BINDING OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN TO THE PRESYNAPTIC MEMBRANE IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

Large synaptosome fractions were isolated from the cerebellar and cerebral cortices of rats and were incubated with *Clostridium botulinum* type A neurotoxin in vitro. The binding of the neurotoxin to the synapses was observed by electron microscopy, using the double-sandwich immunocytochemical method. Botulinum neurotoxin was preferentially bound to the presynaptic membrane in the large synaptosome fraction. The binding regions for the neurotoxin were localized on both the extrajunctional and junctional areas of the presynaptic membranes and appeared as patches of various sizes. However, they did not exist on the postsynaptic membranes. Botulinum neurotoxin is proposed to be a useful analytical tool for understanding the characteristics of the presynaptic membranes in the central nervous system.

KEY WORDS synaptosome · botulinum toxin · synaptic membrane · immunocytochemistry · central nervous system

Synapses have two main functions: one is chemical transmission and the other is recognition of partner cells with which to establish synaptic contact during developmental processes. The molecular mechanisms of both impulse transformation and neuronal recognition are not well known to date. To understand these phenomena, it is necessary to clarify the structural organization of the synapses, especially that of the pre- and postsynaptic membranes.

Numerous investigations have been carried out to characterize the structure of the synaptic membranes by electron microscope observations with various kinds of techniques (1, 3, 13). Another approach to this goal is to use specific compounds such as α-bungarotoxin (22) and lectins which bind to membrane components in a specific manner (2, 7, 19). We have examined the structure and function of the synapses with the use of neurotoxins such as *Clostridium botulinum* neurotoxin (17, 21) and β-bungarotoxin (14–16).

Cl. botulinum toxin is well known, on the basis of physiological and pharmacological studies (4–6, 8, 12, 18, 23, 26, 27, 28), to block transmitter release at neuromuscular junctions. The toxin is thought to act at neuromuscular junctions so as to reduce the influx of Ca ions into the nerve terminal or to decrease the efficiency of intracellular Ca ions in eliciting transmitter release, or both (8, 12). Binding of the toxin at neuromuscular junctions has been visualized by fluorescence microscopy (29) and autoradiography of ^{125}I-labeled botulinum toxin (17). Recent studies have shown that *Cl. botulinum* type A neurotoxin was bound to the synaptosome fractions (10, 11, 21) and synaptosomal plasma membranes (21) from brain

homogenates and also that transmitter release from cerebral synaptosomes was affected by the toxin (28). However, it is still unknown whether the binding sites for the toxin are located pre- and/or postsynaptically.

In the present study, we have intended to visualize by means of electron microscope immunocytochemistry the binding region for *Cl. botulinum* type A neurotoxin on the synaptic fraction prepared from rat central nervous tissue.

MATERIALS AND METHODS

Preparation of the Neurotoxin from Cl. botulinum Type A Crystalline Toxin

The botulinum type A crystalline toxin was supplied by Dr. E. J. Schantz (Wisconsin University). The neurotoxin, a toxic subunit (α-fraction) of the type A crystalline toxin of 150,000 mol wt (9), was isolated from the crystalline toxin by diethyaminoethyl (DEAE)-Sephadex chromatography (20) and dialyzed against 10 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl (phosphate-buffered saline [PBS]). The neurotoxin was homogeneous by disc electrophoresis and immuno-diffusion tests (Fig. 1).

Preparation of an Antiserum Directed against the Neurotoxin of Cl. botulinum Type A

The neurotoxin was mixed with 0.4% formalin to obtain a protein concentration of 100 μg/ml, and the mixture was incubated for 4 d at 37°C to prepare the toxoid. It was then emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.), and 4 ml of the toxoid-adjuvant mixture was injected subcutaneously into two rabbits. The second immunization was carried out with the toxoid after 1 mo. The antiserum was collected after 2 wk. The IgG fraction was purified from the sera by gel filtration on Sephadex G 200. The prepared anti-neurotoxin rabbit IgG (anti-neurotoxin-IgG) neutralized the muscle-paralyzing effect of the neurotoxin in mice. The botulinum neurotoxin and the anti-neurotoxin rabbit IgG preparation formed a single precipitation line in an agar gel diffusion test (Fig. 1). The ferritin-conjugated anti-rabbit IgG goat IgG (anti-IgG-Fe) was purchased from Miles Yada Ltd. (Kiryat, Weizmann, Israel).

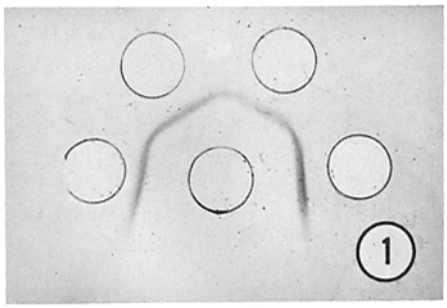


FIGURE 1 Agar gel diffusion test between botulinum neurotoxin and anti-botulinum neurotoxin rabbit IgG. The central well contained botulinum neurotoxin, 70 μg/ml 10 mM PBS, pH 7.2, and outer wells contained anti-botulinum neurotoxin rabbit IgG, 6.1 mg/ml PBS. One precipitation line was observed between central and outer wells.

Preparation of Large Synaptosome Fractions

Large synaptosomes were prepared according to the modified procedure of Kelly et al. (19). After decapitation, the cerebellum and cerebrum of adult rats were cut into slices ~2 mm thick, and the cerebellar cortex and cerebral cortex of the parietal lobe were dissected. About 190 mg wet weight of each tissue preparation was homogenized in 4 ml of 0.32 M sucrose at 0°C, 1 mM phosphate buffer, pH 7.2, containing 1 mM MgCl (SPB), by 10 manual passes in a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). The homogenate was centrifuged at 2,000 *g* for 4 min at 4°C, and the pellet was resuspended in SPB at a final concentration of 7–8 mg protein/ml.

Immunocytochemistry

Three kinds of experiments were carried out under different conditions.

EXP I: 0.2 ml of a suspension of large synaptosomes was incubated with 0.04 ml of botulinum neurotoxin (1,660,000 LD_{50}/ml and 99 μg protein/ml) at 37°C for 30 min. The reacted mixture was centrifuged at 9,950 *g* for 2 min, and then the pellet was washed three times with SPB by centrifugation. For toxicity determinations, 0.1 ml of the supernate from the first centrifugation was taken out, diluted with 0.5 ml of PBS, and 0.1 ml of the diluted supernates was injected into each mouse. Each supernate was injected into four mice to determine the toxicity (Table I). The pellet suspended in 0.1 ml of SPB was reacted with 0.1 ml on anti-neurotoxin rabbit IgG (6.1 mg protein/ml) for 1 h at 37°C. After washing, the pellets were suspended in 0.2 ml of anti-IgG-Fe (5.6 mg protein/ml) for 1 h at 37°C.

For the control studies, the large synaptosomes were incubated with either one of the following: (*a*) 0.04 ml of only SPB; (*b*) 0.04 ml of the toxoid (100 μg/ml) which was denatured by formalin to prepare the antiserum against botulinum neurotoxin; (*c*) 0.04 ml of naturally denatured toxin (100 μg/ml) which was obtained by storing at 4°C for 2 mo, and were subsequently treated as mentioned above.

EXP II: 0.2 ml of the suspension of large synaptosomes was incubated with 0.04 ml of botulinum neuro-

toxin at 37°C for 30 min. Then the sample was centrifuged at 9,950 *g* for 2 min, and the pellet was washed three times with SPB and reacted with 0.1 ml of anti-neurotoxin IgG (6.1 mg protein/ml) for 16 h at 4°C. After washing at 4°C three times, the pellet was suspended in 0.2 ml of anti-IgG-Fe and kept for 12 h at 4°C.

Three kinds of control studies were carried out as described above.

EXP III: 0.2 ml of each suspension of large synaptosomes was fixed with 0.1% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15 min at 20°C. The suspension was centrifuged at 9,950 *g* for 2 min, and the pellet was washed three times with SPB and resuspended in 0.2 ml of SPB. Then the suspension was incubated with 0.04 ml of neurotoxin at 37°C for 30 min and washed three times with SPB. The washed sample was reacted with anti-neurotoxin IgG at 37°C for 1 h. After washing, it was suspended in anti-IgG-Fe at 37°C for 1 h.

The prefixed sample was incubated with the three kinds of solutions used in exp I for control studies and processed as mentioned above.

For the electron microscope observations, the specimens were prepared as follows: The reaction mixtures from exp I and exp III were washed three times with SPB and fixed in 2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 20°C. The samples of exp II were fixed in the same fixative at 4°C for 4 h. All the samples were postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 1.5 h at 4°C and were resuspended in a small volume of warm 4% (wt/vol) agar.

The agar gel was cut into small pieces, stained en bloc in 1% uranyl acetate in Kellenberger's buffer overnight, dehydrated in graded alcohols, and embedded in Araldite. Ultrathin sections were cut with an LKB ultratome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined in a Hitachi 12A electron microscope.

RESULTS

It was demonstrated, by measuring the toxicity remaining in the supernate after incubation of the reaction mixture, that the botulinum neurotoxin binds to the large synaptosomes. More than 99% of the neurotoxin is bound when incubated at 37°C with large synaptosome fractions of cerebral and cerebellar cortices (Table I). However, it does not bind at 0°C, and only a small amount of the toxin binds to synaptosomes at 4°C.

Morphologically, the large synaptosome preparations from cerebellar cortex were observed to contain synaptosomes of various sizes (Figs. 2 and 4), while the synaptosomes from cerebral cortex were of relatively uniform size.

TABLE I

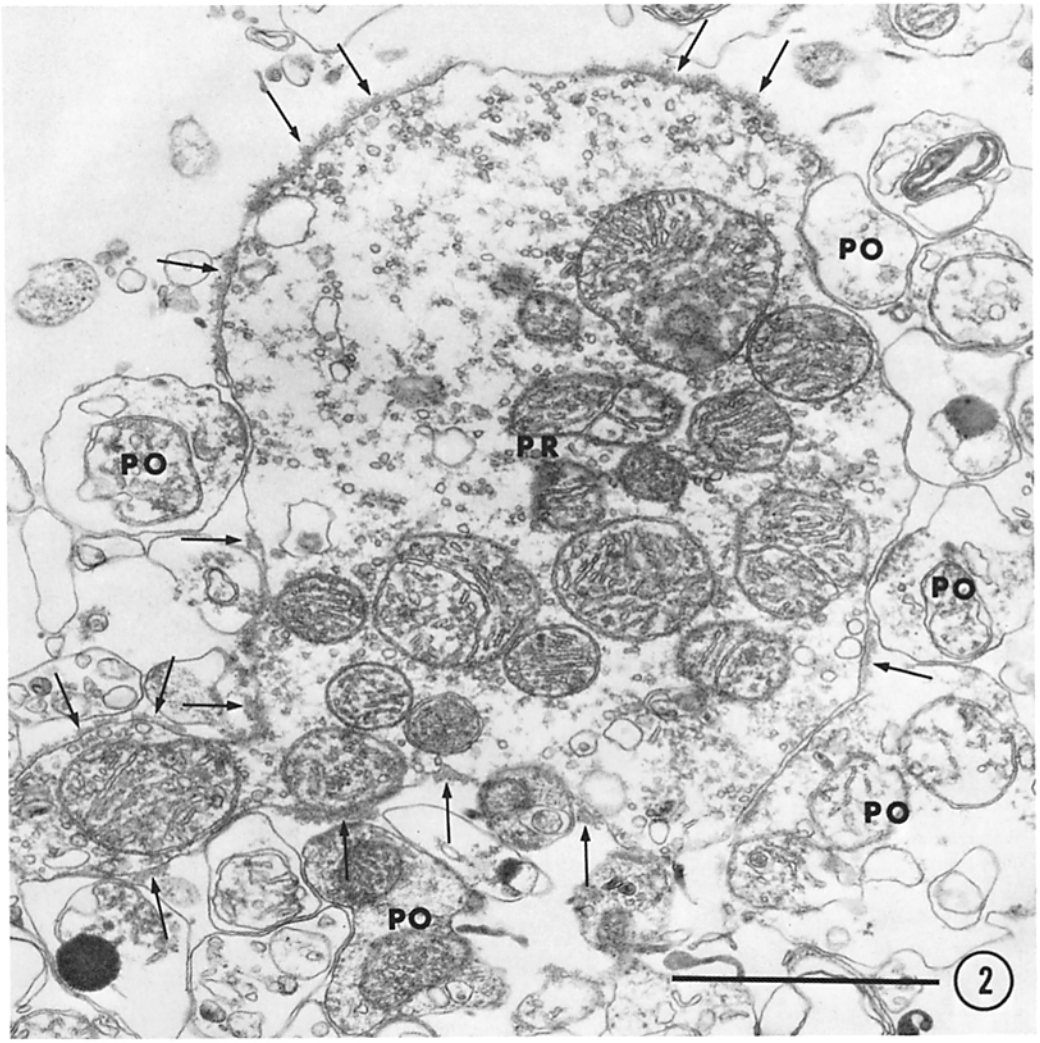
Toxicity of the Incubation Media

Before incubation	1,660,000 LD_{50}/ml
After incubation with cerebral cortex	4,000 LD_{50}/ml
After incubation with cerebellar cortex	4,000 LD_{50}/ml
After incubation without samples	1,645,000 LD_{50}/ml

EXP I: The localization of binding sites for *Cl. botulinum* neurotoxin is observed as neurotoxin, anti-neurotoxin IgG, and anti-IgG-Fe complexes (BoTX-IgG-anti-IgG-Fe) at the surface of the synaptosomes prepared from cerebral and cerebellar cortices (Figs. 2, 3, and 5). The BoTX-IgG-anti-IgG-Fe complexes are localized as patches of various sizes on presynaptic membranes (Figs. 2, 3, and 5). The processes identified as presynaptic are filled with clear vesicles, coated vesicles, and mitochondria and often show presynaptic membrane specializations (Figs. 2 and 5). BoTX-IgG-anti-IgG-Fe complexes are located widely on extrajunctional membranes when the presynaptic processes are attached to the postsynaptic processes (Figs. 2 and 5). When the presynaptic and postsynaptic processes are partially dissociated, the BoTX-IgG-anti-IgG-Fe complexes are often found in the cleft regions of partially opened synapses as well as at extrajunctional membranes of presynaptic processes (see Fig. 6). The immune complexes are sometimes found as a single aggregate on part of the presynaptic membrane; most commonly, however, they are distributed as multiple patches of various sizes over the entire surface of the presynaptic endings (Figs. 2 and 5). The BoTX-IgG-anti-IgG-Fe complexes are not observed on the plasma membrane of either free or attached postsynaptic processes (Figs. 2 and 5), which are identified as the profiles that show postsynaptic membrane thickenings and do not contain vesicles, nor are they found on the membrane of glial cells.

EXP II: The immune complexes are always observed as multiple patches of various sizes all around the circumference of the presynaptic membranes; they never occur as a single aggregate. The complexes also appear in the cleft region of partially opened synapses (Fig. 6), whereas they are not located on postsynaptic membranes (Fig. 6).

EXP III: The results are essentially similar to those obtained in exp II.



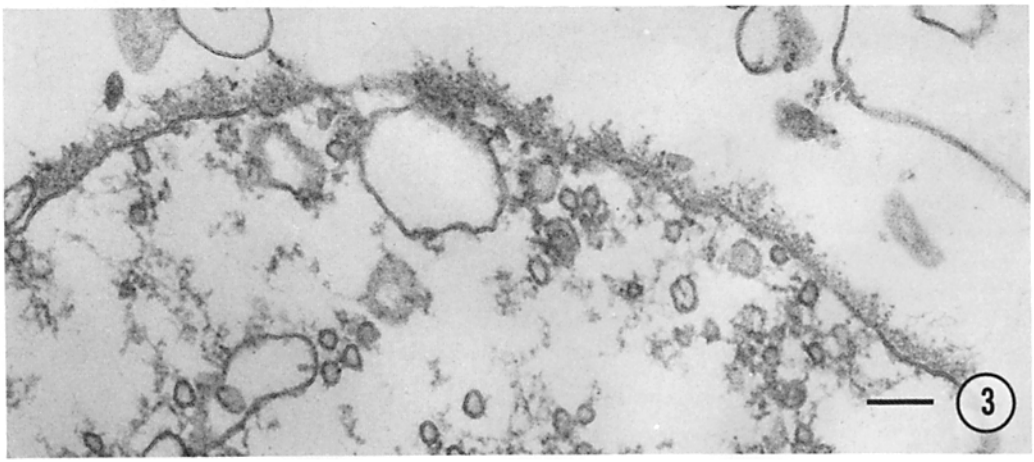


FIGURE 2 A large presynaptic process (*PR*) attached to several postsynaptic processes (*PO*) prepared as described for exp I at 37°C. BoTX-IgG-anti-IgG-Fe complexes occur on the presynaptic membrane as patches (arrows). Note that the complexes are not present on the postsynaptic membranes. Bar, 1 μm. × 35,700.

FIGURE 3 An enlarged view of the upper left part of Fig. 2. The immune complexes are observed on the presynaptic membrane. Bar, 0.1 μm. × 90,000.

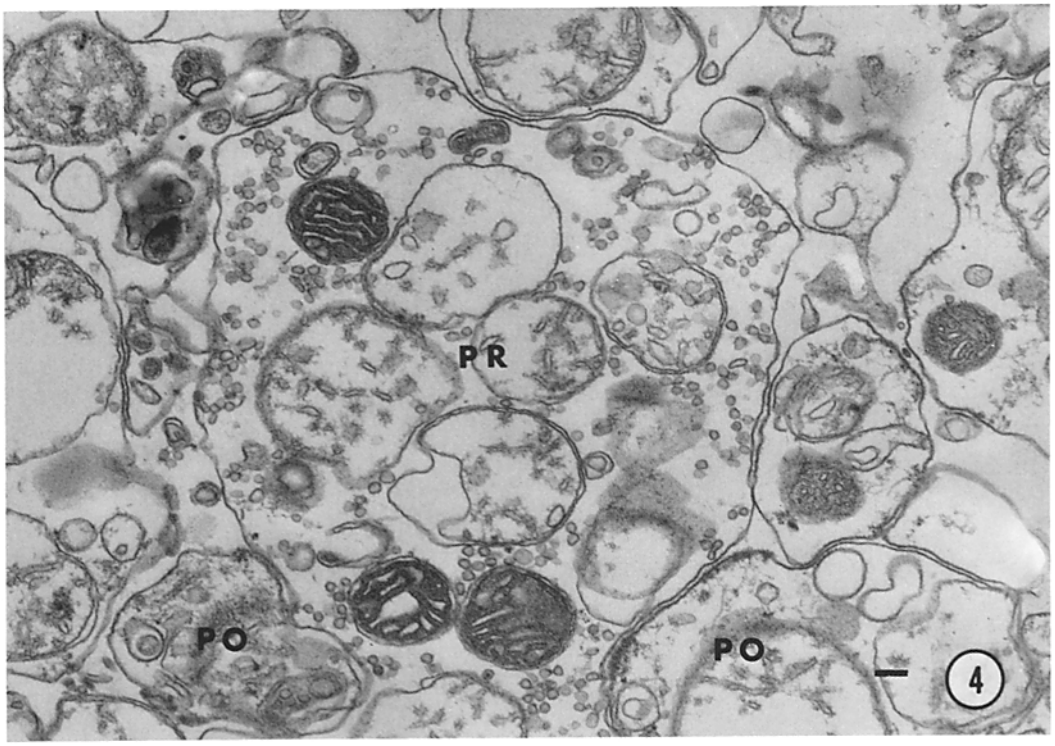


FIGURE 4 A control sample prepared as in exp I and incubated with the toxoid. A presynaptic process (*PR*) with postsynaptic processes (*PO*) in the cerebellar cortex. BoTX-IgG-anti-IgG-Fe complexes are not found on the presynaptic membrane or in any other area. Bar, 0.1 μm. × 43,400.

In the control experiments of Exp. I, II, and III, no BoTX-IgG-anti-IgG-Fe complexes occur on the synaptic membranes (Fig. 4).

DISCUSSION

The present study demonstrates that the binding sites for *Cl. botulinum* neurotoxin do exist widely on the presynaptic membranes of the cerebral and cerebellar cortices, but do not occur on postsynaptic membranes nor on membranes of glial origin in the large synaptosome preparations isolated from rat brain homogenates.

The immune complexes are not found in typical synaptic clefts when the presynaptic processes are not dissociated from the postsynaptic processes, while they occur in the clefts of partially opened synapses in all the samples. However, they have never been observed on free postsynaptic processes which show postsynaptic membrane thickenings. The possibility exists that the double-sandwich immunocytochemical method may not allow for the anti-neurotoxin IgG or anti-IgG-Fe to infiltrate the narrow synaptic clefts. So, the binding sites for the botulinum neurotoxin may be localized to the junctional region as well. In exp I the immune complexes are sometimes observed as a single patch on the surface of the presynaptic endings; this phenomenon, however, is not seen in exp II and III. This result may be due to membrane fluidity (24, 25), because the movement of the membrane molecules in exp. II and III may be inhibited as compared with exp. I. As a result, the immune complexes are found as multiple patches of various sizes over the entire surface of the presynaptic membrane in exp. I, II, and III. The patching process observed in exp. I, II, and III may be due to some extent to cross-linking of the binding sites by the divalent ligands (24).

From these results, we conclude that the binding sites for botulinum neurotoxin are localized widely on the presynaptic membranes of cerebral and cerebellar cortices but not on the postsynaptic membranes. The results of the present study are

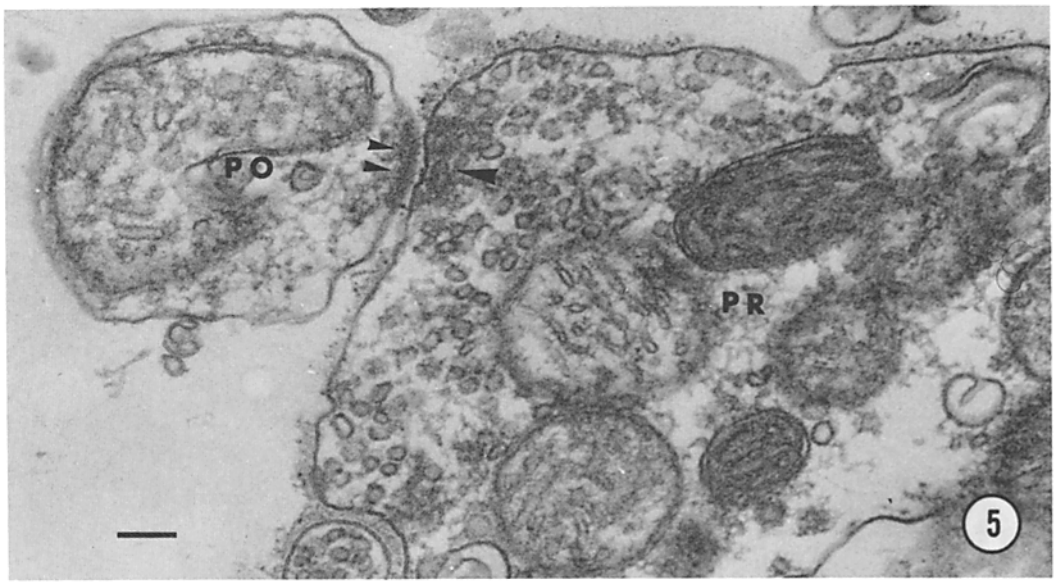


FIGURE 5 A presynaptic process (*PR*) and postsynaptic process (*PO*) from the cerebellar cortex prepared as described for exp I at 37°C. BoTX-IgG-anti-IgG-Fe complexes occur widely on the presynaptic membrane, but the complexes are not present in the narrow synaptic cleft. Small arrowheads: postsynaptic membrane thickening. Large arrowhead: active zone. Bar, 0.1 μm. × 80,200.

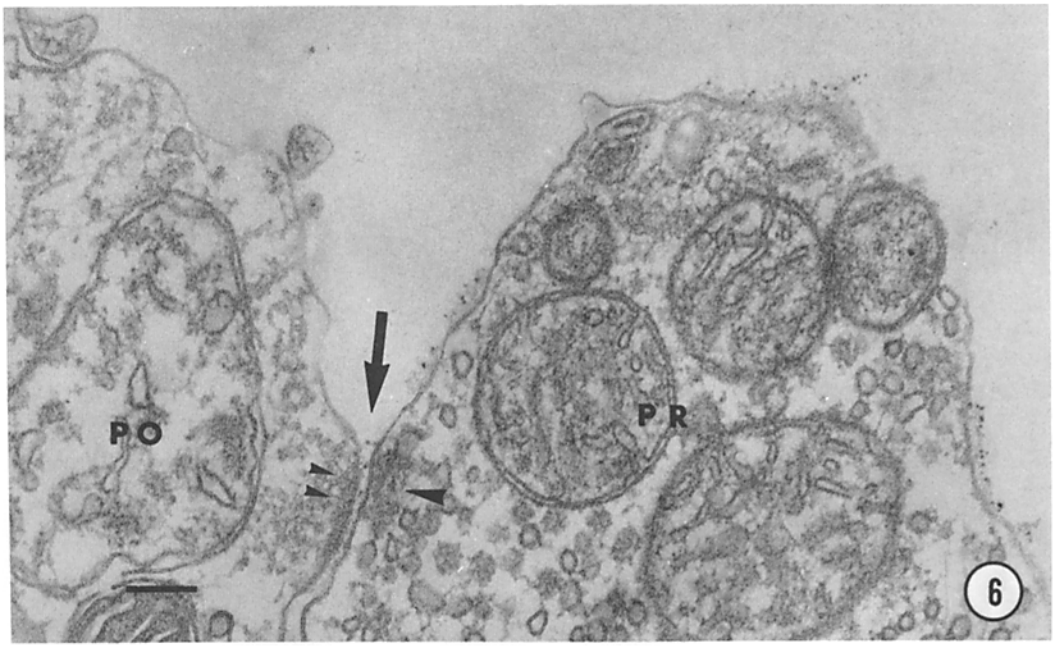


FIGURE 6 A presynaptic process (*PR*) and postsynaptic process (*PO*) from the cerebellar cortex prepared as described for exp II at 4°C. BoTX-IgG-anti-IgG-Fe complexes are found as patches all over the presynaptic membranes and at the cleft region (arrow). Small arrowheads: postsynaptic membrane thickening. Large arrowheads: active zone. Bar, 0.1 μm. × 94,300.

at variance with those obtained with lectins, because the binding sites for lectins exist on both pre- and postsynaptic membranes (2, 7, 19). So far, the receptor for the botulinum toxin is the first molecule by which the membrane of the presynaptic processes can be distinguished from that of the postsynaptic processes in terms of binding properties.

The authors wish to thank Dr. E. J. Schantz, University of Wisconsin for his kind supply of the crystalline toxin, Professors J. Nakai and E. Yamada, Department of Anatomy, Faculty of Medicine, University of Tokyo, and Dr. A. Ohsaka, 2nd Department of Bacteriology, National Institute of Health, for their valuable discussion.

This work is supported by grants from the Japanese Ministry of Education.

This study is dedicated to Professor J. Nakai for his 60th birthday.

Received for publication 16 February 1978, and in revised form 2 November 1978.

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