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Fate of α-Bungarotoxin Bound to Acetylcholine Receptors of Normal and Denervated Muscle

Abstract. *In organ culture, α-[^{125}I]bungarotoxin bound to extrajunctional receptors of denervated muscle is lost from the tissue at a more rapid rate than the toxin bound to the junctional receptors of normal muscle. The rapid loss of toxin from denervated muscle can be blocked by inhibitors of energy production and protein synthesis, and may reflect turnover of the toxin-receptor complex in the membrane.*

The acetylcholine receptor in vertebrate skeletal muscle is a membrane protein whose interaction with the transmitter acetylcholine causes a change in the permeability of the muscle membrane to sodium and potassium ions. Normally the receptors are almost exclusively confined to the region of the muscle surface underlying the nerve terminal (*1*). After denervation, however, the receptors occupy the entire muscle surface although the peak receptor concentration in the extrajunctional membrane never reaches that of the end plate membrane (*2*). Since the receptor is an identifiable component of the muscle membrane, the change in acetylcholine sensitivity that occurs after denervation offers an opportunity to study the mechanism by which a cell regulates the distribution of a particular protein on its surface. We have attempted to follow the fate of acetylcholine receptors in skeletal muscle by binding to them a radioactively labeled, small protein toxin—α-[^{125}I]-bungarotoxin—that binds tightly and specifically to the receptor (*3, 4*). Loss of radioactivity from normal and denervated muscles was then followed in organ culture.

Normal and denervated diaphragms of rats (50 to 70 g) were labeled by injection of α-[^{125}I]bungarotoxin (12 μg per 100 g of body weight) into the thoracic cavity. The extent of labeling was comparable to the maximal labeling obtained by incubation of diaphragms with toxin in vitro and permitted survival of at least 75 percent of the animals (*5*). Three hours after the injection, the diaphragms were removed with a small border of ribs attached and transferred to organ culture (*6*). After three changes of medium at 30-minute intervals to reduce levels of free toxin in the tissue, one hemidiaphragm was taken to determine the amount of radioactivity initially bound (0-hour sample). The other hemidiaphragm was cultured an additional 24 hours before analysis. Because the diaphragm can be conveniently divided into regions containing end plates and regions devoid of end plates, the radioactivity specifically bound to the end plate region could be calculated. To eliminate variation arising from differences in the extent of initial binding among animals, radioactivity bound to the end plate at 24 hours was expressed as a percentage of that present at 0 hour for each animal. The loss of radioactivity from denervated muscles was examined in similar experiments in animals whose left hemidiaphragms had been denervated for 5 days prior to the injection so that extrajunctional acetylcholine sensitivity would be high. Portions of the same denervated hemidiaphragm were analyzed before and after culturing. Only tissue lacking end plates was examined in order to restrict the analysis to those receptors which appear after denervation. Further details of the experiment are given in (*7*).

When hemidiaphragms from normal rats were labeled and cultured, very little loss of radioactivity from the muscles occurred. After 24 hours in culture, 79 ± 7 percent (mean $\pm$ S.E.M.) of the radioactivity specifically associated with the end plate region remained in the tissue. To determine whether the retention of radioactivity by the muscles occurs through readsorption of dissociated toxin, muscles were cultured with and without a large excess (20 μg/ml) of unlabeled toxin in the medium. No difference in the amount of radioactivity lost from the tissue was observed in the two cases. Thus in normal muscles, most of the toxin-receptor complex which is formed appears to be stable over a 24-hour period. In contrast, radioactivity associated with the extrajunctional toxin-receptor complex of denervated muscle is rapidly lost from the tissue. Only 19 ± 2 percent of the radioactivity originally associated with regions devoid of end plates from denervated hemidiaphragms remained after 24 hours in culture. To investigate the mechanism of this rapid loss of radioactivity, we labeled denervated muscles and cultured them in the presence of an inhibitor of protein synthesis. The addition of cycloheximide (10 μg/ml) to the culture medium caused 61 ± 5 percent of the radioactivity to be retained. This concentration of cycloheximide blocks 95 percent of the protein synthesis in rat diaphragm (*8*).

The release of radioactivity from denervated muscle was further examined by measuring its rate of accumulation in the culture medium over the 24-hour period. Denervated left hemidiaphragms were cultured as described above, except that the medium was changed five times at 30-minute intervals, and the final incubation medium contained 2 μg of unlabeled toxin per milliliter. Samples of medium were then taken at 4- to 5-hour intervals, and at the end of the experiment the hemidiaphragms were analyzed for remaining radioactivity. Parallel cultures of the innervated right hemidiaphragms were used as a measure of release from sources other than the extrajunctional receptors. The percentage of radioactivity remaining associated with extrajunctional receptors in the muscle was then calculated for the various times and plotted on semilogarithmic coordinates (Fig. 1). After an initial lag of 1 to 2 hours, the release of radioactivity occurred as a single first-order process. In two such experiments, half-times of 7.8 and 8.4 hours were obtained for the rate of release, and 86 and 85 percent, re-

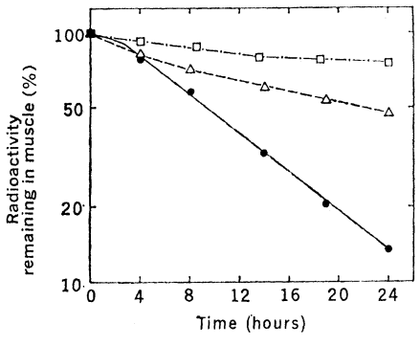

Fig. 1. Loss of radioactivity from extrajunctional receptors of denervated muscle. In each experiment two denervated left hemidiaphragms were cultured in 13 ml of medium. At the times indicated, duplicate 0.10-ml samples were withdrawn, and their radioactivity was determined. At the end of the experiment the hemidiaphragms were analyzed for radioactivity remaining in the tissue. The two corresponding normal right hemidiaphragms were cultured together in a separate dish as a blank to determine the amount of release from sources other than extrajunctional receptors. This background did not exceed 10 percent of the release obtained with the denervated hemidiaphragms and was subtracted from the appropriate values. Similarly, residual binding to the right hemidiaphragms was subtracted from that obtained with the left in order to remove any contribution from binding to end plate receptors and from nonspecific binding to extrajunctional regions. The total amount of radioactivity initially associated with extrajunctional receptors of the denervated muscle was calculated as the sum of the total radioactivity released into the medium and that remaining in the muscle. ●, No additives; □, 20 m*M* NaCN present throughout; △, cycloheximide (10 μg/ml) present throughout.

spectively, of the radioactivity initially bound was released in the 24-hour period. The release of radioactivity was decreased by inhibitors of energy production. Thus, 20 m*M* NaCN (Fig. 1) or 2 m*M* dinitrophenol allowed only 25 percent of the radioactivity to be released in 24 hours. Addition of the protein synthesis inhibitors cycloheximide (Fig. 1) or puromycin to the cultures also reduced the rate of release.

To establish that the radioactivity originally associated with normal and denervated muscles in these experiments represented toxin-receptor complex, we homogenized muscles in detergent, centrifuged the homogenate, and analyzed the high-speed supernatant by density gradient zone centrifugation. In all cases, about 70 percent of the radioactivity in the gradient migrated with a sedimentation constant characteristic of toxin-receptor complex (*4, 5*), while most of the remainder occurred in a position characteristic of free toxin. Sedimentation profiles of the radioactivity retained by normal muscles after 24 hours in culture were essentially unchanged from those obtained at 0 hour.

The radioactive components released into the medium by denervated muscle were examined by P-2 polyacrylamide column chromatography, paper chromatography, and paper electrophoresis. In each case approximately 60 percent of the radioactivity coincided with a monoiodotyrosine marker while another 20 to 30 percent migrated with unidentified low-molecular-weight components. To determine whether association with the tissue plays any role in degradation of the toxin, the experiments were repeated with denervated hemidiaphragms from animals injected with unlabeled toxin and cultured with a trace amount of α-[^{125}I]bungarotoxin added to the unlabeled toxin normally present in the medium. Under these conditions more than 95 percent of the radioactivity was recovered with intact toxin, an indication that little degradation had occurred. Thus, native toxin in the medium is not extensively degraded.

These experiments in organ culture demonstrate a difference between the rates of loss of α-[^{125}I]bungarotoxin bound to the end plate receptors of normal muscle and of that bound to the extrajunctional receptors that appear after denervation. Additional experiments have shown that loss of toxin from junctional receptors in denervated muscles resembles that from junctional receptors in normal muscles. We have also observed a similar difference between junctional and extrajunctional receptors in experiments in which loss of toxin from muscles in vivo was examined (*5*). Consistent with these observations is the finding that extrajunctional regions of denervated muscles have a more rapid recovery of acetylcholine sensitivity after α-bungarotoxin blockade as compared to recovery by the end plate regions of normal muscle (*9*).

It is not known whether normal receptors and those that appear after denervation are the same. The difference in loss of toxin from the two, however, cannot be explained by a difference in the dissociation constants of the respective toxin-receptor complexes. The decrease in toxin loss from denervated muscle produced by inhibitors of energy production and of protein synthesis excludes a mechanism of simple dissociation and indicates that toxin loss from the extrajunctional receptors in this tissue involves an active metabolic process, possibly linked to protein synthesis. The radioactivity that is lost from denervated muscle is recovered in the medium largely as free iodotyrosine, indicating that the toxin has been degraded (*10*). Furthermore, this degradation appears to require association with the tissue. Loss of toxin from the muscle does not involve a net loss of receptors, because we have observed that the acetylcholine sensitivity of denervated muscles that have been blocked with toxin increases during the 24-hour incubation and new toxin-binding sites become available (*5*).

It is possible that toxin loss occurs without affecting the receptor. For example, an extracellular protease, secreted by an energy-requiring process from muscle or connective tissue cells, may selectively degrade toxin bound to the membrane in extrajunctional areas. A more attractive hypothesis, however, is that the loss represents intracellular degradation of the toxin-receptor complex. Degradation of cellular proteins in both bacterial and mammalian cells has been shown to require a source of energy and to be blocked under certain conditions by inhibitors of protein synthesis (*11*). Degradation of the toxin-receptor complex could either reflect normal receptor turnover in the membrane or could occur because binding of the toxin produces an abnormal receptor structure (*12*). If loss of toxin does represent receptor turnover, the observed differences between junctional and extrajunctional receptors could help explain how extrajunctional sensitivity of muscle can vary several orders of magnitude during denervation and subsequent reinnervation while junctional sensitivity remains relatively constant (*2, 13*).

DARWIN K. BERG*
ZACH W. HALL
Department of Neurobiology,
Harvard Medical School,
Boston, Massachusetts 02115

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7. The tissue was divided into innervated and noninnervated portions, homogenized (50 mg of tissue per milliliter) in 0.02*M* tris-HCl, *p*H 7.0, containing 1 percent Triton X-100 and 0.05*M* NaCl, and the radioactivity of the homogenates was measured by liquid scintillation counting. For normal muscles (eight animals), binding to noninnervated regions was assumed to be nonspecific and was subtracted (on a weight basis) from binding to innervated regions (*4*). Nonspecific binding never exceeded 20 percent and did not vary significantly between 0- and 24-hour samples. The amount of end plate–specific [^{125}I]toxin initially bound ranged from 0.15 to 0.25 pmole per hemidiaphragm. In six of the experiments unlabeled α-bungarotoxin (20 μg/ml) was added to the medium at 0 hours. For denervated tissue (six animals), half of the left and all of the right hemidiaphragms were analyzed at 0 hours, as described above, except that only noninnervated regions were taken. The radioactivity bound to the right hemidiaphragm was assumed to be nonspecific and was subtracted from the value obtained at 0 and 24 hours with the left. This correction was less than 10 percent of the total binding at 0 hours. The amount of [^{125}I]toxin initially bound to noninnervated regions of denervated tissue varied from 10 to 31 fmole/mg (wet weight). In the experiments with cycloheximide (six animals), 10 μg/ml was included in the medium during the rinsing and subsequent culture.
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* Present address: Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115.

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Heavy Metal Concentrations in Museum Fish Specimens: Effects of Preservatives and Time

Abstract. *Specimens of myctophid fish preserved for 1 month in formalin, ethyl alcohol, and isopropyl alcohol had higher concentrations of cadmium, copper, zinc, and sometimes lead, and lower concentrations of mercury and sometimes lead, than did unpreserved frozen specimens. Properties of the preservatives and species differences in fish tissues both influence these metal concentrations. Maximum concentrations of some metals in preserved specimens appear to be attained within a month, while concentrations of others may continue to increase for years. Metal tags or other materials in the preservative may cause higher maximum concentrations than the preservatives alone. Comparisons of concentrations of metals between museum specimens and unpreserved (frozen) specimens must be considered unreliable until the changes resulting from preservation are understood.*

Despite suggestions that preserved museum specimens of fishes can provide reliable estimates of naturally occurring heavy metal concentrations, especially mercury (*1*), evidence supporting this assumption has not been forthcoming. We have found that preservation can alter heavy metal concentrations in fish to a considerable extent—enough to cast doubt on any study that is based on preserved material.

A large frozen sample of lantern fish (family Myctophidae) taken in a single midwater-trawl haul was used to determine the effects of current techniques of museum specimen preservation on concentrations of six heavy metals in the specimens. The sample was obtained during regular operations of the Ocean Acre program (*2*): Cruise 12, Station 87, 32°07′N, 64°17′W, 24 August 1971, 1947 to 2104 hours Bermuda Standard Time, 0 to 60 m (60 minutes at 60 m), 1400-mesh Engel trawl. Specimens were frozen in plastic bags in a trunk-type food freezer. Eight species from the sample were analyzed for mercury, cadmium, copper, lead, zinc, and arsenic. When the results of these analyses had been ascertained, specimens of the two most abundant species, *Hygophum hygomi* and *Ceratoscopelus warmingi*, each species in a separate glass container, were fixed in 10 percent formalin solution (about 4 percent formaldehyde). About 1 week was allowed for adequate fixation, and then two-thirds of the specimens of each species were removed from formalin and soaked in several changes of tap water for another week. Half of the soaked specimens of each species were then transferred directly to 40 percent isopropyl alcohol, while the other half were placed for 2 days each in 30 percent and 50 percent ethyl alcohol before reaching the final 70 percent solution. The resulting subsamples of each species in formalin, ethyl alcohol, and isopropyl alcohol were then subjected to the same heavy metal analyses as the frozen subsample. The time from initial fixation to analysis was about 1 month.

To add the element of time, five samples of *H. hygomi* collected in 1969, 1968, 1958, 1914, and 1885 were analyzed. All were from Bermuda or west of Bermuda in the western North Atlantic. The 1969, 1968, and 1958 samples had been fixed in formalin and transferred to isopropyl alcohol. The 1914 and 1885 samples probably were fixed in 70 percent ethyl alcohol, as was the custom in those years, and remained in ethyl alcohol, with periodic additions or changes of fluid to an extent that cannot be determined precisely.

All analyses of frozen and preserved specimens were accomplished at the Skidaway Institute of Oceanography at Savannah, Georgia, by either flame (Cd, Cu, Pb, Zn, As) or flameless (Hg) atomic absorption spectrometry after digestion in HNO_3 (Cd, Cu, Pb, Zn, As) or in H_2SO_4 (Hg). Samples consisted of midsections of fish, minus heads and tails.

The analyzed specimens of each species were relatively uniform in size, within a range of 10 to 15 mm from tip of snout to base of caudal fin (standard length) (Table 1). In seven of the eight species, most of the specimens were subadults, in which females had visible, but small ovarian eggs, and males had developing, but not large testes; the remainder were adults. Most of the specimens of *Bolinichthys indicus* were adults, a few subadults. Therefore, analyses were of specimens at a similar life-history stage, although the sizes were different among species. We suspect that seven of the eight species have a life cycle of less than 2