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Binding of α-Bungarotoxin to Acetylcholine Receptors In Mammalian Muscle

(snake venom/denervated muscle/neonatal muscle/rat diaphragm/SDS–polyacrylamide gel electrophoresis)

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ABSTRACT **Experiments were performed to determine the specificity of [^{125}I]α-bungarotoxin binding to skeletal muscle. In adult rat diaphragm, [^{125}I]α-bungarotoxin was found to bind almost exclusively to those regions of the muscle that contain endplates and are known to be sensitive to acetylcholine. In contrast, chronically denervated adult muscle and muscle from neonatal rats, both of which are sensitive along their entire lengths, bound substantial amounts of toxin in all regions. Toxin binding to all muscles was inhibited by *d*-tubocurarine and by carbamylcholine, but not by atropine. The bound [^{125}I]toxin was solubilized by homogenization of the tissue in 1% Triton X-100 and was recovered as a single band, distinct from free toxin, after zone sedimentation. Treatment of the solubilized, toxin-bound complex with 2-mercaptoethanol and sodium dodecyl sulfate resulted in the recovery of free toxin. A toxin-bound complex was also obtained when toxin was incubated directly with extracts of muscle endplate regions prepared by homogenization in Triton X-100. No such complex was observed with extracts prepared from muscle lacking endplates. These results are consistent with the interpretation that α-bungarotoxin binds specifically to the acetylcholine receptor of mammalian skeletal muscle.**

At the mammalian neuromuscular junction, stimulation of the presynaptic nerve causes the release of the transmitter acetylcholine from the nerve terminals. Released acetylcholine diffuses across the gap separating nerve and muscle cells and interacts with specific receptors associated with the postsynaptic muscle membrane to produce an increase in membrane permeability to sodium and potassium ions (1). The relative amounts of this acetylcholine receptor and its distribution along the muscle have been estimated by measurement of the depolarization produced by the iontophoretic application of acetylcholine onto discrete regions of the muscle surface (2). Using this method, Axelsson and Thesleff (3) and Miledi (4, 5) have shown that adult vertebrate muscle fibers are highly sensitive to acetylcholine only in the region of the neuromuscular junction. After denervation, the muscle fibers become sensitive to acetylcholine along their entire length. Muscle fibers of fetal and neonatal rats are also sensitive to acetylcholine in regions outside the neuromuscular junction (6).

The recent characterization of several snake toxins has suggested another method for estimating the amount and distribution of acetylcholine receptors. Toxins purified from the venoms of *Bungarus multicinctus*, *Lacticauda semifasciata*, and *Naja naja* (cobra) disrupt neuromuscular transmission by blocking the postsynaptic response to acetylcholine (7–9). In addition, α-bungarotoxin blocks the response to acetylcholine of denervated muscle fibers (10). Autoradiographic studies on muscles to which [^{131}I]α-bungarotoxin has been bound have shown that binding occurs preferentially to endplate regions of normal fibers and to the entire length of denervated fibers (11). These experiments suggest that the binding of α-bungarotoxin may allow direct measurement of the acetylcholine-receptor content in muscle preparations.

Both cobra toxin and α-bungarotoxin, which block cholinergic transmission in the electric organs of the marine ray and eel, have been used in studies on the isolation and characterization of the acetylcholine receptor from these tissues (12–14). Muscle preparations, which are relatively poor in synaptic components, are less attractive as a source of acetylcholine receptor for biochemical studies, but may offer special advantages for the study of the synthesis of the receptor and its control. In order to develop an assay for the acetylcholine receptor in this system, we have further examined the specificity of α-bungarotoxin binding in mammalian muscle. We report here that [^{125}I]α-bungarotoxin binds to a component of muscle that has the distribution and pharmacology expected of the acetylcholine receptor.

MATERIALS AND METHODS

Preparation of [^{125}*I*]*α-Bungarotoxin.* Crude *Bungarus multicinctus* venom was fractionated by passage over a Sephadex G-50 column in 0.1 M ammonium acetate (pH 5.0). The protein peak eluted at about 0.6 column volume was applied directly to a carboxymethyl Sephadex column, and the column was developed with a linear gradient of 0.05 M ammonium acetate (pH 5.0) to 1.0 M ammonium acetate (pH 7.0) and concentrated by ultrafiltration with an Amicon Diaflo filter. After 10% polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) buffer, as described below, more than 95% of the purified toxin was found to migrate as a single component, with a molecular weight of about 9×10^3 (15). The purified toxin had a toxicity comparable to that reported by Chang and Lee (7). No loss of toxicity was observed after 3 months of storage at 0°C in 0.05 M sodium phosphate (pH 7.5). The postsynaptic action of the purified toxin was confirmed by demonstrating that it depressed the amplitude of miniature endplate potentials and blocked the depolarization caused by iontophoretically applied acetylcholine.

α-Bungarotoxin was labeled with [^{125}I]iodide by the general

Abbreviations: SDS, sodium dodecyl sulfate; +EP, containing endplates; −EP, without endplates; EGTA, [Ethylenebis(oxyethylenenitrilo)]tetraacetic

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procedure of Greenwood, Hunter, and Gloves (16). To 0.4 mg of α-bungarotoxin in 0.15 ml of 0.05 M sodium phosphate (pH 7.5) was added 0.10 ml of 2 M potassium phosphate buffer (pH 7.5) followed by 10 mCi of carrier-free $Na^{125}I$ in 0.10 ml of 0.1 M NaOH. Iodination was begun by addition of 0.01 ml of a freshly prepared solution of 25 mg/ml of Chloramine T in 0.05 M sodium phosphate (pH 7.5) to the chilled reaction solution and mixing. The reaction was terminated 2–3 sec later by the addition of 0.01 ml of a freshly prepared solution of 50 mg/ml of sodium metabisulfite in 0.05 M sodium phosphate (pH 7.5). [^{125}I]α-Bungarotoxin was separated from $Na^{125}I$ by passage through a Sephadex G-25 column in 0.05 M sodium phosphate (pH 7.5). SDS–polyacrylamide gel electrophoresis (10% gels) of the labeled toxin demonstrated that 85–90% of the ^{125}I label migrated as a single component, with a molecular weight of about 9×10^3. Recovery of protein was 60–90%, as measured by absorbance at 280 nm. With the assumption that this material had an average molecular weight of 9×10^3, specific activities were calculated to be $1.5–1.7 \times 10^5$ cpm per pmol (three preparations). This is equivalent to 0.05 mol of ^{125}I label per mol of toxin. The relative biological activity of [^{125}I]α-bungarotoxin preparations was determined by dilution of aliquots with various amounts of untreated α-bungarotoxin, followed by measurement of the maximum amount of labeled toxin that could bind to muscle in the standard binding assay (see below). The [^{125}I]α-bungarotoxin (two different preparations) was 60% as active as untreated α-bungarotoxin. Since labeled molecules comprise only 5% of the [^{125}I]α-bungarotoxin preparation, dilution experiments of this kind serve to define the limits of activity of only the unlabeled population of molecules in the preparation.

Binding [^{125}I]α-Bungarotoxin to Muscle. Rat diaphragms were removed with ribs attached and incubated with [^{125}I]α-bungarotoxin at room temperature in Kreb's solution, at pH 7.3, containing 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, and 12 mM $NaHCO_3$. The rate at which toxin was bound to diaphragm muscle depended on the age and concentration of the [^{125}I]α-bungarotoxin preparation. The maximum extent of binding was independent of these parameters. With freshly prepared toxin, 2 hr of incubation with 1.4 μg/ml was adequate to achieve maximum binding. In all experiments reported here, [^{125}I]α-bungarotoxin was used within 10 days of preparation; with the exception of the experiments described in Table 2, incubation conditions were always chosen to yield maximum binding of toxin to adult diaphragm muscle.

After incubation with labeled toxin, the muscle was washed overnight in several changes of a solution containing 0.15 M NaCl–0.4 mM EGTA–0.02 M Tris·HCl (pH 7.4) (standard buffer). The muscle was then divided under a dissecting microscope into regions containing endplates (+EP) and regions lacking endplates (−EP). The +EP regions consisted of a central strip of the muscle containing over 95% of the endplates (17). The −EP regions consisted of peripheral strips lacking nerve branches. The dissection was done so that +EP and −EP portions were of about equal weight. Care was taken to trim away any fibers that had been damaged before incubation. The strips were then weighed and homogenized, at a concentration of 100 mg/ml in standard buffer containing 1% Triton X-100, with a Kontes Duall glass homogenizer. Aliquots of the whole homogenate were counted in a Packard scintillation counter, with a scintillation fluid containing one part Triton X-100 to two parts of a solution of 0.2% 2,5-diphenyloxazole (PPO) and 0.01% *p*-bis 2-(5-phenyloxazolyl)-benzene (POPOP) in toluene.

SDS–Polyacrylamide Gel Electrophoresis. SDS gels were prepared as described by Davies and Stark (18). Protein samples were incubated with a 10-fold excess by weight of SDS and 2-mercaptoethanol at 100°C for 5 min, followed by incubation at 37°C for 10 hr. Electrophoresis was at 8 mA/tube, with 0.1% mercaptoacetic acid in the upper reservoir buffer. Pyronin Y was used as the tracking dye. Gels were stained by the method of Weber and Osborn (19) and destained in 42.5% methanol, which contained 7.5% acetic acid. Destained gels were scanned with a Gilford model 240 spectrophotometer and model 2410 linear transport attachment. The distribution of radioactivity in the gels was determined by the method of Young and Fulhorst (20).

Zone Sedimentation. 0.20-ml samples were layered onto 4.8-ml gradients in polyallomer tubes containing 5–20% sucrose in standard buffer and 1% Triton X-100. Zone centrifugation was performed in a Spinco model L-2 preparative ultracentrifuge using an SW 50.2 rotor at 49,000 rpm for 8 hr at 2°C. Protein was determined by the method of Lowry *et al.* (21), with bovine serum albumin as a standard. Since samples containing Triton X-100 gave a precipitate upon addition of the phenol reagent, all such samples, including appropriate controls with albumin, were filtered through a Millipore filter before measurement of the absorption at 750 nm.

Materials. White rats were obtained from Charles River Breeding Co. Adults weighed 170–220 g. Rats were denervated by transection of the left phrenic nerve in the thorax (22). Neonatal diaphragms were taken from rats on the day of birth.

Crude *Bungarus multicinctus* venom was purchased from Sigma Chemical Co. Carrier-free Na ^{125}I was purchased from New England Nuclear Corp. Chloramine T was obtained from Eastman Kodak Co., atropine sulfate from Calbiochem., *d*-tubocurarine from Mann Research Lab., and carbamylcholine chloride from K. and K. Laboratories, Inc.

TABLE 1. *Binding of [^{125}I]α-bungarotoxin to rat diaphragm*

Tissue	Femtomoles of toxin per mg of tissue	
	−EP	+EP
Adult	0.1 ± 0.1 (7)	2.4 ± 0.4 (7)
Denervated	8 ± 3 (4)	15 ± 3 (4)
Neonatal	2.4 ± 0.3 (3)	7 ± 2 (3)

Diaphragms were incubated with labeled toxin under conditions that gave maximal binding for adult tissue. Two neonatal diaphragms were stained for acetylcholinesterase (23), in order to visualize +EP regions as models for dissection. Each value is a mean ±SD and the number in parentheses refers to the number of determinations. For adult and denervated adult muscle, each determination represents the +EP or −EP regions from a single hemidiaphragm. For neonatal muscle, several diaphragms were pooled for each determination. Denervated adult diaphragms were obtained from rats denervated for 5 days.

TABLE 2. *Inhibition of* [^{125}I]*α-bungarotoxin binding*

Additions	Concentration (M)	Percent of control value: Adult EP-specific	Adult −EP	Denervated Adult −EP	Neonatal
None	—	100	100	100	100
d-Tubocurarine	10^{-5}	53	89	—	—
	10^{-4}	42	92	—	—
	10^{-3}	32	118	57	35
Carbamylcholine	10^{-5}	75	—	—	—
	10^{-4}	44	—	—	—
	10^{-3}	21	—	39	31
Atropine	10^{-5}	86	—	—	—
	10^{-4}	97	—	—	—
	10^{-3}	88	—	118	101
None; Prior incubation with carbamylcholine	10^{-4}	83	—	—	—

Adult and denervated adult hemidiaphragms and neonatal whole diaphragms were incubated 1 hr in Kreb's solution with the additions shown. [^{125}I]α-Bungarotoxin was added and the tissues were incubated 2 hr more. The tissues were then washed in standard buffer with the same additions and were homogenized. Prior incubation with carbamylcholine was for 3 hr in Kreb's solution containing 0.1 mM carbamylcholine, followed by three 20-min rinses in Kreb's solution to remove the carbamylcholine. Tissue was then incubated 2 hr in Kreb's solution with [^{125}I]α-bungarotoxin, followed by washing in standard buffer with 0.1 mM carbamylcholine and homogenization. Values represent the average of duplicate samples and are expressed as percentages of the controls. Incubation conditions were chosen such that controls achieved 50–80% of maximal binding. For adult tissue, the inhibition of "endplate-specific" binding was determined as described in the text. For neonatal muscle, the inhibition of binding to the whole diaphragm was determined. Denervated diaphragms were obtained either from rats denervated for 6 days (*d*-tubocurarine experiments) or 3 days (carbamylcholine and atropine experiments). In each case, binding was compared to that with controls denervated for the corresponding amount of time.

RESULTS

As an initial test of the specificity of α-bungarotoxin binding to muscle, we examined the amount of [^{125}I]toxin bound to diaphragms of normal adult, denervated adult, and neonatal rats. Each of the three tissues was incubated under conditions that gave maximal values for the adult, and was washed extensively to remove free toxin. The muscles were then divided into regions containing endplates (+EP) and those lacking endplates (−EP), and the amount of bound toxin in each region was determined. For convenience, the results are presented in Table 1 as femtomoles (10^{-15} mol) of bound toxin (see *Discussion*). In the normal adult, the +EP regions of the diaphragm bound 20-times more toxin per unit weight than did the −EP region. Since the endplate occupies only a small fraction of the surface of each fiber, the +EP regions obtained by dissection necessarily contain much nonendplate tissue. Assuming that the amount of labeled toxin bound to the −EP regions is representative of binding to nonendplate tissue throughout the diaphragm, one can subtract this value on a per weight basis from the value obtained in the +EP regions to determine the amount of "endplate-specific" binding that occurs. By this method, 90% of the toxin that binds to adult diaphragm is found to be "endplate-specific".

A dramatic increase in the amount of toxin bound to −EP regions was found in diaphragms that had been denervated 5 days before the binding assay (Table 1). The +EP regions of denervated muscle showed a smaller relative increase compared to normal muscle. Amounts of binding substantially higher than those of normal adult muscle were also found in both the +EP and −EP regions of diaphragms of neonatal rats.

The binding of [^{125}I]α-bungarotoxin could be blocked by agents known to interact reversibly with acetylcholine receptors. Table 2 shows that both carbamylcholine, an acetylcholine analogue that is a depolarizing agent, and *d*-tubocurarine, a competitive inhibitor of acetylcholine binding, decreased the amount of "endplate-specific" binding that occurred in 2 hr in adult muscle. The binding of toxin to neonatal muscle and to the −EP regions of denervated muscle was also inhibited by these agents. The slight binding at −EP regions of adult muscle was not decreased by *d*-tubocurarine. Adult muscle that had been treated with carbamylcholine bound normal amounts of [^{125}I]α-bungarotoxin once the carbamylcholine was removed, indicating that the inhibition of binding produced by this drug was reversible. The binding of toxin to all three types of muscle was not significantly affected by 1 mM atropine, a compound that blocks the response to acetylcholine in tissues innervated by parasympathetic nerves, but that is relatively ineffective at the neuromuscular junction.

To investigate further the specificity of α-bungarotoxin binding, the properties of the toxin-bound material in the +EP regions of adult muscle were examined. The amount of radioactivity bound by −EP regions of adult muscle was too small to analyze quantitatively. After homogenization of the labeled +EP regions in standard buffer and centrifugation at 28,000 × *g* for 30 min, 50–60% of the ^{125}I label was recovered in the particulate fraction. The addition of 1% Triton X-100 to the homogenization medium, however, resulted in recovery of 80–90% of the ^{125}I label in the supernatant liquid. Analysis of the supernatant fraction by zone sedimentation through a sucrose gradient (Fig. 1*a*) revealed that 50% of the recovered radioactivity migrated as a component with a sedimentation coefficient of 9 S; 15% migrated as free toxin. No faster-sedimenting components were ob-

served when sedimentation was performed for shorter times. Similar results were obtained with the +EP and −EP regions of both denervated adult muscle and neonatal muscle. In each case, the peak contained 40–60% of the recovered ^{125}I label and sedimented at 9 S. No other radioactive components distinct from free toxin were detected.

Experiments were also done to determine whether toxin can bind directly to a component in homogenates of adult diaphragm muscle to form the 9S complex. The +EP and −EP regions were separately homogenized in standard buffer containing 1% Triton X-100 and were centrifuged 30 min at 28,000 × *g*. The supernatant fractions were then incubated with [^{125}I]α-bungarotoxin and the mixtures were analyzed by zone centrifugation. A large fraction of the radioactivity migrated as free toxin, near the top of each gradient. In addition, a small amount of radioactivity, sedimenting at 9 S, was observed in the +EP incubation mixture (Fig. 1*b*). No detectable ^{125}I-labeled complex was formed in −EP extracts, either with comparable incubation conditions (Fig. 1*c*), with longer times of incubation, or with various amounts of [^{125}I]toxin.

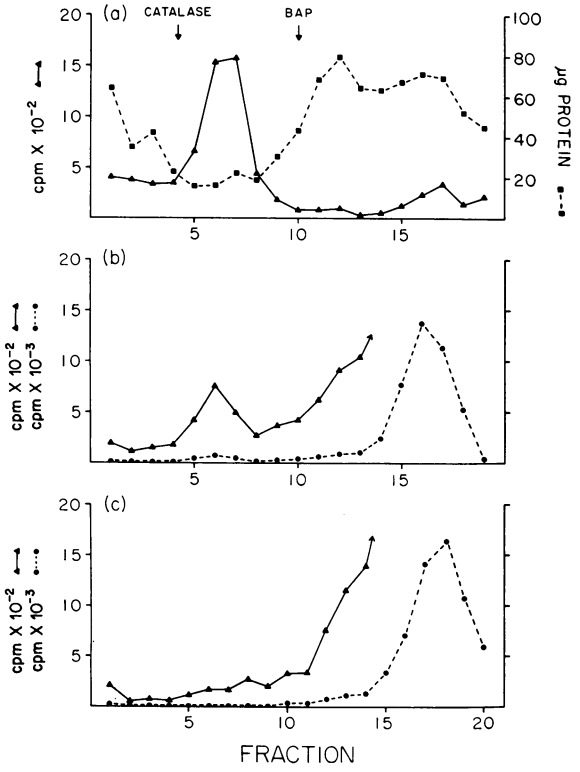

FIG. 1. Zone sedimentation of toxin-bound complexes. Samples were analyzed by zone sedimentation. In all cases fractions were counted for 10 min and a background of 45 cpm was subtracted. Results are expressed as cpm × 10^{-3} (●---●) or, on an expanded scale, as cpm × 10^{-2} (▲——▲). In part (*a*) the distribution of protein was also determined, and is presented as μg of protein per fraction (■---■). Bacterial alkaline phosphatase (BAP), 6.1 S, and bovine liver catalase, 11.3 S, were added as sedimentation markers. Sedimentation is from *right* to *left*. (*a*) Binding to Intact Diaphragm. The +EP region of an adult rat diaphragm labeled with [^{125}I]α-bungarotoxin was homogenized in standard buffer with 1% Triton X-100. The homogenate was centriguged 30 min at 28,000 × *g* and the supernatant liquid was analyzed by zone sedimentation. Recovery of radioactivity was 86%; recovery of protein, determined on a separate gradient, was 93%. (*b*) Binding in +EP Extracts. The +EP region of an adult rat diaphragm was homogenized in 10 vol of standard buffer with 1% Triton X-100. The homogenate was centrifuged 30 min at 28,000 × *g*. To 0.30 ml of the supernatant fraction was added 15 μl of 0.28 μg/ml [^{125}I]α-bungarotoxin containing 8.4 × 10^4 cpm in 0.05 M sodium phosphate (pH 7.5) with 1 mg/ml BSA. The mixture was then incubated 15 hr at 2°C before zone sedimentation. Recovery of radioactivity was 91%. (*c*) Binding in −EP Extracts. The −EP region of an adult rat diaphragm was treated as described in (*b*), except that 16 μl of the toxin solution was added, and the mixture was incubated for 30 hr at 2°C. Recovery of radioactivity was 116%.

The nature of the isolated 9S complex was examined by polyacrylamide gel electrophoresis in SDS buffer, after treatment of the complex with SDS and 2-mercaptoethanol. All of the recovered ^{125}I label migrated as free toxin (Fig. 2).

DISCUSSION

The distribution of bound [^{125}I]α-bungarotoxin in various rat diaphragm preparations corresponds to the distribution of acetylcholine sensitivity as determined by electrophysiological methods. Substantial binding occurs in +EP regions of adult muscle and in both the +EP and −EP regions of denervated adult and neonatal diaphragm muscle. In contrast, only small amounts of toxin are bound in adult −EP regions. This low extent of binding could represent either a low concentration of receptor in −EP regions or "nonspecific" binding. The second possibility is favored by the failure of *d*-tubocurarine to block binding in these regions.

Binding of [^{125}I]α-bungarotoxin to +EP regions of adult diaphragm, to −EP regions of denervated adult diaphragm, and to whole neonatal diaphragm muscle was inhibited by either *d*-tubocurarine or carbamylcholine. No significant inhibition was observed in the presence of atropine, which is only a weak inhibitor at the neuromuscular junction (23). These data are consistent with the interpretation that most of the [^{125}I]α-bungarotoxin binds to rat diaphragm at the acetylcholine receptor.

Most of the bound ^{125}I label could be solubilized with Triton X-100. Zone centrifugation of the solubilized material revealed in each case a small amount of free toxin plus a major radioactive peak, which sedimented at 9 S. Free [^{125}I]α-bungarotoxin could be recovered from the 9S material after denaturation in SDS and 2-mercaptoethanol, suggesting that the toxin is bound to other components of the 9S complex by noncovalent bonds, or possibly by disulfide linkages. The observation that the bound toxin occurs in a single peak during zone sedimentation cannot be taken as evidence that it is bound to a single component. Given the well-known tendency of membrane proteins to aggregate, it is possible that the single band conceals heterogeneity in the binding sites or that it contains muscle components that are nonspecifically associated with the binding material.

The finding that the 9S complex can be isolated from all regions of muscle with high acetylcholine sensitivity is evidence that it contains acetylcholine receptor. In addition, when [^{125}I]toxin is incubated directly with muscle extracts, 9S complex is formed from +EP extracts, but not from −EP extracts. To establish unequivocally that the 9S complex contains acetylcholine receptor will require purification of the toxin-binding component and a demonstration that inhibition of toxin binding by pharmacological agents corresponds quantitatively to that predicted from the *in vivo* activity of these agents.

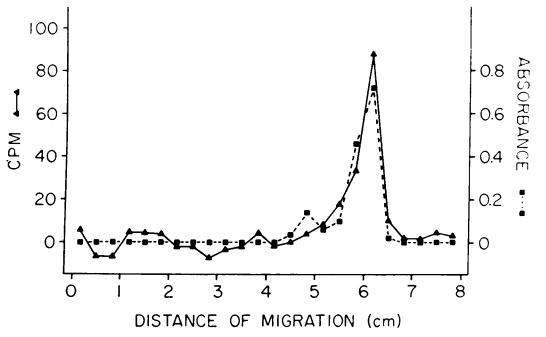

FIG. 2. SDS–gel electrophoresis of 9S complex. Zone sedimentation was as described in Fig. 1*a*, and peak fractions of radioactivity that sedimented at 9 S were pooled. This pooled material was then analyzed by SDS–polyacrylamide gel electrophoresis. The sample added to the top of the 4% gel contained 25 μl of [^{125}I]toxin-bound complex (300 cpm), 20 μl of 0.5 mg/ml unlabeled α-bungarotoxin, 5 μl of 0.1% pyronin Y, and 75 μl of 15% sucrose. Gels were scanned for stain at 592 nm. Dissolved gel fractions were counted for 10 min and backgrounds were subtracted. α-Bungarotoxin and radioactivity from [^{125}I]toxin-bound complex, when subjected to electrophoresis on separate gels, had the same mobilities as above. Recovery of radioactivity from the gel was 54%. Recovery of radioactivity from SDS gels after electrophoresis of [^{125}I]α-bungarotoxin and unlabeled α-bungarotoxin was normally 45–55%.

Studies of toxin binding to the electric organs of *Torpedo* (13) and eel (25) have also revealed a toxin-bound complex that can be solubilized with detergents; in the case of the eel, the complex sediments at 9–10 S (25). In contrast to our results, the toxin-bound complex was reported to migrate as a single component, distinct from free toxin, after gel electrophoresis in SDS buffer (25). Since the exact conditions of the experiment were not specified, in particular, whether or not a reducing agent was present, the results are difficult to compare with ours.

In normal adult muscle, 90% of the binding that occurred was calculated to be "endplate-specific". This calculation does not take into account the extensive membrane folding at the endplate, which would introduce a small correction. Also, no attempt was made to assess how much, if any, of the "endplate-specific" binding might be associated with presynaptic structures. At least 50% of the "endplate-specific" binding corresponds to 9S complex. If this complex is taken to represent toxin bound to receptor, 0.2–0.4 pmol of receptor sites for toxin occur per diaphragm for 170–220 g rats. Preliminary experiments in which [^{125}I]α-bungarotoxin was incubated directly with muscle extracts gave up to 0.2 pmol of [^{125}I]toxin sedimenting as 9S material per diaphragm. These results are in reasonable agreement and make it unlikely that there are additional binding sites that are inaccessible to toxin in intact muscle. Since the experiments with extracts, however, were performed with supernatant fractions prepared by low-speed centrifugation, it is still possible that hidden sites exist that are not solubilized or that become inactive under these conditions.

In calculating the pmoles of bound toxin, we assumed that active and inactive molecules of toxin in the [^{125}I]α-bungarotoxin preparation were labeled to the same extent. In the dilution experiments described in *Methods*, the unlabeled molecules in the [^{125}I]α-bungarotoxin preparation were found to be 60% as active as α-bungarotoxin not subjected to the iodination procedure. We have no direct measure of the activity of the labeled molecules. Preliminary experiments in which the [^{125}I]α-bungarotoxin preparation was incubated with extracts indicate that under appropriate conditions a minimum of 25% of the labeled molecules are capable of forming a 9S complex. Thus, estimates of the specific activity of [^{125}I]α-bungarotoxin may not be in error by more than a factor of three. Nevertheless, the values calculated for the number of toxin-binding sites in diaphragm must be regarded as tentative.

Note Added in Proof

Experiments on the binding of (^{131}I)α-bungarotoxin to frog sartorius and rat diaphragm muscle have recently been reported by Miledi and Potter (1971) *Nature* **233**, 599–603. Their results on the specificity of toxin binding and the value that they obtain for the number of toxin binding sites per diaphragm are in general agreement with those reported here.

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