HISTOCHEMISTRY OF BUNGAROTOXIN BINDING SITES AT ELECTRON MICROSCOPIC LEVEL

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For the demonstration of acetylcholine receptor (Ach R) at neuromuscular junction using α -bungarotoxin-horseradish peroxidase (α -BT-HRP) conjugate, a modified conjugation method was described to keep the better binding activity to Ach R. For the tissue processing, three methods were compared regarding the binding specificity and preservation of ultrastructure. The best result was obtained when EPON sections were made from glutaraldehyde-fixed tissue after incubation with α -BT-HRP conjugate. The preliminary examination of cryostat sections and subsequent embedding for electron microscopic examination was useful but unsatisfactory for the preservation of membrane structure. Electron microscopic examination revealed that α -BT was localized both on the presynaptic and on the postsynaptic membrane.

Despite many attempts to determine the localization of acetylcholine receptor (Ach R) including histochemical demonstration of acetylcholine esterase and radioautographic or immunohistochemical demonstration of neurotoxin bound to Ach R, established techniques for localization of Ach R at electron microscopic level have been limited. We have tried and compared several methods to obtain the localization of Ach R at neuromuscular junction by electron microscopy. At first, we tried the indirect immunoperoxidase method using α -bungarotoxin (α -BT), antibungarotoxin rabbit IgG and anti-rabbit IgG goat F (ab')₂ labelled with horseradish peroxidase (HRP) according to Ringel *et al.* (2). Although this method allowed for good preservation of fine structure when the first and second antibodies were applied after PLP fixation, the contrast was insufficient probably due to small amounts of reaction products at toxin binding sites. The method utilizing α -BT conjugate with HRP according to Engel *et al.* (1) gave the best localization of Ach R at the electron microscopic level. We modified the procedure for HRP conjugation according to the method described by Wilson and Nakane (3) and obtained good results when it was applied to tissue blocks instead of teased muscle fibers. Further substitution of α BT by cobrotoxin did not improve results either by the immunohistochemical or by the direct conjugate method. In the present report, we describe a reliable method to demonstrate Ach R of neuromuscular junction by electron microscopy applied to tissue blocks.

MATERIALS AND METHODS

Conjugation of horseradish peroxidase to α -bungarotoxin

Neurotoxins were purified and kindly provided by Dr. Kyoze Hayashi, Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University. Briefly, α -BT was purified from crude toxin of *Bungarus multicinctus* and cobrotoxin from that of *Naja naja atra* using gel filtration with Sephadex G-50 and ion exchange chromatography with CM-cellulose. For conjugation, according to Wilson and Nakane (3), 4 mg of Sigma HRP type VI were dissolved in 1 ml of distilled water and activated by stirring with 0.2 ml of 0.1 M sodium metaperiodate at room temperature. The solution was dialyzed against 1 mM acetate buffer pH 4.5 at 4°C overnight, and 2 mg of α -BT or cobrotoxin in 1 ml of 0.02 M carbonate buffer pH 9.5 was added and stirred for 2 hr at room temperature. After further stirring with 0.05 ml of NaBH₄ 4 mg/ml for 1 hr at 4°C and dialysis against phosphate buffered saline (PBS) at 4°C overnight, the conjugate was purified by Sephadex G-100 column (Fig. 1). The solution contained α -BT-HRP at a concentration of 0.017 mg/ml and was presumed to contain HRP and α -BT at 1 : 3.2 molar ratio.

FIG. 1. Separation of α -BT-HRP conjugate on Sephadex G-100 column. Straight line: absorption at 280 nm showing protein content. Dotted line: absorption at 403 nm showing HRP content. Column size 45 $\times$ 2.4 cm. Elution buffer PBS 12 ml/hr.

Toxicity assay

The obtained toxin-HRP conjugate was given intravenously to adult ICR mice and lethal activity was confirmed.

Binding of toxin to Ach R

The diaphragm muscle of ICR and BALB/c adult mice was cut into small blocks measuring $1 \times 1 \times 3$ mm and incubated with α -BT-HRP conjugate at a concentration of 1×10^{-7} M in Tyrode's solution for 2 hr at 20°C. As a control for binding specificity, the muscle blocks were incubated in the following medium.

- a. Preincubation in the 1×10^{-4} M tubocurarine chloride (Amerizol®) in Tyrode's solution for 20 min at room temperature, followed by the reaction medium containing toxin conjugate.
- b. Incubation with non-conjugated HRP.
- c. Incubation in Tyrode's solution.

Procedure for electron microscopy

After the incubation with toxin-HRP conjugate, tissue blocks were washed in Tyrode's solution for 2 hr and fixed for 30 to 40 min at 4°C with 2% glutaraldehyde or with 4% paraformaldehyde in 0.1 M cacodylate buffer pH 7.4. Subsequently, DAB reaction, dehydration and embedding were performed according to two different procedures.

a. Embedding after light microscopic observation. Muscle blocks were washed in PBS containing sucrose at 5%, 10%, 15% for 4 hr in each solution, and finally in PBS with 20% sucrose and 5% glycerol for 2 hr at 4°C. Subsequently, frozen sections were cut at 4 μ m with a cryostat and fixed on albumin-coated slides. After washing in PBS, sections on slide glasses were pre-incubated in diaminobenzidine solution without H_2O_2 for 30 min, and then incubated in complete Karnovsky's solution containing H_2O_2 for 30 min (4). Sections were osmified for 45 min with 2% osmium tetroxide. After light microscopic observation, sections were dehydrated in graded alcohol and embedded in EPON using the inverted gelatin capsule method to make blocks from selected areas.

b. Direct embedding. Muscle blocks washed in PBS were preincubated in DAB solution for 30 min, and then incubated in complete Karnovsky's solution for 5 min. After washing in PBS, blocks were osmified for 2 hr with 2% cold osmium tetroxide to obtain adequate osmium black formation. Blocks were then dehydrated and embedded in EPON. For electron microscopy, 1 μ m EPON sections were cut with an LKB 2000 ultramicrotome using a diamond knife, and sections without counterstain were observed with a JEOL 100-C electron microscope.

FIG. 2. Binding of α -BT-HRP conjugate at the neuromuscular junction of mouse diaphragm muscle. $\times 600$

A. Localization of α -BT-HRP after 2 hr incubation at 20°C (arrow). B. No reaction products are noted except for red cells after pretreatment of muscle with 10^{-4} M d-tubocurarine prior to toxin binding.

FIG. 3. Electronmicrograph of neuromuscular junction fixed in 2% glutaraldehyde after incubation with toxin showing linear localization of α -BT-HRP on the postsynaptic membrane. $\times 13,000$

FIG. 2.

FIG. 3.

RESULTS

1. Toxic activity of α -BT-HRP conjugate

All mice died after intravenous administration of non-conjugated α -BT or α -BT-HRP conjugate containing the same molar amount of toxin, although the mouse group given conjugated toxin showed delayed death compared with those given non-conjugated toxin.

2. Binding specificity of α -BT-HRP conjugate

The control blocks treated with HRP solution or Tyrode's solution showed no localization of peroxidase reaction products at neuromuscular junction. The preincubation of muscle blocks with 1×10^{-4} M tubocurarine for 20 min completely inhibited the subsequent binding of α -BT-HRP conjugate at neuromuscular junction (Fig. 2).

3. Electron microscopic localization of α -BT-HRP conjugate

The α -BT-HRP conjugate was observed on the postsynaptic membrane as a

FIG. 4. Higher power view of Fig. 3 showing α -BT-HRP localized on the postsynaptic and to a lesser extent on the presynaptic membrane. Note the excellent preservation of ultrastructure. $\times 40,000$

FIG. 5. Electron micrograph of neuromuscular junction fixed in 4% paraformaldehyde after incubation with toxin. Reaction products are seen on cytoplasmic granules in addition to postsynaptic membrane. $\times 40,000$

FIG. 6. Localization of α -BT-HRP in the blocks prepared from a cryostat section cut after toxin-binding and fixation in paraformaldehyde. Preservation of postsynaptic membrane is insufficient and diffusion of reaction products are seen in the cytoplasm. $\times 40,000$

FIG. 5.

FIG. 6.

continuous deposition of osmium black and to a lesser extent on the presynaptic membrane (Figs. 3, 4).

The preservation of fine structure was best when ultrathin sections were prepared from tissue blocks fixed with glutaraldehyde (Figs. 3, 4). Samples fixed with paraformaldehyde showed a slight diffusion of reaction products into the cytoplasm (Fig. 5). Electron microscopic samples made from cryostat sections gave relatively poor preservation of membrane structure and showed a moderate diffusion of reaction products (Fig. 6).

DISCUSSION

For detecting localization of Ach R using labelled bungarotoxin, the critical point is binding activity of toxin-HRP conjugate. Since we have often experienced a loss of toxin activity resulting in a diminished lethal effect *in vivo* and binding activity *in vitro*, we used a smaller amount of sodium borohydride than the original method used by Engel *et al.* (1) and Wilson and Nakane (3) to protect the disulphate bond in the toxin molecule. The binding specificity of thus obtained α -BT-HRP conjugate to Ach R was shown by competitive inhibition with d-tubocurarine and the concentration of DAB reaction products was sufficient to give a clear contrast in electronmicrograph. These results suggest that the preservation of the configuration of bungarotoxin is important to keep binding activity to Ach R.

For electron microscopic histochemistry, the preservation of ultrastructure and that of the binding activity of receptor sites are frequently incompatible. Our study showed that the incubation and washing of fresh muscular tissue up to 4 hr at 20°C in Tyrode's solution had no adverse effects on the preservation of ultrastructure if fixed with 2% glutaraldehyde immediately after incubation.

For the electron microscopic study of neuromuscular junction, it is convenient and often necessary to confirm toxin binding sites by light microscope in advance. To detect the receptor sites, our method of preparing cryostat sections was confirmed to be effective, although membrane ultrastructure was not adequately preserved probably due to freeze and thaw effects. Therefore, further improvement in preservation is desirable in cases where preliminary checking of cryostat sections is necessary.

Finally, the localization of α -BT-HRP on the postsynaptic membrane needs consideration. It is well known that crude bungarotoxin contains β -toxin which binds to the presynaptic site. Engel *et al.* (1) also reported post- as well as presynaptic localization of α -BT at the electron microscopic level. It may be necessary to utilize other tracers such as antibodies to Ach R for obtaining specific binding to the postsynaptic membrane.

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