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Endocytosis of *Clostridium botulinum* Type B Neurotoxin into Rat Brain Synaptosomes

Tomoko KOHDA[1)]*, Yoichi KAMATA[1)] and Shunji KOZAKI[2)]**

[1)]*Laboratories of Veterinary Public Health and* [2)]*Veterinary Epidemiology, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan*

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ABSTRACT. *Clostridium botulinum* type B neurotoxin cleaves VAMP (vesicle-associated membrane protein)/synaptobrevin into two fragments, which results in inhibition of neurotransmitter release. The induced fragment did not react to the antibody raised against the synthetic peptide of the amino-terminal 20 residues of VAMP-2, suggesting that the toxin treatment has caused antigenical alteration in the amino-terminal region of VAMP-2. In rat brain synaptosomes, type B neurotoxin was reduced presumably with sulfhydryls in the membrane and detected in the synaptic vesicle fraction which involved the degradation of VAMP-2 and the inhibition of neurotransmitter release. The light chain in a free form was present in the cytosol fraction. These findings suggest a possibility that type B neurotoxin endocytoses into synaptic vesicles by the recycling pathway and the light chain is penetrable through synaptic vesicle membrane. However, the amount of type B neurotoxin entrapped into synaptic vesicles appears to be extremely small, which may be attributed to a lower sensitivity of the toxin to brain synaptosomes than to peripheral nerve endings.

KEY WORDS: *Clostridium botulinum*, endocytosis, neurotoxin, synaptosome, VAMP.

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Clostridium botulinum produces a highly potent neurotoxin which causes flaccid paralysis. The neurotoxin (BoNT), which is serologically classified into seven types A through G, is released from the bacteria as a single polypeptide chain of 150 kDa [24, 29]. It is subsequently activated by an endogenous bacterial or digestive protease to generate nicked toxin, in which the light chain (50 kDa) remains linked to the heavy chain (100 kDa) by a disulfide bond [29]. It has been proposed that the action of BoNT involves the following steps; binding of the toxin to the type-specific receptor on the presynaptic membrane, sequent internalization, and translocation into the cytosol, where the light chain acts by inhibiting neurotransmitter release [28]. Recent studies have reported that the light chains of all types exhibit protease activities toward one of the three neural proteins such as VAMP (vesicle-associated membrane protein) /synaptobrevin, syntaxin/HPC-1, and SNAP-25 (synaptosomal protein of 25 kDa) [19, 20]. A recent model of synaptic vesicle fusion proposes that the vesicle protein VAMP, termed a v-SNARE (vesicle-SNAP receptor), and the plasma membrane-associated protein syntaxin and SNAP-25, t-SNARE (target-SNAP receptor) form a core complex for the subsequent cascade of protein-protein interaction for exocytosis to occur [7]. Cleavage of these SNAREs by BoNT causes a failure of their precise assembly, suggesting that the formation of the synaptic core complex is essential for synaptic vesicle exocytosis [8].

* PRESENT ADDRESS: KOHDA, T., Nara Prefectural Livestock Hygiene Service Center, Yamatokoriyama, Nara 639–1123, Japan.

**CORRESPONDENCE TO: KOZAKI, S., Laboratory of Veterinary Epidemiology, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan.

Recently, we have demonstrated that synaptotagmin, a synaptic vesicle membrane protein, exerts the binding activity for type B BoNT (BoNT/B) when associated with ganglioside GT1b or GD1a [12, 21]. There is a confirmed evidence that synaptotagmin not only functions as Ca^{2+} sensor at the site of exocytosis [3, 6, 26] but also binds to clathrin adaptor protein 2 with high affinity [32], suggesting a role of synaptotagmin in receptor-mediated endocytosis that follows exocytosis [17, 26]. BoNT/B was found to recognize the amino-terminal region of synaptotagmin [22]. Although the amino-terminus of synaptotagmin is oriented toward the interior of synaptic vesicles, it is exposed outside the nerve terminal after synaptic vesicle exocytosis [23, 27]. These findings provide a consideration that BoNT/B can easily be entrapped into synaptic vesicles by the receptor-mediated endocytosis and causes proteolysis of VAMP which exists on the surface of synaptic vesicle membrane. In the present study, we suggested that BoNT/B endocytosed into synaptosomes is present in the synaptic vesicles and causes degradation of VAMP-2.

MATERIALS AND METHODS

Toxin and antibodies: BoNT/B in a nicked form was purified according to the method previously described [15]. The purified BoNT/B was concentrated by untrafiltration with YM-10 membrane (Amicon Corporation, Danvers, MA) and dialyzed against 50 mM phosphate buffer, pH 7.5. No significant decrease in the toxicity was observed for at least 6 months at −80°C. Rabbit polyclonal antibodies (PAbs) against a synthetic peptide corresponding to the amino-terminal 20 residues of synaptotagmins I and II, the carboxyl-terminal 11 residues of SNAP-25, and the amino-terminal 20 residues of VAMP-2 and mouse monoclonal antibodies

(MAbs) against synaptophysin, syntaxin, and Na^+/K^+-ATPase were kindly provided by Dr. Masami Takahashi, Mitsubishi Kasei Institute of Life Science, Tokyo. Two MAbs reacting to each of the heavy and light chains of BoNT/B (designated as B17 and B101, respectively) were prepared as described [14]. All antibodies were purified by Protein A- or G- coupled Sepharose (Pharmacia Biotech, Tokyo) chromatography. Protein content was measured according to the method of Bradford [2] with bovine gammaglobulin as a standard.

Inhibition of noradrenaline release by BoNT/B: Rat brain synaptosomes (P2 fraction) were prepared as described [31]. Synaptosomes (3 mg of protein/m*l*) were incubated at 37°C for 90 min with [^{3}H] noradrenaline (^{3}H-NA) (Amersham International, Buckinghamshire, UK) at a final concentration of 0.1 μM (1 μCi/m*l*) in the following physiological buffer; 125 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES (4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid, Dojindo, Kumamoto), pH 7.5. BoNT/B treatment of synaptosomes was performed simultaneously during the incubation with ^{3}H-NA. No significant difference in the amount of ^{3}H-NA incorporated into synaptosomes was observed in the absence or presence of BoNT/B (data not shown). All buffers used in NA release were oxygenated with 95% O_2/5% CO_2 for 20 min at 30°C prior to addition to synaptosomes and all through incubations. After incubation, synaptosomes were washed three times with Ca^{2+}-free buffer by centrifugation at 9,000 × g for 1 min at 4°C. The pellet was suspended in Ca^{2+}-free buffer of the original volume, which was divided into 0.3-m*l* aliquots. For K^+-stimulated and Ca^{2+}-dependent NA release, 0.6 m*l* of K^+-containing buffer (final concentration of 5 mM or 25 mM) was added in the presence of 1.2 mM Ca^{2+}. After incubation for 5 min at 30°C, samples were centrifuged at 9,000 × g for 1 min and ^{3}H-NA released in each supernatant was determined with a scintillation counter. K^+-stimulated release during a 5-min period was calculated by subtracting the amount of resting release (5 mM K^+) from that obtained in the corresponding sample in the presence of 25 mM K^+.

Subcellular fractionation of BoNT/B-treated synaptosomes: The synaptosomes (15 mg of protein in 5 m*l*) treated with 100 nM BoNT/B as described above were washed three times with the chilled physiological buffer by centrifugation at 12,000 × g for 5 min at 4°C. The washed pellet was suspended in 5 mM MOPS (3-morpholino-propanesulfonic acid; Dojindo)-NaOH buffer, pH 7.4 containing 10 mM sucrose and protease inhibitors (5 μg/m*l* leupeptin, 10 μl/m*l* pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride), which was allowed to stand on ice for 20 min. The suspension was centrifuged at 25,000 × g for 20 min at 4°C to collect the crude membrane fraction. The membrane fraction (LP1) was washed further three times by centrifugation at 25,000 × g for 20 min at 4°C. The supernatant was then centrifuged at 165,000 × g for 60 min at 4°C. The resultant supernatant (LS2) was collected, and the pellet (LP2) containing synaptic vesicles was resuspended in 0.2 m*l* of 5 mM MOPS-NaOH buffer, pH 7.4, containing 10 mM sucrose. The suspension was loaded on top of a linear continuous gradient in 5 m*l* of 5 to 30% sucrose. After centrifugation at 150,000 × g for 2 hr at 4°C, 0.3-m*l* fractions were collected from the bottom. Each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described below.

SDS-PAGE and immunoblotting: SDS-PAGE was performed by the method of Laemmli [16]. For immunoblotting, samples were transferred electrophoretically to polyvinylidene difluoride membrane (Immobilon-PTM, Nihon Millipore Ltd., Tokyo) [4]. The blots were washed with 25 mM Tris-HCl buffer, pH 7.5 containing 0.14 M NaCl and 0.05% Tween 20 (TBST) and blocked for 1 hr with TBST containing 10% skimmed milk (Difco Laboratories, Detroit, MI). The blots were incubated at room temperature for 1 hr with PAbs or MAbs. After washing, the membranes were treated for 1 hr with the second antibody conjugate coupled with alkaline phosphatase or horseradish peroxidase. The immunoreactive bands were visualized by the ProtoBlot Western blot AP system (Promega Corporation, Madison, WI) or enhancement chemiluminescence (Amersham). The intensity of immunoreactive band was determined by scanning with a dual-wavelength densitometer (Shimadzu CS-9000).

In vitro cleavage of VAMP-2 with BoNT/B: After reduction of BoNT/B (0.3 μM) for 30 min at 37°C in 5 mM MOPS-NaOH buffer, pH 6.8, containing 0.3 M glycine and 10 mM DTT, reduced BoNT/B (a final concentration of 10 nM) was incubated with small synaptic vesicles (500 μg protein/m*l*) which had freshly been prepared from rat brain by the method of Huttner *et al.* [10], from which omitted was chromatography on controlled pore glass beads. After incubation for 1 hr at 37°C, the reaction was terminated by addition of SDS-sample buffer and the mixture was subjected to SDS-PAGE and immunoblotting as described above.

RESULTS

We confirmed that BoNT/B in a reduced and nicked form exerted a proteolytic activity on 18-kDa protein in synaptic vesicles, which resulted in induction of two fragments with molecular masses of 12 and 6 kDas (Fig. 1A). In immunoblotting, however, the 18-kDa protein reacted to anti-VAMP-2 recognizing the amino-terminal region whereas either fragment induced appeared to have lost mostly or completely their immunoreactivity (Fig. 1B), although the large fragment was considered to retain the amino-terminus of VAMP-2 [25]. In the following experiments, we therefore determined the residual intensity of VAMP-2 by immunoblotting to calculate the degree of degradation by BoNT/B proteolytic activity.

Synaptosomes were treated with various amounts of BoNT/B before ^{3}H-NA release has been triggered by depolarization. The ^{3}H-NA release was inhibited in a dose-dependent manner; 100 nM BoNT/B caused about 30% decrease (Fig. 2). No significant degradation of VAMP-2 was

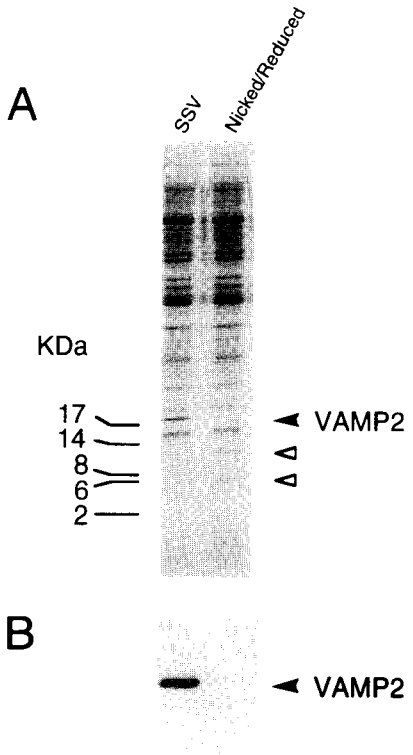

Fig. 1. SDS-PAGE (A) and immunoblotting (B) of synaptic vesicles before and after the *in vitro* treatment with nicked and reduced BoNT/B. (A) SSV, untreated synaptic vesicle; Nicked/reduced, toxin-treated synaptic vesicles. The positions of molecular mass (in kilodaltons) are indicated on the left. The closed and open arrowheads indicate the migration positions of VAMP-2 and BoNT/B-induced fragments, respectively. (B) Immunoblotting analysis of equivalent protein samples described in panel A by using rabbit antiserum against the synthetic peptides of amino-terminal 20 residues of VAMP-2.

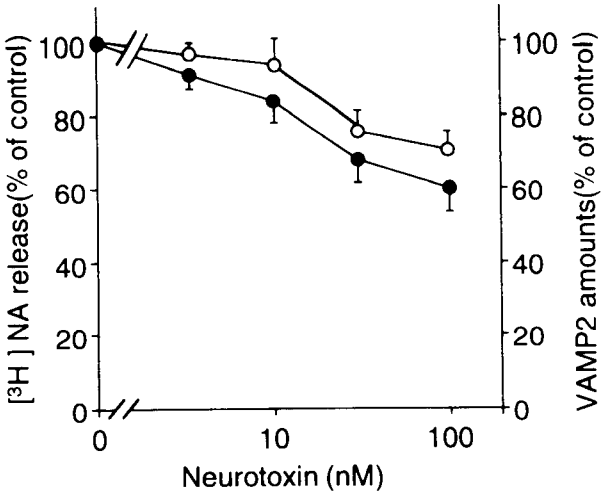

Fig. 2. Effect of BoNT/B on noradrenaline release and degradation of VAMP-2 in brain synaptosomes. Release of ^{3}H-noradrenaline (○) is expressed in percentage of that released from BoNT/B-untreated synaptosomes (16,500 dpm/mg protein). After SDS-PAGE and immunoblotting with crude synaptic vesicles (LP2) isolated from the respective BoNT/B-treated synaptosomes, the amount of VAMP-2 (●) normalized to those of synaptophysin are expressed in percentage of the amount of VAMP-2 in BoNT/B-untreated synaptosomes. Data presented are means ± S.E. from four separate experiments.

observed in direct determination with synaptosomes before or after BoNT/B treatment (data not shown). However, after separation of LP2 from LP1 with hypoosmotic shock followed by centrifugation, the VAMP-2 content of LP2 was found to have decreased to about 60% of their control values by the treatment with 100 nM BoNT/B when the values were normalized to the respective amounts of synaptophysin, the other synaptic vesicle protein (Fig. 2). These results support the previous observation that blockade of neurotransmitter by BoNT/B bears a parallel to the degradation of VAMP-2 [25].

To determine whether BoNT/B was really entrapped into synaptic vesicles to exert the proteolytic activity, we examined the localization of BoNT/B and its relation to synaptic vesicle proteins by using LP2 obtained from 100 nM BoNT/B-treated synaptosomes. As shown in Fig. 3, the data on sucrose density gradient centrifugation followed by SDS-PAGE and immunoblotting revealed that VAMP-2, synaptotagmin I and synaptotagmin II sedimented to the position corresponding to that of synaptophysin, suggesting that the fractions including these proteins contained synaptic vesicles. Aside from the synaptic vesicle-associated proteins, syntaxin and SNAP-25, t-SNAREs were also detected, which agreed with the previous observation [30]. But little Na^+/K^+-ATPase, a plasma membrane protein marker, was detected, although it sedimented to near the bottom. The amount of VAMP-2 detected in the synaptic vesicle fractions appeared to have decreased by BoNT/B treatment. Immunoblotting with MAbs against the heavy and light chains showed that a single immunoreactive band with a molecular mass of 150 kDa was seen only in the synaptic vesicle fraction. The observations imply that the whole toxin molecule is entrapped into synaptic vesicles and causes mere cleavage of VAMP-2 associated with synaptic vesicle membrane. We obtained also the results from the subsequent *in vitro* experiments that BoNT/B did not bind spontaneously to synaptic vesicles in LP2 (data not shown). However, they are inconsistent with the previous results that the proteolytic activity of BoNT/B appears only after reduction of interchain disulfide bond [25].

In order to reconcile with the above issues, we attempted to find whether BoNT/B was reduced after binding to synaptosomal membrane and whether the free chains were liberated into cytosol to target VAMP-2. When the LP1 isolated from 100 nM BoNT/B-treated synaptosomes was subjected to SDS-PAGE and immunoblotting, the light and heavy chains, as well as the intact toxin, were detected under an unreducing condition, suggesting that reduction of BoNT/B had occurred in the membrane after binding (Fig. 4B). The amounts of BoNT/B and the derived chains associated with

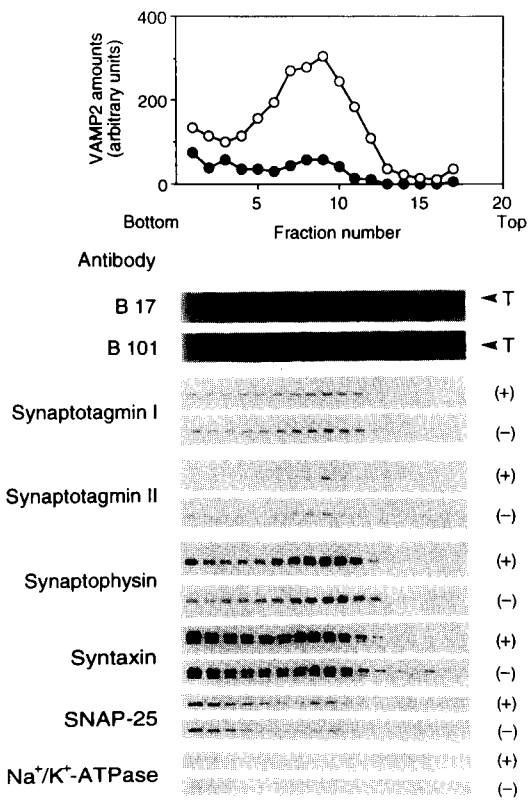

Fig. 3. Sucrose density gradient of crude synaptic vesicles obtained from BoNT/B-treated or -untreated synaptosomes. VAMP-2 is expressed relative to the amount after normalized to that of synaptophysin. The immunoreactive bands recognizing B17 and B101 against the heavy chain and the light chain were visualized with enhanced chemiluminescence, while the other bands were detected with Proto-Blot Western blot AP system. T, BoNT/B; ● and +, BoNT/B-treated; ○ and −, untreated.

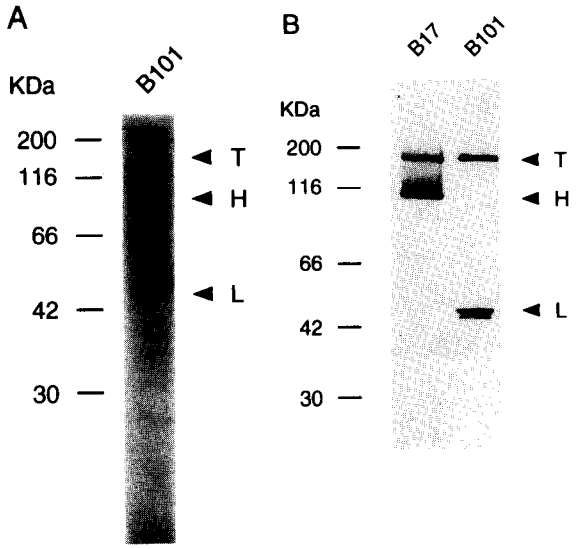

Fig. 4. Detection of the heavy and light chains in the cytosol fraction (A) and plasma membrane (B) obtained from BoNT/B-treated synaptosomes. The positions of molecular mass (kilodaltons) are indicated on the left. The migration positions of BoNT/B (T), the heavy chain (H), and the light chain (L) are shown on the right.

LP1 were estimated at about 0.2% of BoNT/B used. When the proteins containing LS2 were precipitated with 7% trichloroacetic acid and the precipitate was subjected to SDS-PAGE and immunoblotting with MAbs B17 and B101, a faint 50-kDa band reacting to MAb B101 was detected, although the amount calculated from the intensity of the immunoreactive band was only about 0.001% of BoNT/B used in this experiment (Fig. 4A).

DISCUSSION

There are many definite evidences showing that the light chain of BoNT attacks SNAP receptors to disturb their function in synaptic vesicle exocytosis [19, 20]. In the present study, we confirmed that BoNT/B cleaves VAMP-2 into two 12- and 6-KDa fragments, as described by Schiavo *et al.* [25]. However, the immunoblotting analysis showed that neither of the induced fragments reacted to the antibody against the synthetic peptide of amino-terminal region of VAMP-2. From this finding, we deduced that the cleavage of VAMP-2 may involve antigenic change of the 12-kDa fragment, especially in the amino-terminal region.

The inhibition by BoNT/B of ^{3}H-NA release from synaptosomes appeared to parallel the degradation of VAMP-2 contained in synaptic vesicles. In agreement with the previous paper [1], a relatively high concentration of BoNT/B is required to block neurotransmitter release from synaptosomes in comparison with peripheral neuromuscular junction [28]. This is enigmatic in understanding the BoNT/B action. One relevant explanation is that the low potency of BoNT/B on synaptosomes may reflect a low concentration of the highly specific receptor. In fact, we recently demonstrated that there are much abundant low-affinity binding sites and less high-affinity binding sites for BoNT/B on synaptosomes, which comprise synaptotagmin isoforms I and II, respectively [22]. The present data show that the amount of BoNT/B entrapped into synaptic vesicles seems to be extremely small, which may be due to the low rate of endocytosis during the treatment of synaptosomes with BoNT/B. Since it is generally accepted that stimulation promotes paralysis occurring by BoNT in the peripheral nerve ending and that the rate of endocytosis is proportional to the rate of exocytosis [28], it is likely that quiescent nerves such as synaptosomes under resting condition show endocytosis at a slow rate.

BoNT/B detected in synaptic vesicles was found to be mainly in an intact form. However, it is probable that a small amount of BoNT/B entrapped into synaptic vesicles have changed to a reduced form since VAMP-2 associated with these synaptic vesicles degraded to a significant level. This would be supported by the results that free light chains were

detected in the cytosol fraction in BoNT/B-treated synaptosomes. The findings raised the question of how reduction of BoNT occurs after the receptor binding. Sulfhydryls present in the membrane may be involved in the reduction. In fact, we found that BoNT/B bound to synaptosomal membrane (LP1) was partly in a reduced form. Even if the reduction of BoNT/B occurs on the membrane just after the receptor binding, BoNT/B does not release free chains since the two chains appear to be held together by noncovalent bonds [13]. Thus, both of reduced and unreduced BoNT/B enter into synaptic vesicles and only free light chain may penetrate through the membrane by the aid of acidic pH-dependent pore formation with the heavy chain [5, 9, 18] or itself [11]. In contrast with the light chain, the complementary heavy chain was not found in the synaptic vesicles or the cytosol fraction, presumably due to the sensitivity to the detection of the heavy chain or causing degradation of the heavy chain inside synaptosomes. Further experiments should be carried out to clarify this point.

Here we provide a possibility that BoNT/B endocytoses into synaptic vesicles in brain synaptosomes even with a relatively low efficiency. Therefore, this ability may make it a valuable marker in investigating the mechanism of endocytosis.

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