

# Specific Localization of the $\alpha$ -Latrotoxin Receptor in the Nerve Terminal Plasma Membrane

F. VALTORTA, L. MADEDDU, J. MELDOLESI, and B. CECCARELLI

*Department of Medical Pharmacology, University of Milan, National Research Council Center of Cytopharmacology and Center for the Study of Peripheral Neuropathies and Neuromuscular Diseases, 20129 Milan, Italy*

**ABSTRACT** The receptor for  $\alpha$ -latrotoxin, the major protein component of the black widow spider venom, was investigated by the use of the purified toxin and of polyclonal, monospecific anti- $\alpha$ -latrotoxin antibodies. Experiments on rat brain synaptosomes (where the existence of  $\alpha$ -latrotoxin receptors was known from previous studies) demonstrated that the toxin-receptor complex is made stable by glutaraldehyde fixation. At saturation, each such complex was found to bind on the average five antitoxin antibody molecules. In frog cutaneous pectoris muscles, the existence of a finite number of high-affinity receptors was revealed by binding experiments with  $^{125}\text{I}$ - $\alpha$ -latrotoxin ( $K_d = 5 \times 10^{-10}$  M;  $b_{\text{max}} = 1.36 \pm 0.16$  [SE]  $\times 10^9$  sites/mg tissue, dry weight). Nonpermeabilized muscles were first treated with  $\alpha$ -latrotoxin, and then washed, fixed, dissociated into individual fibers, and treated with anti- $\alpha$ -latrotoxin antibodies and finally with rhodamine-conjugated sheep anti-rabbit antibodies. In these preparations, muscle fibers and unmyelinated preterminal nerve branches were consistently negative, whereas bright specific fluorescent images, indicative of concentrated  $\alpha$ -latrotoxin binding sites, appeared in the junctional region. These images closely correspond in size, shape, and localization to endplates decorated by the acetylcholinesterase reaction. The presynaptic localization of the specific fluorescence found at frog neuromuscular junctions is supported by two sets of findings: (a) fluorescent endplate images were not seen in muscles that had been denervated; and (b) the distribution of fluorescence in many fibers treated with  $\alpha$ -latrotoxin at room temperature was the one expected from swollen terminal branches. Swelling of terminals is a known morphological change induced by  $\alpha$ -latrotoxin in this preparation. When muscles were treated with either proteolytic enzymes (trypsin, collagenase) or detergents (Triton X-100) before exposure to  $\alpha$ -latrotoxin, the specific fluorescent endplate images failed to appear. Taken together these findings indicate that the  $\alpha$ -latrotoxin receptor is an externally exposed protein highly concentrated in the nerve terminal plasma membrane. Its density (number per unit area) at the frog neuromuscular junction can be calculated to be  $\sim 2,400/\mu\text{m}^2$ .

The presynaptic membrane is the portion of the neuronal plasmalemma that surrounds synaptic terminals, where the release of neurotransmitters occurs by fusion of synaptic vesicles (exocytosis). A number of other important functions (e.g., the recycling of the vesicle membrane, the high-affinity uptake processes of neurotransmitters or transmitter precursors, and the initiation of retrograde transport) are also localized at this level. It is generally agreed that these functional specializations of the presynaptic membrane occur as a consequence of its molecular dissimilarity with respect to the rest

of the plasma membrane. However, a large gap of knowledge still exists between these specifically localized functions and the identification of the molecules involved in these functions or otherwise specific for the presynaptic membrane (for a recent review see reference 39).

In recent years natural neurotoxins have been widely used in neurobiological studies (5, 15, 22, 27). Among the most interesting features of these molecules are the high selectivity and high affinity of interaction with unique membrane components, their specific receptors. Thus, neurotoxins can be

invaluable tools for the study of their receptor molecules. A class of neurotoxins is known to act presynaptically (5, 15, 22, 27). Among these,  $\alpha$ -latrotoxin ( $\alpha$ -LTx),<sup>1</sup> from the venom of the black widow spider, was found to act on many, and possibly all, types of vertebrate synapses, where it induces massive stimulation of quantal release of neurotransmitter accompanied by parallel depletion of synaptic vesicles (9, 16, 22, 24, 29, 41, 45). In contrast,  $\alpha$ -LTx is completely inactive on axons and on nonneural cells, irrespective of their specific properties (secretion, excitability, etc.) (16, 24, 34).

The mechanisms that mediate the widespread stimulatory action of  $\alpha$ -LTx at vertebrate synapses have been elucidated only in part. A small number of specific, high-affinity binding sites (henceforth referred to as the  $\alpha$ -LTx receptors) have been found in synaptosomes and pheochromocytoma cells (PC12) (18, 34, 35, 42). Since the occupancy of these receptors correlated in various experimental conditions with the subsequent stimulation of transmitter release (34, 35), a causal relationship between these two processes (possibly mediated by the influx of divalent cations) appears quite likely. However, knowledge about  $\alpha$ -LTx receptors is still very limited and their presynaptic localization had never been demonstrated. Here we report findings obtained by the use of monospecific anti- $\alpha$ -LTx polyclonal antibodies in rat cerebral cortex synaptosomes and frog neuromuscular junctions (NMJs). In agreement with the exclusively presynaptic effect of the toxin, our results indicate that the high-affinity  $\alpha$ -LTx receptor is an externally exposed component of the nerve terminal plasma membrane.

## MATERIALS AND METHODS

### Purification and Iodination of $\alpha$ -LTx and Anti- $\alpha$ -LTx Ig

$\alpha$ -LTx was purified from homogenates of venom glands dissected out from cephalothoraces of female European black widow spiders, *Latrodectus mactans tredecimguttatus*, as described by Frontali et al. (16). The purity of the toxin preparations was routinely assessed by SDS PAGE.  $\alpha$ -LTx affinity columns were prepared by coupling the purified toxin to activated Sepharose 4B.

Anti- $\alpha$ -LTx Ig were raised in rabbits by three successive injections of  $\alpha$ -LTx (25  $\mu$ g, emulsified with complete Freund's adjuvant) given intradermally at weekly intervals. Booster injections (50  $\mu$ g of  $\alpha$ -LTx emulsified with incomplete adjuvant) were given in the subscapular space. Anti- $\alpha$ -LTx sera were characterized by Ouchterlony double diffusion, by crossed immunoelectrophoresis (1), and by immunolabeling of SDS PAGE gels. For the latter test, 7.5–12% polyacrylamide gradient slab gels bearing triplicate samples of crude venom and purified  $\alpha$ -LTx were cut longitudinally into three portions, one of which was stained with Coomassie Brilliant Blue. The other two were fixed, exposed to either the preimmune or to the anti- $\alpha$ -LTx serum and then to <sup>125</sup>I-protein A ( $1 \times 10^6$  cpm) as described by De Camilli et al. (12). Immunolabeled gels were dried and autoradiographed on Kodak x-ray films for 20 h at  $-80^\circ\text{C}$ .

Anti- $\alpha$ -LTx Ig were purified from immune sera by absorption onto  $\alpha$ -LTx affinity columns (overnight incubation at 2–3°C in a rotating apparatus) followed by extensive washing in PBS and then by elution at low pH (200 mM glycine-HCl buffer, pH 2.8).

$\alpha$ -LTx and anti- $\alpha$ -LTx Ig were iodinated by the Bolton-Hunter procedure (3). As shown elsewhere (34), the labeled toxin retains full biological activity.

### Analytical Procedures

Protein was measured according to Lowry et al. (30). SDS PAGE slabs were prepared and run as described by Maizel (32). The biological activity of  $\alpha$ -LTx was tested routinely on crude synaptosome preparations obtained from Sprague-Dawley rat striatum. The synaptosomes were loaded in vitro with [<sup>3</sup>H]-dopamine, exposed for 10 min at 37°C to various concentrations of the toxin,

<sup>1</sup> Abbreviations used in this paper:  $\alpha$ -LTx,  $\alpha$ -latrotoxin; mKR, modified Krebs-Ringer medium; NMJ, neuromuscular junction; R, frog Ringer's medium.

and then centrifuged for 5 min at 10,500 g in an Eppendorf microcentrifuge (34). The pellets were washed once with incubation medium and their radioactivity was counted in a Beckman SL 30 liquid scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, CA) (34).

### Experiments on Rat Brain Synaptosomes

Pellets of purified synaptosomes, prepared from the cerebral cortex of rats (20), were resuspended in a modified Krebs-Ringer solution (mKR) containing (in mM): NaCl, 125; KCl, 5; MgSO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2; HEPES-NaOH buffer, pH 7.4, 25; glucose, 6; BSA, 0.02. Aliquots of these suspensions were incubated under 100% O<sub>2</sub> with  $\alpha$ -LTx, <sup>125</sup>I- $\alpha$ -LTx, or <sup>125</sup>I-anti- $\alpha$ -LTx Ig as described in Results and in the figure legends. <sup>125</sup>I radioactivity was measured in a Beckman GAMMA 4000 spectrometer (Beckman Instruments, Inc.).

### Experiments on the Frog NMJ

Experiments were made with cutaneous pectoris nerve-muscle preparations from frogs, *Rana pipiens* (~20 g body wt). Muscles were mounted in lucite chambers previously described (23) and bathed in Ringer's solution (R), pH 7.0, containing (in mM): NaCl, 116; KCl, 2.1; CaCl<sub>2</sub>, 1.8; NaH<sub>2</sub>PO<sub>4</sub>, 1; Na<sub>2</sub>HPO<sub>4</sub>, 2. When the ionic composition of the R solution was modified, the concentration of NaCl was adjusted to keep the tonicity constant. To denervate muscles, we cut the II and III spinal nerves of anesthetized frogs near the spinal cord and removed 1 cm of the common trunk. After 18–20 d the success of denervation was evaluated in all muscles by visual inspection under a dissecting microscope. Some muscles were also mounted in the lucite chamber and observed under a Zeiss microscope equipped with Nomarski optics. In other muscles, fibers were impaled near remnants of nerve branches to ascertain the absence of miniature endplate potentials.

### Electrophysiology

Intracellular recording of miniature endplate potentials at the endplate region was used to test the activity of  $\alpha$ -LTx and <sup>125</sup>I- $\alpha$ -LTx. Specific activity was defined as previously described (23). In these experiments a modified R containing 0.7 mM Ca<sup>2+</sup> and 4 mM Mg<sup>2+</sup> was used. To determine the effect of anti- $\alpha$ -LTx sera, we recorded the indirectly evoked compound action potentials in cutaneous pectoris muscles exposed to  $\alpha$ -LTx as previously described (8).

### Immunocytochemistry

The muscles, mounted in lucite chambers, were incubated for 1 h either at 2–3°C or at room temperature in R solution containing BSA, 1 mg/ml, and  $\alpha$ -LTx, 2.5 or 7.5  $\times 10^{-9}$  M. These concentrations roughly corresponded to 3.5 and 10 times the threshold dose for  $\alpha$ -LTx at the frog NMJ. The chamber was then drained and refilled with the same solution without toxin every 5 min (six to eight washes). Subsequently the muscles were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3. After fixation the muscle fibers were teased apart under a dissecting microscope by means of bent insect pins mounted on wooden sticks. Single fibers were then transferred to plastic Eppendorf tubes.

The bound toxin was revealed by indirect immunofluorescence using rabbit anti- $\alpha$ -LTx antisera and rhodamine-conjugated sheep anti-rabbit antibodies. To quench the aldehyde groups of fixatives, the muscle fibers were first soaked in glycine (0.1 M, pH 7.4, 10 min), washed with phosphate buffer (0.1 M, pH 7.3), then soaked in sodium borohydride (1%, 10 min, at 0°C), and finally extensively washed with phosphate buffer. Anti- $\alpha$ -LTx serum was diluted 1:10 with phosphate buffer (20 mM, pH 7.3) containing 0.5 M NaCl and 17% normal goat serum. After incubation at 37°C for 1 h, the fibers were washed for an additional hour with the 0.5 M NaCl, 20 mM phosphate buffer solution. Subsequently, rhodamine-conjugated sheep anti-rabbit antibodies (diluted 1:2.5 with the same solution used for the first antiserum) were applied at room temperature for 1 h. After being washed overnight with the 0.5 M NaCl–20 mM phosphate buffer solution containing 0.1% Triton X-100, the fibers were mounted on microscope slides in 95% glycerol–5% PBS and examined with a Zeiss Photomicroscope III equipped with fluorescence epillumination. Two sets of controls were run concomitantly with the experimental samples. In the first, the anti- $\alpha$ -LTx serum was replaced by preimmune serum; in the second, the muscle was not exposed to the toxin before treatment with anti- $\alpha$ -LTx serum.

### Cholinesterase Staining

Cutaneous pectoris muscles were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.3, and

processed according to Karnovsky and Roots (26). The staining of the nerve terminals was followed under a dissecting microscope and the reaction was stopped by extensive washing in 0.44 M sucrose.

## Morphometric Analyses

**DETERMINING THE NUMBER OF MUSCLE FIBERS IN THE CUTANEOUS PECTORIS MUSCLE:** Five muscles pinned to a Sylgard layer in the bottom of a Petri dish were fixed for 1 h with a cold (4°C) solution of 2% OsO<sub>4</sub> in 0.1 mM phosphate buffer, pH 7.3. Each muscle was longitudinally cut into three parts which were dehydrated and stacked for embedding in Epon. Semithin sections were cut perpendicular to the major axis of the fibers, stained with toluidine blue, and photographed in a Zeiss photomicroscope III. Fibers were then counted on the printed micrographs. The average number of fibers in individual muscles was found to be 517 ± 43 (SD).

**DETERMINING THE SURFACE AREA OF THE NERVE TERMINAL BRANCHES:** To obtain a rough estimate of the total surface area of terminal branches in one end plate, we considered the entire terminal arborization a cylinder with the height being the sum of the length of the individual branches. The length and number of the branches were determined on the photomicrographs of 10 terminals revealed by the cholinesterase reaction. The average length of the total arborization per terminal was 639 μm ± 180 (SD). This estimate is in good agreement with previous findings (28). The value of the circumference used to compute the surface area was the average of 20 perimeters of cross-sectioned resting terminals measured on printed electron micrographs (4.0 μm ± 1.1, SD). Fixation and processing of the muscles for electron microscopy are described elsewhere (4).

**MEASUREMENT OF THE AVERAGE TRANSVERSE DIMENSION OF FLUORESCENT NERVE TERMINALS:** In order to assess the degree of swelling induced by α-LTx (9, 16, 24), the transverse dimension of immunofluorescent terminals that had been exposed to α-LTx at either 2–3°C or room temperature was measured on printed micrographs (× 400) of front viewed terminals (see Fig. 8, C and D). Measurements were made normal to the major axis of individual branches, at 2-mm intervals. For the two experimental conditions a total of 300 and 270 measurements were made in portions of branches from 8 and 7 NMJs, respectively.

## α-LTx Binding

Entire cutaneous pectoris muscles were incubated for 90 min at 0°C in Eppendorf plastic tubes containing 200 μl of R solution supplemented with BSA (1 mg/ml), with a fixed amount of <sup>125</sup>I-α-LTx and various amounts of nonradioactive α-LTx. Final concentrations of the toxin ranged from 10<sup>-10</sup> to 4 × 10<sup>-9</sup> M. After this incubation the muscles were washed extensively (3 h; 10 washes) at 0°C, first with R solution containing BSA, then with double distilled water. Subsequently, the muscles were freeze-dried, weighed in a Cahn analytical microbalance (Cahn Instruments, Inc., Cerritos, CA), and finally counted in a Beckman GAMMA 4000 spectrometer. Unspecific binding was estimated by counting radioactivity in muscles exposed for 1 h to a large excess of unlabeled toxin (5 × 10<sup>-8</sup> M) before incubation with <sup>125</sup>I-α-LTx. Specific binding is defined as the difference between total and unspecific binding, expressed as femtomoles of α-LTx per milligram of muscle tissue dry weight.

## Materials

The following materials were purchased from the sources indicated below: Bolton-Hunter reagent, *N*-succinimidyl 3-(4-hydroxy-5-[<sup>125</sup>I]iodophenyl)propionate (specific radioactivity: 1,500 Ci/mmol) and ethylamine 1,2-[<sup>3</sup>H]-dopamine HCl (specific radioactivity: 3.2 Ci/mmol): The Radiochemical Centre, Amersham, Bucks, U.K.; <sup>125</sup>I-Protein A, various Sephadex gels, and activated Sepharose 4B: Pharmacia Inc., Uppsala, Sweden; low *M<sub>r</sub>* agarose, high and low gel temperature: Bio-Rad Laboratories, Richmond, CA; 8% glutaraldehyde vials, electron microscopy grade: Polysciences, Inc., Warrington, PA; trypsin: Boehringer, Darmstadt, Federal Republic of Germany; soybean trypsin inhibitor: Sigma Chemical Co., St. Louis, MO; rhodamine-labeled sheep anti-rabbit Ig, purchased from Dako, Copenhagen, Denmark, were bound onto a Sephadex DE52 column and eluted with phosphate buffer (10 mM, pH 7.4) containing 100 mM NaCl. Crude collagenase (type III, Sigma Chemical Co.) was purified as described by Schultz et al. (40). All other chemicals were reagent grade.

## RESULTS

### Characterization of Anti-α-LTx Sera and Purified Ig

The specificity of the anti-α-LTx sera and Ig was investigated by various methods. Ouchterlony double diffusion of

antisera against total black widow spider venom and purified α-LTx yielded single precipitation lines, which appeared at serum dilutions up to 1:16 and 1:32 (not shown). Crossed immunoelectrophoresis of the sera against total venom gave rise to two arcs of precipitation which, however, were continuous with each other (Fig. 1), indicating that the two responsible antigens had common determinants. This result confirms the microheterogeneity of α-LTx, which is known to contain at least two closely related components, similar in size but slightly different in charge (16). The monospecificity of the antibody was also shown by immunolabeling of SDS PAGE slabs, first soaked in the immune serum and then overlaid with <sup>125</sup>I-protein A. As can be seen in Fig. 2, the immune serum, when applied to the total venom homogenate (which gives a gel pattern of over 20 major bands) only bound to the large α-LTx band and to a minor doublet of slightly smaller *M<sub>r</sub>*. The latter is probably a degradation product of α-LTx, since it was found only in some preparations and tended to increase with storage. No radioactive bands appeared in the control gels treated with the preimmune serum.

The ability of anti-α-LTx sera and purified Ig to interfere with the action of α-LTx was studied in both synaptosomes and NMJs. Immune sera and the purified Ig, when mixed with the toxin in appropriate concentrations, were found to prevent the release response from synaptosomes and to prevent transmission block at the NMJ (Fig. 3). The block of the indirectly evoked compound action potential induced by a supramaximal dose of α-LTx was almost completely prevented by the anti-α-LTx immune serum. In contrast, the preimmune serum, as well as the immune serum that had been depleted of anti-α-LTx Ig by immunoadsorption onto an α-LTx-Sepharose column, were without effect.

### Experiments on Rat Brain Synaptosomes

The experiments on synaptosomes were carried out with the following two purposes: (a) to obtain information on the interaction of α-LTx with its receptor and with the anti-α-

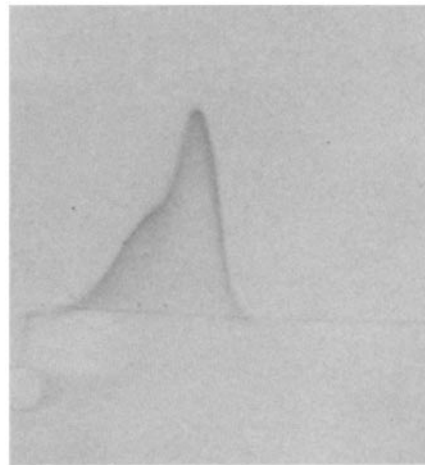
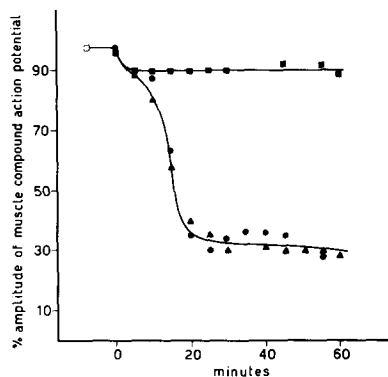
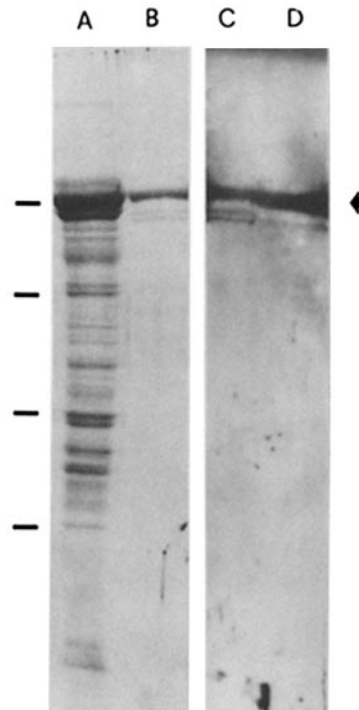


FIGURE 1 Crossed immunoelectrophoresis of anti-α-LTx Ig against total black widow spider venom gland homogenate. An aliquot (40 μg) of the gland homogenate prepared according to Frontali et al. (16) was electrophoresed in the first dimension. The separated antigens were then electrophoresed in the Ig-containing gel (3.5 ml of 1.1% agarose with 150 μl of rabbit anti-α-LTx serum). Material was run toward the anode because preliminary experiments showed no precipitation areas when electrophoresis was performed in the opposite sense. Coomassie Blue staining.

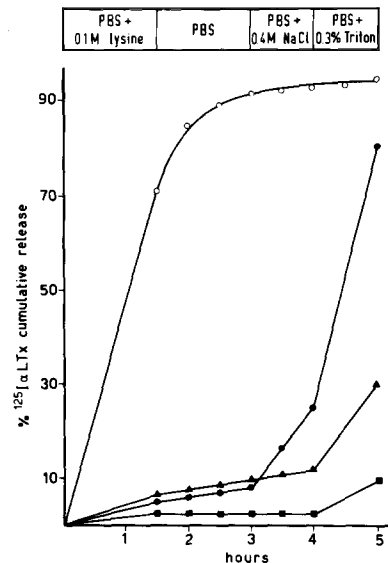
**FIGURE 2** Coomassie Blue staining and anti-body- $^{125}\text{I}$ -protein A radioimmunolabeling of an SDS polyacrylamide gel. A 7.5–12% polyacrylamide gradient slab gel was loaded with duplicate samples of black widow spider venom gland homogenate (60  $\mu\text{g}$  protein, lanes A and D) and purified  $\alpha$ -LTx (4  $\mu\text{g}$  protein, lanes B and C). After electrophoresis the gel was cut longitudinally. One part (lanes A and B) was stained with Coomassie Blue, another part was processed for immunolabeling with anti- $\alpha$ -LTx serum followed by  $^{125}\text{I}$ -protein A (lanes C and D). The arrow on the right marks the position of  $\alpha$ -LTx. Horizontal bars correspond to the positions of four standards: from top to bottom,  $\beta$ -galactosidase, BSA, ovalbumin, and chymotrypsinogen.



**FIGURE 3** Effects of  $\alpha$ -LTx (3 nM) on neuromuscular transmission when applied in R solution together with different sera (33  $\mu\text{l}/\text{ml}$ ). Ordinate: amplitude of the compound action potential of the muscle evoked by indirect stimulation normalized to the amplitude recorded immediately before  $\alpha$ -LTx was added ( $\square$ ). Abscissa: time in minutes after adding  $\alpha$ -LTx.  $\blacktriangle$ ,  $\alpha$ -LTx plus preimmune serum;  $\blacksquare$ ,  $\alpha$ -LTx plus anti- $\alpha$ -LTx serum;  $\bullet$ ,  $\alpha$ -LTx plus anti- $\alpha$ -LTx serum depleted of anti- $\alpha$ -LTx Igs by immunoabsorption onto an  $\alpha$ -LTx-Sepharose column.

LTx Ig and (b) to set up experimental conditions for the studies at the NMJ.

Central to the problem of receptor localization by means of anti- $\alpha$ -LTx is the stability of the toxin binding to its receptor. Therefore we first addressed our experiments in this direction. Synaptosome suspensions were exposed to  $^{125}\text{I}$ - $\alpha$ -LTx and then embedded in thin agarose films (10) in order to facilitate transfer through a series of washing solutions. The results are illustrated in Fig. 4. In agreement with previous findings (34, 42), we observed that the binding of  $\alpha$ -LTx to its receptor is very stable during incubation in physiological



**FIGURE 4** Effects of various treatments on the dissociation of  $^{125}\text{I}$ - $\alpha$ -LTx. Freshly prepared synaptosome suspensions in mKR (1 ml, 4.62 mg protein) were labeled with  $^{125}\text{I}$ - $\alpha$ -LTx ( $10^{-10}$  M;  $9 \times 10^5$  cpm) for 30 min at  $0^\circ\text{C}$ , then centrifuged (10,500 g, 5 min), washed, and resuspended in the same volume of mKR. 100- $\mu\text{l}$  aliquots (containing 38,500 cpm) were mixed with 10  $\mu\text{l}$  of either 20% formaldehyde, 20% glutaraldehyde (both in 125 mM phosphate buffer, pH 7.4), or plain phosphate buffer. Additional samples were prepared by diluting 100  $\mu\text{l}$  aliquots of  $^{125}\text{I}$ - $\alpha$ -LTx in mKR (40,000 cpm), containing no synaptosomes, with 10  $\mu\text{l}$  of either formaldehyde, glutaraldehyde, or mKR. After 30 min at room temperature the samples were heated at  $38^\circ\text{C}$  and mixed with 100  $\mu\text{l}$  of 3% fluid agarose, and the mixtures were poured in the space remaining between two coverslips sandwiched between two microscope slides held in position by spring clips. After gelation (30 min at  $0^\circ\text{C}$ ) the agar layer was recovered, sliced with a razor blade, and incubated at  $0^\circ\text{C}$  in the solutions indicated at the top of the figure.  $\circ$ , free, unfixed  $\alpha$ -LTx;  $\bullet$ , unfixed synaptosomes;  $\blacktriangle$ , synaptosomes fixed with formaldehyde;  $\blacksquare$ , synaptosomes fixed with glutaraldehyde. The data obtained with free  $\alpha$ -LTx exposed to fixatives before agar embedding were almost identical to those for the free unfixed  $\alpha$ -LTx samples ( $\circ$ ).

media. However, when agar strips containing synaptosomes were transferred to high salt medium, 20% of the bound toxin was readily released. Most of the remaining toxin was released during a further incubation in a medium supplemented with the non-ionic detergent Triton X-100, which can be used for permeabilization and washing in immunofluorescence techniques. Different results were obtained with synaptosomes that, after  $\alpha$ -LTx treatment and washing, had been fixed before embedding in agar. When synaptosomes were fixed with formaldehyde, the high salt-induced release disappeared, and the Triton-induced release was decreased; after glutaraldehyde, even the latter was almost entirely prevented. Thus, glutaraldehyde fixation appeared as an adequate procedure for preserving the  $\alpha$ -LTx-receptor complex.

A second point to be investigated was the kinetics of the anti- $\alpha$ -LTx Ig interaction with the  $\alpha$ -LTx-receptor complex. We found that synaptosomes that had been previously exposed to the toxin were able to specifically bind  $^{125}\text{I}$ -anti- $\alpha$ -LTx Ig, whereas untreated synaptosomes were not (Fig. 5 left). Moreover, this Ig binding was not appreciably affected by fixation of  $\alpha$ -LTx-treated synaptosomes with either formaldehyde or glutaraldehyde (data not shown). Fig. 5 illustrates

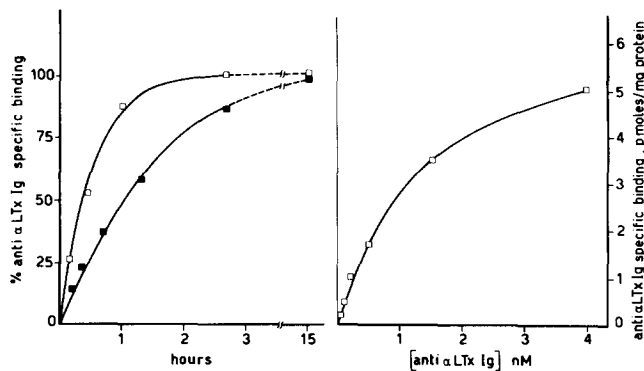


FIGURE 5  $^{125}\text{I}$ -anti- $\alpha$ -LTx Ig binding to  $\alpha$ -LTx-treated synaptosomes: time course (left) and concentration dependence (right). Pellets of freshly prepared purified synaptosomes were resuspended in mKR (11 mg protein/ml) and treated with  $\alpha$ -LTx ( $8 \times 10^{-9}$  M) at  $0^\circ\text{C}$  for 30 min. After centrifugation (10,500 g, 5 min), washing, and resuspension in mKR, they were divided into aliquots (55  $\mu\text{g}$  of protein, 200  $\mu\text{l}$ ) which were exposed to affinity-purified  $^{125}\text{I}$ -anti- $\alpha$ -LTx Ig. (Left) In these experiments 20 ng (18,000 cpm) of  $^{125}\text{I}$ -anti- $\alpha$ -LTx Ig was used; the incubations were carried out for the indicated time periods first at either  $37^\circ\text{C}$  ( $\square$ ) or  $22^\circ\text{C}$  ( $\blacksquare$ ) and then at  $4^\circ\text{C}$  (dashed lines). 100% specific binding = 9,200 cpm. (Right) In these experiments the concentration of  $^{125}\text{I}$ -anti- $\alpha$ -LTx Ig varied in the range indicated in the abscissa; the incubations were carried out at  $37^\circ\text{C}$  for 90 min. For both panels, values shown are differences between total binding and unspecific binding, i.e., the  $^{125}\text{I}$ -anti- $\alpha$ -LTx binding measured in the presence of 4.5  $\mu\text{g}$  of unlabeled anti- $\alpha$ -LTx Ig (averages of two experiments). During the experiments shown on the left, the unspecific binding increased from 750 to 2,250 cpm; in the experiments on the right, it varied between 7 and 16% of the specific binding.

the time course (left panel) and the concentration dependency (right panel) of the  $^{125}\text{I}$ -Ig specific binding. At  $37^\circ\text{C}$  the binding interaction saturated after  $\sim 90$  min, while at room temperature a longer time was needed. In synaptosomes exposed to saturating concentrations of first  $\alpha$ -LTx and then anti- $\alpha$ -LTx Ig, almost 5 pmol of the latter was found to bind per milligram of synaptosomal protein. Since the maximal specific binding of  $\alpha$ -LTx to rat cerebral cortex synaptosomes is 0.88 pmol/mg protein (34), these results indicate that at saturation, five molecules of Ig are bound per each  $\alpha$ -LTx-receptor complex.

### Experiments on Frog NMJ

**BINDING OF  $\alpha$ -LTx:** The frog cutaneous pectoris nerve-muscle is the experimental preparation in which the presynaptic effect of  $\alpha$ -LTx was originally discovered and then extensively investigated (6, 7, 9, 16, 17, 24, 29). However, until now, no direct information was available on the binding of the toxin in this preparation. Biochemical experiments were therefore carried out as a necessary complement to the immunofluorescence data reported below.

Intact muscles (which are three to four fibers thick) were incubated at  $0^\circ\text{C}$  with labeled  $\alpha$ -LTx (Fig. 6). A specific binding was detected which approached saturation at concentrations in the nanomolar range. When analyzed according to Scatchard, the data fell along a straight line, suggesting the existence in the preparation of a single class of high-affinity receptors ( $b_{\text{max}}$ :  $1.36 \pm 0.16 \times 10^9$  sites/mg of muscle tissue, dry weight (average  $\pm$  SE of four experiments);  $K_d = 5 \times 10^{-10}$  M). This high-affinity binding was not detected in muscles that had been successfully denervated.

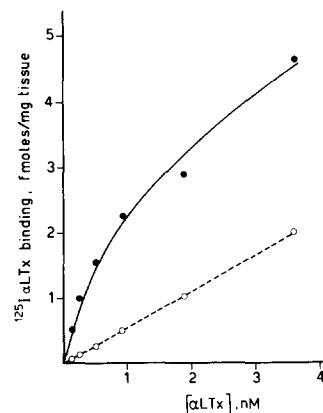


FIGURE 6  $\alpha$ -latrotoxin binding to cutaneous pectoris muscle as a function of toxin concentration. Entire muscles were incubated for 90 min at  $0^\circ\text{C}$  in 200  $\mu\text{l}$  of R solution supplemented with BSA and containing a fixed amount of  $^{125}\text{I}$ - $\alpha$ -LTx (150,000 cpm; 40 pM) as well as nonradioactive  $\alpha$ -LTx, to the final concentrations indicated in abscissa. Total ( $\bullet$ ) and unspecific ( $\circ$ ) binding are shown. Each symbol represents the average of values obtained in two muscles during a single experiment.

### Immunofluorescence

In the frog cutaneous pectoris muscle, NMJs are easy to identify because of their characteristic structure (14, 31, 38). The motor axon loses its myelin sheet at some distance from the muscle fiber. The preterminal unmyelinated axon divides each side into several branches which make contact with the muscle fiber after running free for many micrometers. The true terminal branches, which are a few hundred micrometers long, run almost parallel to the major axis of the muscle fiber. In Fig. 7 the typical aspect of the terminal branching is revealed by the acetylcholinesterase reaction. The preterminal arborization occupies the cholinesterase-negative region towards the middle of the endplate (31).

Fig. 8 shows fibers from preparations that had been treated first with  $\alpha$ -LTx, then with anti- $\alpha$ -LTx serum, and finally with rhodamine-conjugated sheep anti-rabbit Ig. Two experimental conditions were investigated. In the first (Fig. 8, A-C) the muscle was exposed to the toxin at  $2-3^\circ\text{C}$ , in the second at room temperature. The immunofluorescence pattern on the fibers of muscles treated in the cold closely resembled the images revealed at rest by the cholinesterase reaction (Fig. 7). The muscle fibers were uniformly negative, and very little or no fluorescence was observed on either the preterminal branches (Fig. 8, A and B) or the residual portions of motor axons (Fig. 8, C and D). In contrast, a bright fluorescence appeared over the entire terminal arborization. In different endplates, and even in different portions of the same endplate, the distribution of the label was appreciably variable. At some sites the fluorescence appeared almost uniformly distributed (Fig. 8, A and B), while at some others it showed a punctate pattern (Fig. 8 C) or was concentrated in two parallel rows marking the edges of the terminal branches (Fig. 8 C).

In the fibers of muscles that had been exposed to  $\alpha$ -LTx at room temperature, the overall pattern of terminal branching revealed by immunofluorescence (Fig. 8 D) was analogous to that described for the muscles treated in the cold. However, the prominence of the fluorescence along the edges of the terminals was frequent and very marked in the fibers exposed at room temperature. Moreover, the transverse dimension of these terminal branches was obviously increased. This change,

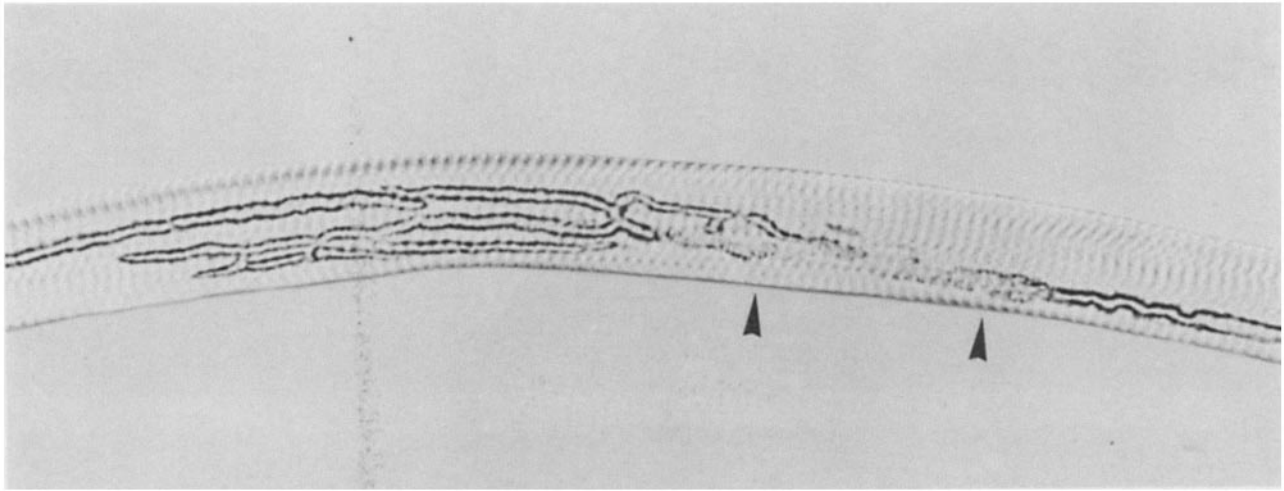


FIGURE 7 Light micrograph of an NMJ on a single fiber teased from cutaneous pectoris muscle of *Rana pipiens*. The terminal branching of the motor nerve is revealed by the cholinesterase reaction according to Karnovsky and Roots (26). Note the lack of cholinesterase activity in the region between the two arrowheads. This region is where the unmyelinated preterminal axon branches to form the terminal arborization. Compare this figure with Fig. 8.  $\times 330$ .

however, was not uniform along the entire length of the branches, and swollen portions were frequently continuous with portions of smaller size, where fluorescence appeared more uniformly distributed (Fig. 8D). The difference in apparent transverse dimension of fluorescent terminal branches between preparations exposed to  $\alpha$ -LTx at either 2–3°C or room temperature was further substantiated by the results of a morphometric analysis. The average dimensions ( $\mu\text{m} \pm \text{SD}$ ) were  $5.3 \pm 1.3$  in the preparations treated in the cold, and  $10 \pm 2.2$  in those treated at room temperature. These dimensions are 5–10 times the diameters of terminals measured from electron micrographs. They must overestimate the true diameters since they include the fluorescence halo. They were measured to provide a rough index of swelling that occurred in the two conditions.

Two types of controls were used in order to ascertain the specificity of the immunofluorescence images so far described. In the first, the muscles were fixed and exposed to the antibodies without prior treatment with  $\alpha$ -LTx; in the second,  $\alpha$ -LTx treatment was made as usual, but the first serum was not the anti- $\alpha$ -LTx but the rabbit preimmune serum. Under both these conditions the preparations appeared uniformly negative.

Since the immunofluorescence images illustrated in Fig. 8 were suggestive of a specific localization of  $\alpha$ -LTx binding at the endplate region, it seemed imperative to perform experiments in muscles that had their motor nerve trunks removed 20 d before the experiments and that were therefore denervated according to the criteria specified in Materials and Methods. In these muscles no specific immunofluorescence images were ever observed.

A further series of experiments aimed to unravel the nature of the  $\alpha$ -LTx receptor was carried out by pretreatment of the normally innervated muscle preparations with proteolytic enzymes before exposure to  $\alpha$ -LTx. Both trypsin (100  $\mu\text{g}/\text{ml}$ ; 1 h, room temperature, followed by soybean trypsin inhibitor, 200  $\mu\text{g}/\text{ml}$ , to block the reaction) and purified collagenase (0.3 mg/ml, 1 h, room temperature) entirely prevented the appearance of the specific immunofluorescent images. In contrast, normal images were seen in muscles preincubated with trypsin together with its inhibitor. Finally, pretreatment

with detergent was also studied. In muscles preincubated with Triton X-100 (0.5%, 1 h, room temperature) and then extensively washed with R, exposed to  $\alpha$ -LTx in the cold, and processed for immunofluorescence as described above, no labeled endplate images were ever observed.

## DISCUSSION

In the present paper we report biochemical, immunochemical, and immunocytochemical studies on the  $\alpha$ -LTx receptor, carried out by the use of the purified toxin in conjunction with monospecific antibodies against  $\alpha$ -LTx. Two experimental systems were investigated: rat cerebral cortex synaptosomes and frog NMJ. Our initial purpose was to obtain complementary data on the two systems. These systems appear to be similar because (a) the effects produced by the toxin are analogous (compare references 9, 24, and 29 with references 2, 19, 34, and 41); (b) the two systems are sensitive to approximately the same concentrations of  $\alpha$ -LTx (16, 34, 41, 45); and (c) the high-affinity receptors have similar kinetic features (compare the data on  $\alpha$ -LTx binding in this paper with references 34 and 42).

### Stability of Antibody-Toxin-Receptor Complex

Part of the experiments we carried out on synaptosomes were aimed to characterize the receptor- $\alpha$ -LTx-anti- $\alpha$ -LTx Ig interaction and thus to define conditions appropriate for the immunocytochemical studies. In particular, from these studies we learned that the stability of the  $\alpha$ -LTx-receptor binding could be preserved by glutaraldehyde fixation without impairment of the subsequent recognition of the bound toxin by the specific Ig. This observation was important in setting up the experimental protocol that was successfully used for the immunofluorescence studies at frog NMJ. However, when this protocol was applied to synaptosomes (cryosections of centrifugation pellets or synaptosomes attached to polylysine-coated slides), the results were disappointing because of the low ratio between specific signal and background fluorescence. This failure is probably due to the low density of  $\alpha$ -LTx receptors in the rat synaptosome plasma membrane (34).

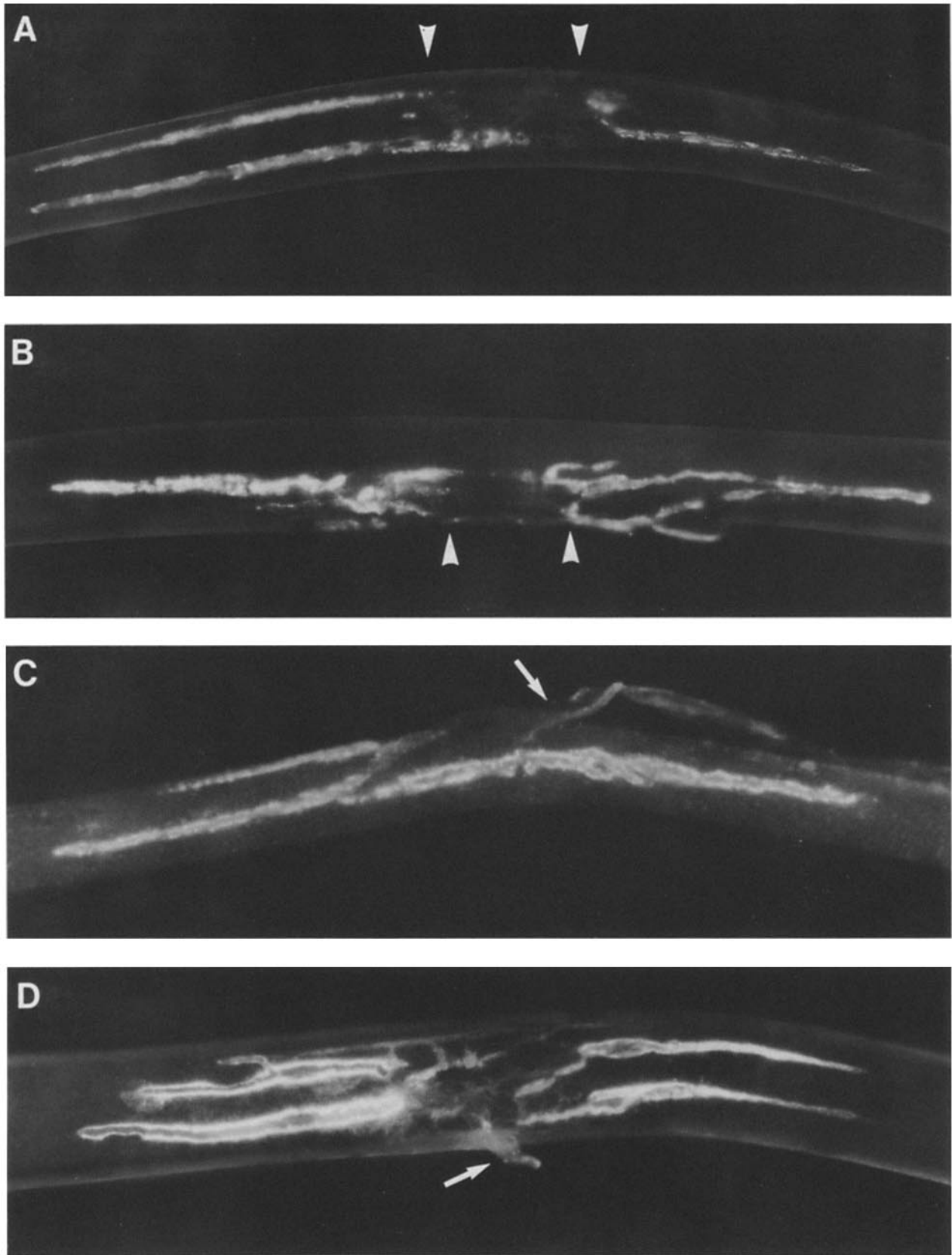


FIGURE 8  $\alpha$ -LTx immunofluorescence in NMJs on single fibers teased from cutaneous pectoris muscle of *Rana pipiens*. The muscles were incubated at 2–3°C (A–C) or at room temperature (D) for 1 h in R solution containing  $\alpha$ -LTx ( $7.5 \times 10^{-9}$  M), washed, and fixed in 2% glutaraldehyde. Then the fibers were teased apart. The bound toxin was revealed by indirect immunofluorescence. The regions of intense fluorescence are arranged in a branching pattern characteristic of frog motor nerve endings. In A and B note the paucity of fluorescence in the region between the arrowheads corresponding to the location of the unmyelinated preterminal branches (compare with Fig. 7). In C and D the residual portions of motor axons are evident (arrows). The prominence of fluorescence along the edges of the terminal is particularly evident in the preparation treated with the toxin at room temperature (D). Little fluorescence is associated with the muscle fibers and with the residual portions of the axons.  $\times 350$ .

## Experiments on the NMJ

Biochemical experiments with  $\alpha$ -LTx demonstrated the existence of a single class of high-affinity  $\alpha$ -LTx receptors in innervated frog muscles. Occupancy of these receptors occurred in the  $10^{-10}$ – $10^{-9}$  molar range of  $\alpha$ -LTx. This finding is in good agreement with the concentration dependency of the toxin-induced release of quanta of acetylcholine (see references 16 and 24). This high-affinity binding disappeared after denervation.

The bound toxin was revealed by immunofluorescence only in the end plate region of the muscle. The muscle fibers, as well as the myelinated axons, were consistently negative. Little or no fluorescence was found in the central region of the endplate. This area, which is also negative for acetylcholinesterase and which contains no postsynaptic folds when observed at the electron microscope level, is the site where the preterminal unmyelinated fibers branch before making the synaptic contact with the muscle fiber.

In our experimental conditions  $\alpha$ -LTx as well as Ig were applied before any membrane permeabilization of the tissue. Thus, the receptor responsible for toxin binding was exposed at the external surface, and could not be a secluded component of the cytoplasm. The lack of immunofluorescence observed when the muscles were treated with Triton X-100 before the application of  $\alpha$ -LTx indicates that the receptor is an integral membrane component, and its sensitivity to proteolytic enzymes suggests that it is probably a protein. This conclusion is in line with previous data on synaptosomes and PC12 cells (34, 35, 42).

The resolution of immunofluorescence is not adequate to answer directly the question of whether the  $\alpha$ -LTx receptor is localized in the membrane of terminal branches or in other structures of the endplate region (postsynaptic membrane, Schwann cells). Indeed, a presynaptic binding has been suggested in the past for several reasons: (a) the massive stimulation of secretion of quanta of acetylcholine, which ultimately results in the depletion of the total quantal store of the terminals; (b) the lack of effect of  $\alpha$ -LTx on muscle electrophysiology and contractile properties; and (c) the striking ultrastructural changes, which are restricted to the nerve terminals, with no obvious changes of muscle fibers and Schwann cells (for review see reference 24). The present work provides two further pieces of evidence supporting the  $\alpha$ -LTx binding to the presynaptic membrane. These are (a) the concomitant disappearance of specific, high-affinity binding and immunofluorescence after denervation, and (b) the swelling of the immunofluorescent terminal branches, which was particularly evident in the preparations exposed to  $\alpha$ -LTx at room temperature. These results are in good agreement with those of previous ultrastructural studies on the frog NMJ exposed to the toxin, which showed depletion of synaptic vesicles accompanied by profound terminal swelling (9, 16). Further support for the identification of the room-temperature fluorescence figures as swollen presynaptic terminals comes from the sharp local differences in transverse dimension observed in adjacent portions of individual branches. Since the increase in the surface area, accompanied by a swelling of the terminal, is due to local incorporation of vesicle membrane into the axolemma, the sharp differences in the transverse dimension might be the consequence of the abrupt variation in the vesicles concentration which is known to exist in the different portions of individual terminal branches (4).

Suggestions have been made about a possible localization of  $\alpha$ -LTx receptors at discrete sites of the presynaptic membrane (42). The resolution of immunofluorescence is certainly too low to definitely set this issue. However, we wish to emphasize that the images we have obtained failed to reveal a constant, ordered pattern of fluorescence, as would be expected in the case of a nonrandom distribution of the  $\alpha$ -LTx receptors. To calculate the density (number per unit area) of the receptors, we have therefore assumed that they are uniformly distributed over the entire plasma membrane of the synaptic terminal. Our measurements indicate that one cutaneous pectoris muscle (average dry weight: 2.36 mg) contains  $517 \pm 43$  (SD) fibers. Each of these fibers is endowed with one endplate. The surface area of an individual terminal arborization (estimated as described in Materials and Methods) is on the order of  $2,500 \mu\text{m}^2$ . Thus, the high-affinity binding sites that we have found ( $3.21 \times 10^9$  sites per muscle) should be distributed in a total surface area of  $1.32 \times 10^6 \mu\text{m}^2$ , with a density of  $\sim 2,400/\mu\text{m}^2$ . Although unexpected, this high density does not seem unreasonable in view of two simple considerations: (a) in nerve-muscle preparations, the total surface area of nerve terminals is a small fraction of the total membrane surface area, and thus it would be difficult to detect high-affinity binding sites with low density; (b) the intense immunofluorescent specific signal obtained from our single muscle fibers is only compatible with a relatively high receptor density. Indeed, in rat brain synaptosomes and in PC12 cells where the  $\alpha$ -LTx receptor density was found to be much lower ( $20\text{--}30/\mu\text{m}^2$  and  $12/\mu\text{m}^2$ , respectively) (34, 35), the immunofluorescent signal was undetectable.

In conclusion, the data that we have reported support the view that the  $\alpha$ -LTx receptor is a protein highly concentrated at the plasma membrane of synaptic terminals, where it is exposed at the external surface. Other molecules specific for the presynaptic compartment have been recently described. Synapsin I, which is a substrate for both  $\text{Ca}^{2+}$ - and cAMP-dependent protein kinases, is now recognized to be a peripheral membrane protein located at the cytoplasmic surface of synaptic vesicles in many (and possibly all) synapses (10, 11, 25). Another widely distributed protein exposed at the cytoplasmic surface of synaptic vesicles has been recognized by the use of monoclonal antibodies (33), and one antigen has been localized at the inner surface of the same organelles by polyclonal antibodies (21). Moreover, an internally exposed glycosaminoglycan has been recognized in cholinergic synaptic vesicles (43, 44). In the presynaptic membrane of synaptosomes from *Torpedo* electric organ, one specific protein band has been identified by SDS PAGE analysis (36) and one antigen (possibly a ganglioside) by immunocytochemistry (43). Very recently it has been reported that two other neurotoxins bind specifically to the plasma membrane of nerve terminals: botulinum toxin to mouse NMJ (13) and glycerotoxin to synaptosomes from *Torpedo* electric organ (37). Our data and these recent findings indicate that toxin receptors are specific components of the plasma membrane of synaptic terminals. Thus it seems that neurotoxins are important tools for the investigation of not only the process of transmitter release, but also the topological specialization of the nerve terminal plasma membrane with respect to the remaining portions of the axolemma.

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## REFERENCES

1. Axelsen, N. H., J. Krol, and B. Weeke. 1973. Quantitative Immunoelectrophoresis. Methods and Applications. Universitetsforlaget, Oslo. 169 pp.
2. Baba, A., and J. R. Cooper. 1980. The action of black widow spider venom on cholinergic mechanisms in synaptosomes. *J. Neurochem.* 34:1369-1379.
3. Bolton, A. E. 1977. Radioiodination Techniques. Review 18. The Radiochemical Centre, Amersham, Bucks, U.K. 70 pp.
4. Ceccarelli, B., W. P. Hurlbut, and A. Mauro. 1973. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57:499-524.
5. Ceccarelli, B., and F. Clementi, editors. 1979. Neurotoxins: Tools in Neurobiology. *Adv. Cytopharmacol.* 3:1-461.
6. Ceccarelli, B., F. Grohovaz, and W. P. Hurlbut. 1979. Freeze-fracture studies of frog neuromuscular junctions during intense release of transmitter. I. Effects of black widow spider venom and  $Ca^{2+}$ -free solutions on the structure of the active zone. *J. Cell Biol.* 81:163-177.
7. Ceccarelli, B., and W. P. Hurlbut. 1980. Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* 60:396-441.
8. Ceccarelli, B., and W. P. Hurlbut. 1980.  $Ca^{2+}$ -dependent recycling of synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 87:297-303.
9. Clark, A. W., A. Mauro, H. E. Longenecker, Jr., and W. P. Hurlbut. 1970. Effects of black widow spider venom on the frog neuromuscular junction. Effects on the fine structure. *Nature (Lond.)*, 225:703-705.
10. De Camilli, P., R. Cameron, and P. Greengard. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* 96:1337-1354.
11. De Camilli, P., S. M. Harris, Jr., W. B. Huttner, and P. Greengard. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96:1355-1373.
12. De Camilli, P., T. Ueda, F. E. Bloom, E. Battemberg, and P. Greengard. 1979. Widespread distribution of Protein I in the central and peripheral nervous system. *Proc. Natl. Acad. Sci. USA* 76:5977-5981.
13. Dolly, J. O., J. Black, R. S. Williams, and J. Melling. 1984. Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. *Nature (Lond.)*, 307:457-460.
14. Dreyer, F., and K. Peper. 1974. A monolayer preparation of innervated skeletal muscle fibers of the cutaneous pectoris muscle of the frog. *Pfluegers Arch.* 348:257-262.
15. Eaker, D., and T. Wadström, editors. 1979. Natural Toxins. Pergamon Press, Oxford, U.K. 709 pp.
16. Frontali, N., B. Ceccarelli, A. Gorio, A. Mauro, P. Siekevitz, M. C. Tzeng, and W. P. Hurlbut. 1976. Purification from black widow spider venom of a factor causing the depletion of synaptic vesicles at neuromuscular junctions. *J. Cell Biol.* 68:462-479.
17. Gorio, A., L. L. Rubin, and A. Mauro. 1978. Double mode of action of black widow spider venom on frog neuromuscular junction. *J. Neurocytol.* 7:193-205.
18. Grasso, A., M. Pelliccia, and S. Alemá. 1982. Characterization of  $\alpha$ -latrotoxin interaction with rat brain synaptosomes and PC12 cells. *Toxicol.* 20:149-156.
19. Grasso, A., and M. I. Senni. 1979. A toxin purified from the venom of black widow spider affects uptake and release of radioactive aminobutyrate and *N*-epinephrine from rat brain synaptosomes. *Eur. J. Biochem.* 102:337-344.
20. Hajos, F. 1975. An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* 93:485-489.
21. Hooper, J. E., S. S. Carlson, and R. B. Kelly. 1980. Antibodies to synaptic vesicles purified from *Narcine* electric organ bind a subclass of mammalian nerve terminals. *J. Cell Biol.* 87:104-113.
22. Howard, B. D., and C. B. Gundersen. 1980. Effects and mechanisms of polypeptide neurotoxins that act presynaptically. *Annu. Rev. Pharmacol. Toxicol.* 20:307-336.
23. Hurlbut, W. P., and B. Ceccarelli. 1974. Transmitter release and recycling of synaptic vesicle membrane at the neurotransmitter junction. *Adv. Cytopharmacol.* 2:141-154.
24. Hurlbut, W. P., and B. Ceccarelli. 1979. Use of black widow spider venom to study the release of neurotransmitters. *Adv. Cytopharmacol.* 3:87-115.
25. Huttner, W. B., W. Schiebler, P. Greengard, and P. De Camilli. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374-1388.
26. Karnowsky, M. J., and L. Roots. 1964. A direct "coloring" technique for cholinesterases. *J. Histochem. Cytochem.* 12:219-221.
27. Lee, C. Y., editor. 1979. Snake Venoms. Handbook of Experimental Pharmacology, Vol. 52. Springer Verlag, Berlin. 3-1130.
28. Letinsky, M. S., K. H. Fischbeck, and U. J. McMahan. 1976. Precision of reinnervation of original postsynaptic sites in frog muscle after a nerve crush. *J. Neurocytol.* 5:691-718.
29. Longenecker, H. E., W. P. Hurlbut, A. Mauro, and A. W. Clark. 1970. Effects of black widow spider venom on the frog neuromuscular junction. *Nature (Lond.)*, 225:701-703.
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
31. Mc Mahan, U. J., N. C. Spitzer, K. Peper. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. B Biol. Sci.* 181:421-430.
32. Maizel, J. V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:179-246.
33. Matthew, W. D., L. Tsavaler, and L. F. Reichhardt. 1981. Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol.* 91:257-269.
34. Meldolesi, J. 1982. Studies on  $\alpha$ -latrotoxin receptors in rat brain synaptosomes. Correlation between toxin binding and stimulation of transmitter release. *J. Neurochem.* 38:1559-1569.
35. Meldolesi, J., L. Madeddu, M. Torda, G. Gatti, and E. Niutta. 1983.  $\alpha$ -latrotoxin action in the neurosecretory PC12 cell line. Studies on toxin binding and stimulation of neurotransmitter release. *Neuroscience*. 10:997-1009.
36. Morel, N., R. Manaranche, M. Israël, and T. Gulik-Krzywicki. 1982. Isolation of a presynaptic plasma membrane fraction from *Torpado* cholinergic synaptosomes: evidence for a specific protein. *J. Cell Biol.* 93:349-356.
37. Morel, N., M. Thieffry, and R. Manaranche. 1983. Binding of *Glycera convoluta* neurotoxin to cholinergic nerve terminal plasma membranes. *J. Cell Biol.* 97:1737-1744.
38. Peper, K., F. Dreyer, C. Sandri, K. Akert, and H. Moor. 1974. Structure and ultrastructure of the frog motor end plate. *Cell Tissue Res.* 149:437-455.
39. Reichhardt, L. F., and R. B. Kelly. 1983. A molecular description of nerve terminal function. *Annu. Rev. Biochem.* 52:871-926.
40. Schultz, G. S., M. P. Sarras, G. R. Gunther, B. E. Hull, H. A. Alicea, F. S. Gorelick, and J. D. Jamieson. 1980. Guinea pig pancreatic acini prepared with purified collagenase. *Exp. Cell Res.* 130:49-62.
41. Tzeng, M. C., R. S. Cohen, and P. Siekevitz. 1978. Release of neurotransmitters and depletion of synaptic vesicles in cerebral cortex slices by  $\alpha$ -latrotoxin from black widow spider venom. *Proc. Natl. Acad. Sci. USA* 75:4016-4020.
42. Tzeng, M. C., and P. Siekevitz. 1979. The binding interaction between  $\alpha$ -latrotoxin from black widow spider venom and a dog cerebral cortex synaptosomal membrane preparation. *J. Neurochem.* 33:263-274.
43. Walker, J. H., R. T. Jones, J. Obrocki, G. P. Richardson, and H. Stadler. 1982. Presynaptic plasma membrane and synaptic vesicles of cholinergic nerve endings demonstrated by means of specific antisera. *Cell Tissue Res.* 223:101-116.
44. Walker, J. H., J. Obrocki, and C. W. Zimmermann. 1983. Identification of a proteoglycan antigen characteristic of cholinergic synaptic vesicles. *J. Neurochem.* 41:209-216.
45. Watanabe, O., and J. Meldolesi. 1983. Effect of  $\alpha$ -latrotoxin of black widow spider venom on synaptosome ultrastructure. Morphometric analysis and correlation with the effects on transmitter release. *J. Neurocytol.* 12:517-531.