Binding of Clostridium botulinum Type C and D Neurotoxins to Ganglioside and Phospholipid

NOVEL INSIGHTS INTO THE RECEPTOR FOR CLOSTRIDIAL NEUROTOXINS

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Clostridium botulinum neurotoxins (BoNTs) act on nerve endings to block acetylcholine release. Their potency is due to their enzymatic activity and selective high affinity binding to neurons. Although there are many pieces of data available on the receptor for BoNT, little attempt has been made to characterize the receptors for BoNT/C and BoNT/D. For this purpose, we prepared the recombinant carboxyl-terminal domain of the heavy chain (HC) and then examined its binding capability to rat brain synaptosomes treated with enzymes and heating. Synaptosomes treated with proteinase K or heating retained binding capability to both HC/C and HC/D, suggesting that a proteinaceous substance does not constitute the receptor component. We next performed a thin layer chromatography overlay assay of HC with a lipid extract of synaptosomes. Under physiological or higher ionic strengths, HC/C bound to gangliosides GD1b and GT1b. These data are in accord with results showing that neuraminidase and endoglycoceramidase treatment decreased HC/C binding to synaptosomes. On the other hand, HC/D interacted with phosphatidylethanolamine but not with any ganglioside. Using cerebellar granule cells obtained from GM3 synthase knock-out mice, we found that BoNT/C did not elicit a toxic effect but that BoNT/D still inhibited glutamate release to the same extent as in granule cells from wild type mice. These observations suggested that BoNT/C recognized GD1b and GT1b as functional receptors, whereas BoNT/D induced toxicity in a ganglioside-independent manner, possibly through binding to phosphatidylethanolamine. Our results provide novel insights into the receptor for clostridial neurotoxin.

Seven types of Clostridium botulinum strains (A through G) are distinguished by differences in the antigenic specificity of their pharmacologically similar neurotoxins. Botulinum neurotoxins (BoNTs) are synthesized as single chain peptides with a molecular mass of ~150 kDa that are proteolytically activated into a light chain (L-chain; 50 kDa) and a heavy chain (H-chain; 100 kDa) linked by a disulfide bond. It has been possible to assign some functional activities to certain domains of BoNT. The L-chain acts as a zinc-dependent endopeptidase and cleaves soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein families (1, 2), whereas upon the Ca2+-triggered fusion of a synaptic vesicle with the presynaptic membrane is disrupted. BoNT/B, BoNT/D, BoNT/F, and BoNT/G specifically cleave vesicle-associated membrane protein (VAMP), a membrane protein of small synaptic vesicles, at different single peptide bonds. The other BoNTs attack specific proteins of the presynaptic membrane. BoNT/A and BoNT/E cleave the 25-kDa synaptosome-associated protein (SNAP-25) at two different sites located within the carboxyl terminus (1), whereas the specific target of BoNT/C is syntaxin. BoNT/C may also cleave the carboxyl terminus of SNAP-25 (3). The H-chain is composed of two remaining domains and serves as the vehicle that delivers the L-chain into the cytosol of neuronal cells. Therefore, the extreme toxicity has to be largely ascribed to the specific binding to nerve terminals at the neuromuscular junction. The amino-terminal domain of the H-chain (HN) is responsible for translocating the L-chain from the lumen of an acidic intracellular compartment into the cytosol subsequent to cell binding and receptor-mediated endocytosis. The carboxyl-terminal domain of the H-chain (HC) exhibits highly selective binding for neurons, in particular for those of the cholinergic system (4). The receptor for BoNT has not been fully identified. Current evidence suggests that the receptors are composed of a certain specific ganglioside and of proteins that cooperate to form high affinity toxin-binding sites. Previously, we demonstrated the first time that BoNT/B binds specifically to synaptotagmins I and II in the presence of gangliosides GT1b and GD1a (5–7). These proteins are homologous synaptic vesicle membrane proteins thought to function as Ca2+ sensors for exocytosis (8). Since then, reliable data that synaptotagmin is a functional receptor protein for BoNT/B have accumulated (9). Furthermore, BoNT/G was found to interact with synaptotagmin in the absence of ganglioside (10).

In contrast to human botulism mainly involving BoNT/A, BoNT/B, and BoNT/E, BoNT/C and BoNT/D are causative agents for animal and avian botulism (11). Although BoNT/C and BoNT/D genes of several strains have been sequenced, these data appeared to be rather complicated. Some type C strains possess a unique BoNT gene structure that comprises two thirds of the BoNT/C gene and one third of the BoNT/D gene corresponding to the HC portion (12, 13). Recently, we described how the gene for the mosaic form of BoNT is harbored in the isolates related to avian botulism, whereas the authentic BoNT/C may be implicated in the disease affecting mammals, including cattle, mink, and...
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protease inhibitor mixture (Sigma). After treatment with 0.1 mg/ml lysosome for 1 h at 4 °C, the cells were solubilized on ice by sonication and centrifuged at 27,000 g for 15 min at 4 °C. The supernatant was subjected to puromycin-resistant tagged H₃ with a nickel-nitriolactoferrin Superflow column (Qiagen, Chatsworth, CA) as recommended by the instruction manual. The recombinant H₃ was further purified with an anion exchange column, Mono-Q HR 5/5 (Amersham Biosciences).

**Binding of Neurotoxins to Synaptosomes**—The purified recombinant H₃ and BoNT were radioiodinated with Na¹²⁵I (PerkinElmer Life Sciences) by the chloramine-T method as described previously (25). The specific activities of H₃ and BoNTs were 13–17 μCi/μg protein (26.0–34.0 MBq/nmol). After iodination, the ¹²⁵I-BoNTs retained >80% of that of unlabeled BoNTs. Synaptosomes were prepared from rat brain (26) and suspended in 3 mM HEPES-NaOH buffer (HBS), pH 7.0, containing 120 mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, and 2 mM CaCl₂. The binding of ¹²⁵I-H₃ to synaptosomes was measured by filtration assay in the presence or absence of unlabeled H₃ or BoNT (27). The concentration giving 50% inhibition (IC₅₀) was calculated using GraphPad Prism software (GraphPad Software, San Diego, CA).

**Enzyme Treatments**—Synaptosomes were solubilized with HBS containing 15 mM N-ethyl-N-β-d-thioglycolate. Then, the solubilized synaptosomes (20 μg/ml protein) were treated at 37 °C for 30 min with 100 milliunits/ml endoglycoceramidase II (Takara Shuzo Co., Ltd., Tokyo, Japan), 100 milliunits/ml neuraminidase (Seikagaku Corp., Tokyo, Japan), and 10 μg/ml proteinase K (Wako, Osaka, Japan) or boiled for 10 min. After treatment, the remaining toxin binding activity of the solubilized synaptosomes to ¹²⁵I-H₃ was determined by filter-absorbance assay. In brief, the solubilized synaptosomes (100 μl) were applied to absorb on MultiScreen-HA plates (Millipore Corp., Bedford, MA) for 1 h at 37 °C. After filtration, the filters were blocked with HBS and 1% BSA. After washing three times with HBS and 0.5% BSA, 0.2 ml of ¹²⁵I-H₃ (0.5 nM) in HBS plus 0.5% BSA was added to each well and incubated at 37 °C for 1 h. Finally, the filters were washed six times with 0.25 ml of chilled HBS and 0.5% BSA. The radioactivity retained on the filter membrane was measured using a γ-counter (GMI Inc., Ramsey, MN).

**TLC Overlay Assay**—Lipids were extracted from lyophilized synaptosomes by the method described previously (28). Briefly, lyophilized synaptosomes were incubated with chloroform/methanol/water (20:10:1, 10:20:1, and 1:1 v/v), the volume of combined extracts being adjusted with chloroform/methanol (1:1 v/v). Extracted lipids (corresponding to 20 ηg of dry tissue weight), gangliosides (GM₅, GD₁, GT₁a, GD₂, GD₃, GD₁b, GT₁b, GQ₁b, and GT₃; 0.5 nM each), and phospholipids (phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylglycerol and phosphatidylinositol; 1 nmol each) were chromatographed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany) in chloroform/methanol/water (5:4:1 v/v) for synaptosomal lipids and gangliosides or 65:25:4 v/v for phospholipids. The TLC plates were then dried and monitored for separation with resorcinol (for gangliosides) or molybdenum blue (for phospholipids). For the overlay assay, the replica plates were blocked with blocking One (Nacalai Tesque, Kyoto, Japan) containing 1% polyvinylpyrrolidone at 4 °C for 12 h. After blocking, the plates were incubated with 1 nM ¹²⁵I-H₃ for 2 h at room temperature. After washing three times in HBS to remove non-bound H₃, the plates were dried in air and exposed to an imaging plate (LAS-3300; Fuji Photo Film, Tokyo, Japan). The H₃ binding lipids were detected with a FLA-3000 system (Fuji Photo Film).

**EXPERIMENTAL PROCEDURES**

**Purification of Neurotoxins—C. botulinum** type C strains CB-19, 003-9, and type D strain 1873 were used for the purification of neurotoxins (BoNT/CB-19, BoNT/003-9, and BoNT/1873 respectively). BoNT/CB-19 was reported to produce a typical type C toxin, whereas BoNT/003-9 consists of a mosaic form of type C and D neurotoxins (C/D mosaic) (Fig. 1A). BoNTs were purified principally according to the method of Kurazono et al. (22). Toxicity was assayed by the time-to-death method by intravenous injection into mice (23). Samples were also titrated by intraperitoneal injection into mice with serial 2-fold dilutions to obtain a mean 50% lethal dose (LD₅₀) by the calculations of Reed and Muench (24).

**Plasmid Constructions and Recombinant Proteins**—DNA fragments encoding the H₃ (H₃/CB-19, amino acids 863–1291; H₃/003-9, 863–1280; and H₃/1873, 859–1276) of BoNTs were amplified with PCR using the primers 5'-CATGCCATGGCTGAATATTTCAATAGTATTAATGATTCA-3' (reverse) and 5'-CCCAAGCTTTTATTCACTTACAGGTACAAAACC-3' (forward) for H₃/CB-19 and 5'-CATGCCATGGCTGAATATTTCAATAGTATTAATGATTCA-3' (forward) and 5'-CCCAAGCTTTTATTCACTTACAGGTACAAAACC-3' (reverse) for H₃/003-9 and H₃/1873, where the Ncol and HindIII sites were included at the ends of the forward and reverse primers, respectively. These primers were designed based on the respective DNA sequences in GenBank (accession numbers AB200358 for BoNT/CB-19, AB200360 for BoNT/003-9, and AB012112 for BoNT/1873). After digestion with NcoI and HindIII, the PCR products were ligated into NcoI- and HindIII-digested pET-30a vector (Novagen, Madison, WI) and verified by DNA sequencing. The recombinant plasmids were introduced into Escherichia coli BL21 CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). Expression of recombinant H₃ was performed according to the pET system manual (Novagen). Cultures were grown at 37 °C in 100 ml of Luria-Bertani broth containing 25 μg/ml kanamycin until the optical density at 600 nm reached 0.5. After the addition of a 50 μM final concentration of isopropyl-β-d-thiogalactoside, growth was continued at 25 °C for an additional 24 h. The cells were collected and suspended in 4 ml B-PER bacterial protein extraction reagent (Pierce) containing a...
Preparation of Cerebellar Granule Neurons—Following the decapitation of 6–9-day-old C57/BL6J mice, the cerebella were removed and transferred immediately into ice-cold Ca²⁺- and Mg²⁺-free Dulbecco’s phosphate-buffered saline containing 5.6 mM glucose. After eliminating the meninges, the tissues were cut into 1-mm pieces and incubated in phosphate-buffered saline containing 0.25% (w/v) trypsin, 28 mM glucose, and 0.05% DNase for 20 min at 37 °C, with occasional agitation. After the removal of trypsin by brief centrifugation, the tissue was mechanically dissociated by repeated pipetting in Hanks’ balanced salt solution containing 0.25% glucose, 12 mM MgSO₄, and 0.01% DNase. After filtering through a Falcon cell strainer (BD Labware), the cell suspension was collected by centrifugation. The pellet was resuspended in minimal essential medium containing 25 mM K⁺ (HK-MEM) with 5% horse serum and 5% fetal calf serum. The cells were plated on polyethyleneimine-coated four-well culture dishes (Nalge Nunc International, Naperville, IL) at a density of 4.5 × 10⁵ cells/cm² and maintained at 37 °C in 5% CO₂. Between 2 and 4 days the medium was replaced with HK-MEM and 2% B-27 supplement (Invitrogen), 50 units/ml penicillin, 100 µg/ml streptomycin, and 5 µM cytosine arabinofuranoside to inhibit the replication of non-neuronal cells, and, thereafter, no further medium change was carried out before the experiment.

Exposure to BoNT and Assay of Glutamate Release—In the experiments to examine the effects of BoNT on glutamate release various concentrations of BoNT (4 µl) were added to cultures (400 µl) at 7 days and incubated at 37 °C for 18 h. BoNT-treated cerebellar cells were washed with pre-warmed low K⁺ solution (10 mM HEPES-NaOH, pH 7.4, containing 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgSO₄, and 10 mM glucose), and the extracellular solution was changed every 2 min. After changing the low K⁺ solution three times, the K⁺ concentration was elevated from 5 to 50 mM to depolarize the cells (29). The glutamate content of the extracellular solution was determined by reverse-phase high performance liquid chromatography on a Mighysil RP-18 GP 150-4.6 column (Kanto Kagaku, Tokyo, Japan) using precolored derivatization with o-phthalaldehyde and fluorescence detection (FP-2020 Plus, Jasco, Tokyo, Japan).

Immunoblotting Detection of BoNT-cleaved Intracellular Substrates—BoNT-treated cerebellar granule cells were collected and solubilized with SDS sample buffer (0.1 M Tris–HCl, pH 6.8, containing 2% SDS, 4% glycerol, and 0.01% bromphenol blue). The samples were subjected to SDS-PAGE (12.5% acrylamide gels) and immunoblotting. The antibodies against syntaxin or VAMP-2 were obtained according to a method described elsewhere (5, 31). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Biosciences). The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 and then treated with 10 µg/ml rabbit anti-VAMP-2 antibody and mouse anti-syntaxin monoclonal antibody followed by 5 µg/ml goat anti-rabbit (or mouse) IgG conjugated with alkaline phosphatase (Bio-Rad). The reactive bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution (Promega, Madison, WI).

Others—Purified gangliosides (GM1a, GD1a, GT1a, GD2, GD3, GD1b, GT1b, GQ1b, and GT3) were kindly provided by Masao Iwamori, Faculty of Science and Technology, Kinki University. The gangliosides were generated from C57BL/6J mice according to a previously reported method (35). The neomycin resistance gene was inserted in the position of the second exon of the seven-exon GM3 synthase gene. The mutant mice were unable to synthesize GM3 ganglioside, a simple and widely distributed glycosphingolipid. The mutant mice were viable and appeared without major abnormalities. Other detailed characteristics of the mutant mice will be reported elsewhere.

RESULTS

Binding of Recombinant Hc to Rat Brain Synaptosomes—The recombinant proteins (Hc/CB-19, Hc/003-9, and Hc/1873) were successfully obtained in a pure state (Fig. 1B). In the binding experiments we conducted an examination of the binding capability of [125I]-Hc in the presence of unlabeled homologous and heterologous Hc, or BoNT (Fig. 2). The binding of [125I]-Hc/CB-19 was effectively inhibited in the presence of homologous Hc, and the presence of BoNT/CB-19 to the same extent, whereas Hc/003-9 and Hc/1873 had little inhibitory effect on the binding. The binding of [125I]-Hc/CB-19 was completely out-competed by both unlabeled Hc/003-9 and Hc/1873, but not by Hc/CB-19. These data indicate that the recognition site of Hc/003-9 and Hc/1873 is the same, whereas that of Hc/CB-19 is different. When compared with the IC₅₀ of the BoNTs, 003-9/BoNT appeared to possess a binding capability higher than that of BoNT/1873. BoNT/CB-19 showed a binding activity lower than did BoNT/003-9 and Hc/1873. To examine the properties of their Hc binding substrances, we conducted some experiments with enzyme- and heat-treated synaptosomes for the binding of recombinant Hc (Fig. 3). In Hc/CB-19, a drastic decrease of the binding was observed in enzyme treatments with endoglycosidase II and neuraminidase, whereas neither proteinase K nor heat treatment affected the binding. These results may suggest that BoNT/CB-19 requires a sialic acid moiety in binding to synaptosomes, but not a proteinaceous substance. In type D binding with Hc/003-9 and Hc/1873, from Japan SLC, Inc. (Shizuoka, Japan). GM3 synthase knock-out mice were generated from C57BL/6J mice according to a previously reported method (35). The neomycin resistance gene was inserted in the position of the second exon of the seven-exon GM3 synthase gene. The mutant mice were unable to synthesize GM3 ganglioside, a simple and widely distributed glycosphingolipid. The mutant mice were viable and appeared without major abnormalities. Other detailed characteristics of the mutant mice will be reported elsewhere.
there was no significant decrease in their binding after any treatment. These observations indicate that the receptors for BoNT/C and H₄/D possess characteristics different from those for type A and B, because their toxin binding to brain synaptosomes was inhibited by treatment with neuraminidase (36, 37) and boiling (data not shown). It is probable that the binding substance for BoNT/D does not involve a sialic acid moiety.

**Binding of Recombinant H₄ to Gangliosides and Phospholipids**—In the preliminary experiments, we found that BoNT/C and BoNT/D could bind to lipids. There is little available data on the interaction of BoNT/C and BoNT/D with gangliosides and phospholipids. In the binding experiments with lipids, we performed a TLC overlay assay in the presence of 0.3 M NaCl because a nonspecific reaction was observed in the presence of 0.15 M NaCl or low concentrations. H₄/CB-19 bound to gangliosides GD₁b and GT₁b but not to other gangliosides or to any phospholipids. On the other hand, H₄/003-9 and H₄/1873 recognized PE, but not other phospholipids or any gangliosides (Fig. 4A). Analysis of brain gangliosides in the GM3 synthase knock-out mice demonstrated a pattern consistent with an absence of GM3 synthase (Fig. 4B, two left sections). The major brain gangliosides present in wild type mice (GM₁a, GD₁b, and GT₁b) were absent in the knock-out mice. However, the GM3 synthase knock-out mice expressed major ganglioside species that comigrated with GM1b and GD1a gangliosides of the α-series, which do not require the activity of GM3 synthase (38). We then performed similar experiments using lipid extracts from mouse brain to...
confirm the HC binding to lipids. As shown in Fig. 4B, three right sections, H/C/CB-19 bound to two kinds of lipids showing different migration distances on TLC. The migration positions of these lipids corresponded to those of GD1b and GT1b, respectively. HC/003-9 and HC/1873 bound only to a lipid whose migration position in TLC was identical to that of PE. In addition, to elucidate the binding property of HC/003-9 and HC/1873, we determined the degree of binding to saturated fatty acids with different chain lengths of 12 to 20 carbons and found that diphytanoyl PE showed the strongest affinity with HC/003-9 (Fig. 5).

**Inhibition of Exocytosis in Mouse Cerbellar Granule Cells**—To functionally evaluate the binding, we next examined the effects of BoNTs on the exocytotic release of glutamate from cerebellar granule cells prepared from GM3 synthase knock-out mice. Cerebellar granule cells release substantial amounts of glutamate in response to high K⁺ treatment. In the cerebellar granule cells from wild type mice, BoNT/CB-19, BoNT/003-9 and BoNT/1873 inhibited evoked glutamate secretions in a dose-dependent manner (Fig. 6). When cerebellar granule cells from GM3 synthase knock-out mice were subjected to an examination of the effects of BoNTs, the toxic effects of BoNT/003-9 and BoNT/1873 were observed. In contrast, BoNT/CB-19 did not induce a concentration-dependent inhibition of glutamate release in the cells from knock-out mice. The content of syntaxin and VAMP-2 in cerebellar granule cells was detected by immunoblotting (Fig. 7). In the cells from wild type mice, the amount of syntaxin gradually decreased upon the addition of BoNT/CB-19 and BoNT/003-9 in a dose-dependent manner. Cleavage of VAMP-2 by BoNT/1873 was also observed. In the cells from the knock-out mouse, BoNT/003-9 affected the hydrolysis of syntaxin, and BoNT/1873 was shown to decrease the amount of VAMP-2. However, BoNT/CB-19 did not cause syntaxin to be cleaved in the cells from knock-out mice.

**Sensitivity of Knock-out Mice to BoNTs**—Moreover, for the purpose of evaluation of the binding to gangliosides in vivo, we analyzed the toxicity of BoNTs against GM3 synthase-deficient mice. The toxicity of BoNT/CB-19, BoNT/003-9, and BoNT/1873 against ddY mice was determined by intraperitoneal injection into death between 2.1 × 10⁷, 2.6 × 10⁸, and 3.3 × 10⁸ intraperitoneal LD₅₀ per mg of protein. The lethalities of the three BoNTs were also determined by intraperitoneal injection into C57BL/6J mice. The specific toxicities of the three BoNTs were equivalent to those obtained in ddY mice. There was no difference in the relationship between the dose in intraperitoneal LD₅₀ and the survival time with intravenous injection to death between ddY and C57BL/6J mice. When BoNT/CB-19 was injected intravenously into the knock-out mice at an appropriate dose, the survival time was prolonged and the value was decreased to 0.7% of that of the wild type mice, whereas BoNT/003-9 and HC/1873 were still toxic to the knock-out mice and the dose (intraperitoneal LD₅₀) estimated by the survival time was 70% of that of the wild type mice (TABLE ONE).

**DISCUSSION**

The intracellular action of BoNT has been somewhat elucidated at the molecular level. BoNT elicits endoprotease activity in the cell cytosol to specifically cleave proteins of the exocytotic apparatus, thereby blocking neurotransmitter release. In comparison with such intracellular events, the mechanism of receptor recognition remains obscure. In fact, many attempts have been made to identify the receptor for BoNTs, but there are only a few pieces of data reliable for BoNT/B and BoNT/G (5, 9, 10). In this study we characterized the binding of the botulinum type C and D neurotoxins. Our study had major three findings. First, both BoNT/C and BoNT/D did not require proteinaceous components for their binding to the synaptosome. Second, BoNT/C specifically bound to gangliosides GD1b and GT1b under physiological conditions, which is functionally crucial for their toxicity. Third,
BoNT/D specifically bound to PE under physiological conditions, which was in a hydrocarbon tail-dependent manner. We surveyed the binding capability of BoNT/C and BoNT/D to the solubilized fraction from rat brain synaptosomes. Protease treatment and boiling were ineffective in decreasing the toxin binding capability for BoNT/C and BoNT/D, indicating that their receptors were not involved in the proteinaceous property. In fact, the TLC overlay assay revealed that HC/C (CB-19) effectively bound to gangliosides GD1b and GT1b and that HC/D (003-9 and 1873) bound to PE in the presence of 0.3M NaCl. It has been reported that BoNT/A and BoNT/B bound directly to a certain gangliosides, but the binding was strongly affected in the presence of NaCl (17–19). In the in vivo experiments, we confirmed that the knock-out mice were more resistant to BoNT/C than wild type mice because the knock-out mice did eventually succumb to the toxin, but they were still sensitive to BoNT/D. These observations, together with the results of in vitro binding experiments, indicated that GD1b and GT1b were major functional components of the receptor for BoNT/C. It is possible, although perhaps unlikely, that another ganglioside might be the functional receptor. These results also support a notion that BoNT/C and BoNT/D possess quite different properties in regard to ganglioside dependence.

To clarify ganglioside dependence in the binding of BoNT/C and BoNT/D, we examined their toxic effects with cerebellar granule cells from GM3 synthase knock-out mice. In the mice, complex gangliosides including GD1b and GT1b were absent in the extract of brain synaptosomes. As expected, treatment of BoNT/C on the granule cells from the knock-out mice did not cause the reduction of the K⁺-evoked glutamate release. On the other hand, C/D mosaic toxin and BoNT/D still elicited an inhibitory effect on glutamate release in the granule cells from knock-out mice to the same extent as in those from wild type mice. These results suggested that gangliosides require an essential receptor component for BoNT/C, as well as the other type of BoNTs, whereas type D toxin induces toxicity to target cells in a ganglioside-independent manner. The ganglioside dependence on the binding of BoNT/C to neural cells seems to be higher than the other type of BoNTs, because binding of the other BoNT to gangliosides was affected in the presence of NaCl (17–19). In the in vivo experiments, we confirmed that the knock-out mice were more resistant to BoNT/C than wild type mice because the knock-out mice did eventually succumb to the toxin, but they were still sensitive to BoNT/D. These observations, together with the results of in vitro binding experiments, indicated that GD1b and GT1b were major functional components of the receptor for BoNT/C. It is possible, although perhaps unlikely, that another ganglioside might be the functional receptor. These results also support a notion that BoNT/C and BoNT/D possess quite different properties in regard to ganglioside dependence.

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FIGURE 6. Effects of BoNT on depolarization-evoked glutamate release from cultured cerebellar cells. Cerebellar granule cells cultured for 7 days in vitro. The cells were pretreated with increasing concentrations of BoNT/CB-19 (open circle), BoNT/003-9 (open square), or BoNT/1873 (open triangle) at 37 °C for 18 h. After a brief washing, the cells were incubated in a low K⁺ (5 mM) solution and then in the high K⁺ (50 mM) solution for 2 min each. Glutamate (Glu) content was determined by reverse phase high performance liquid chromatography using precolumn derivatization with o-phthalaldehyde and fluorescence detection.

FIGURE 7. BoNT-induced hydrolysis of syntaxin and VAMP-2 in cerebellar granule cells. Cerebellar granule cells were exposed for 18 h in the presence of increasing concentrations of BoNT. The cells were then washed and solubilized. The samples were subjected to SDS-PAGE and immunoblotting. After blocking the nitrocellulose membrane, it was treated with 10 μg/ml rabbit anti-VAMP-2 antibody and mouse anti-syntaxin antibody followed by 5 μg/ml goat anti-rabbit (or mouse) IgG conjugated with alkaline phosphatase. The reactive bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution.
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TABLE ONE

| BoNT      | Mouse | Survival timea | Toxicityb | Percentage |
|-----------|-------|----------------|-----------|------------|
|           |       | min            | $\times 10^5$ ip$^3$ LD$_{50}$/ml |            |
| CB-19     | Wild type | 53 ± 2       | 1,040       | 100        |
|           | Knock-out | 312 ± 13     | 7           | 0.7        |
| 003-9     | Wild type | 49 ± 3       | 1,290       | 100        |
|           | Knock-out | 57 ± 5       | 889         | 69         |
| 1873      | Wild type | 48 ± 1       | 3,330       | 100        |
|           | Knock-out | 54 ± 4       | 2,410       | 72         |

a Data are presented as the mean ± S.D. ($n = 3$).

b The toxicity was determined by the time-to-death method by intravenous injection of 0.1 ml of BoNT/CB-19 (5 μg/ml), BoNT/003-9 (5 μg/ml), and BoNT/1873 (1 μg/ml).

Antral Helicobacter pylori (44). The binding of these bacteria to PE is thought to mediate attachment to host cells and subsequent infection. Because PE is a widely distributed phospholipid in the plasma membrane, being primarily on the cytosolic leaflet of the bilayer (45), it might be difficult to function as a neurospecific receptor. From these observations, two points are worthy of consideration. First, PE is predominantly a component of the inner leaflet of the plasma membrane bilayer (46). However, recent studies have revealed that PE moved to outward of membrane mediated by ATP-binding cassette transporters (47). Second, a wide disparity in the binding ability of H. pylori to PE from different sources was observed (40), suggesting that the hydrocarbon tail in PE plays a role in the possible receptor function of PE. In fact, H$_2$/003-9 strongly bound to PE consisting of a longer hydrocarbon tail (C$_{20}$:0), indicating that hydrophobicity is important for BoNT/D binding. Moreover, it was reported that PE composed of a long hydrocarbon tail mainly existed in neurons (48, 49). Ganglioside-independent binding of BoNT/D seems to accord with the results reported by Knight et al. (50). They found that the type D toxin, but not the type C toxin, blocked catecholamine release in a bovine adrenal medullary cell culture.

In this study we provide a new, informative idea on the receptors of BoNT/C and BoNT/D. Our present data cannot exclude the possibility that the representative substances to be defined as type C or type D-specific binding are still present, but such substances must be extremely resistant to protease and heat treatment. The properties of the receptor for BoNT/D as well as that for the C/D mosaic seem to be quite different from those of the other BoNTs. This indicates a critical issue to be resolved in regard to the question as to why BoNT/D and the C/D mosaic elicit a toxic effect on animals and birds specifically, but not on humans.

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Binding of Clostridium botulinum Type C and D Neurotoxins to Ganglioside and Phospholipid: NOVEL INSIGHTS INTO THE RECEPTOR FOR CLOSTRIDIAL NEUROTOXINS

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