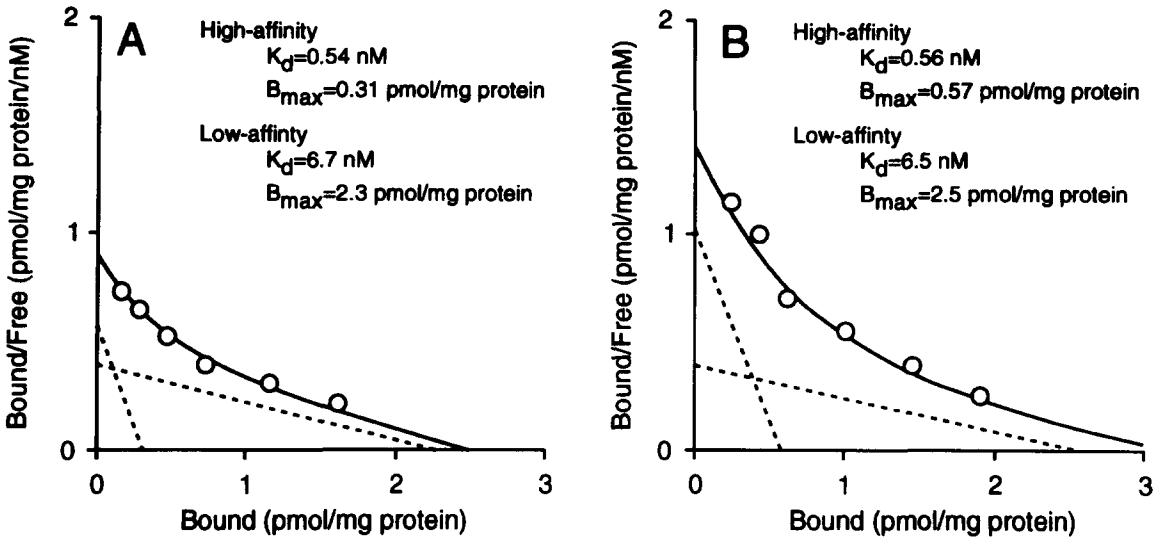

Fig. 5. Effect of mAb ST209 on [^{125}I]BoNT/B binding to the high- affinity site. [^{125}I]BoNT/B binding to synaptosomes was determined in the presence (A) or absence (B) of mAb ST209 (50 μg/ml) as described in Fig. 2. The data are displayed as a Scatchard plot. Each point represents the mean from three experiments. Inset: the K_d and binding capacity (B_{max}) of the toxin binding site.

a complex consisting of synaptotagmin and gangliosides G_{D1a} or G_{T1b}.

Synaptotagmin is highly conserved among a wide range of animal species and considered to be involved in Ca^{2+}-dependent exocytosis of synaptic vesicles at the nerve terminal [25,26]. The homology between synaptotagmin I and II is not uniformly distributed across the molecule, and is highest in the carboxyl-terminal cytoplasmic C_2 domains (88% sequence identity) and lowest in the amino-terminal regions (46% identity) [27]. The lower homology in the amino-terminal regions may confer the

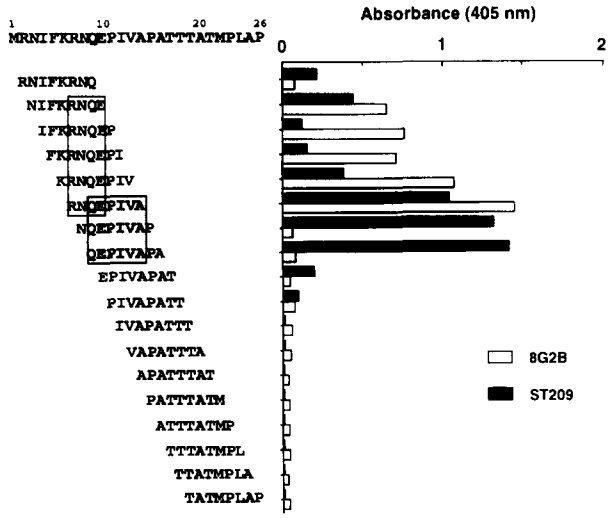

Fig. 6. Immunoreactivity of mAbs ST209 and 8G2b with octapeptides from the amino-terminal region of synaptotagmin II. Overlapping peptides were synthesized in a pin-bound form (amino acid sequences are shown on the left of the figure) were reacted with mAb 8G2b (0.2 μg/ml, open bar) and ST209 (1 μg/ml, closed bar) for 1 h at room temperature. After washing, the bound mAbs were detected with rabbit anti-mouse IgG conjugated with horseradish peroxidase and ABTS as a substrate. The amino acid sequence of the amino-terminal region (residues 1–26) is shown on the top of the figure. The amino acid sequences common to each of the immunoreactive peptides are boxed.

difference in the affinities of the two isoforms for BoNT/B. Synaptotagmin has a single transmembrane region and short amino-terminal domain located in the intravesicular space [28]. However the amino-terminal domain is exposed at the outside of the nerve terminal after synaptic vesicle exocytosis [13], which is compatible with the hypothesis that the amino-terminal region of synaptotagmin constitutes a part of BoNT/B receptor. In order to address the question, we examined the inhibitory effect of anti-synaptotagmin II antibodies on the toxin binding to synaptosomes. Of two mAbs (8G2b and ST209) recognizing 20 amino acid synthetic peptides from the amino-terminus, only mAb ST209 inhibited the toxin binding with high affinity. This mAb was subsequently shown to react with the octapeptides including homologous sequence from Gln^9 to Ala^{14}. These findings support the idea that the amino-terminal region of synaptotagmin II, by association with gangliosides G_{T1b}/G_{D1a}, forms the high-affinity binding site for BoNT/B. The precise structure of the complex of synaptotagmin and gangliosides acting as BoNT/B receptors remains to be established. More recently, cDNA clones encoding other synaptotagmins (III through VIII) have been isolated one after another [29–32]. It is now in progress to determine whether these novel isoforms of synaptotagmin interact with BoNT/B and other BoNTs.

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