Acetylcholine Receptor in Normal and Denervated Slow and Fast Muscle†

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ABSTRACT: The equilibrium binding of [^{125}I]-α-bungarotoxin to muscle fractions derived from normal and denervated, slow and fast muscle was examined. The toxin bound to normal slow and fast muscle with an affinity constant (K) in the order of 10^8 l./mol. The number of receptor sites (N) was approximately 8.5 pmol/g of muscle. The toxin bound to both types of denervated muscle with an affinity constant of 10^9 l./mol. The number of receptor sites (N) was more than tenfold higher than the number of sites in the corresponding normal muscle. Binding of the toxin to the receptor derived from both normal and denervated muscle closely approximated the isotherm predicted from the mass law equation for the interaction of 1 mol of homogeneous ligand with 1 mol of identical and independent sites. Further experiments suggested that the observed difference between normal and denervated muscle in the free energy of binding may be attributed to factors other than the primary structure of the receptor. The long term exposure of both types of normal muscle to the nonionic detergent Triton X-100 converted all binding to a high affinity set of sites (K, 10^9 l./mol) with no change in the total number of sites (N). This conversion went through a complex intermediate state, in which the binding did not fit the model of a ligand interacting with a single set of noninteracting sites. In contrast, the long term exposure of either slow or fast denervated muscle to detergent had no effect on either the total number of binding sites (N) or the binding affinity (K). These data suggest that extra-junctional receptors in denervated muscle may be different than normal junctional receptors and that the difference may reflect different molecular interactions of the receptor with its environment rather than a difference in primary structure of the receptor protein.

The normal and denervated states of slow and fast mammalian skeletal muscle provide a unique system for studying the acetylcholine receptor. In fast muscle, the iontophoretic application of acetylcholine (ACH)[1] to the end-plate results in a change in membrane sodium and potassium conductance which is not noted following application of ACH to non-end-plate regions of the membrane. In slow muscle ACH sensitivity is noted not only at the end-plate but also at the muscle-tendon junction and to a very limited extent over the entire cell surface. In contrast, the application of ACH to any point on the surface of denervated slow or fast muscle results in ion conductance changes almost comparable to the response observed at the end-plate region of normal muscle (Albuquerque and Thesleff, 1968; Albuquerque and McIsaac, 1970).

Recent studies have employed the elapid neurotoxins α-bungarotoxin and cobra α-toxin to investigate the changes in bioelectric responsiveness induced by denervation. These homologous toxins are potent cholinergic antagonists which specifically block the ACH receptor site (Chang and Lee, 1964; Lester, 1970). Following denervation a dramatic increase in α-bungarotoxin binding to rat diaphragm preparations has been observed (Miledi and Potter, 1971; Fambrough, 1970; Berg *et al.*, 1972). In accord with the physiological data, the increased toxin binding has been localized to the non-end-plate region of the muscle membrane (Miledi and Potter, 1971; Berg *et al.*, 1972) and can be partially inhibited by cholinergic antagonists (Berg *et al.*, 1972). Such data suggest that, at least in part, the increase in ACH sensitivity can be explained by an increased number of ACH binding units. However, since the free energy of ligand binding has not been examined with junctional and extra-junctional receptors, it is possible that the enhanced ACH sensitivity may be related to the ACH binding affinity of the receptor as well as the number of such binding units.

The interaction between a ligand and the receptor *in vivo* will depend upon both free energy of ligand binding (K) and the number of receptors (N). The number of receptor sites (N) will be a function of both receptor metabolism and receptor interactions which limit availability. On the other hand, the equilibrium binding affinity (K) of the ligand to the receptor will be a function of both the primary structure of the protein and receptor interactions. The affinity (K) will be influenced not only by competitive interaction with the receptor site but also by interactions which affect the conformation and state of aggregation of the receptor.

In previous studies a single high concentration of the neurotoxin was employed and therefore only an assessment of the number of toxin molecules binding to the preparation with a lower limit of affinity of 10^5–10^7 l./mol could be obtained. Experiments with ACH receptor from neuronal tissue (Greene *et al.*, 1973) and electric tissue (Raftery *et al.*, 1972; Klett *et al.*, 1973) suggest that these previous experiments may have been conducted at toxin concentrations 2.5–4 orders of magnitude above the dissociation constant of the toxin receptor complex. In addition, since previous reports with muscle (Miledi and Potter, 1971; Berg *et al.*,

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[1] Abbreviations used are: ACH, acetylcholine; MEPP, miniature end-plate potentials.

1972; Chiu *et al.*, 1974) employed single relatively high concentrations of toxin and the binding was not totally blocked by other cholinergic ligands, the biological validity of denoting all binding as ACH receptor must be tested. An appropriate test of this validity is to characterize with respect to free energy of binding all associations of the toxin over a wide range and to analyze each thermodynamically defined set of sites within the framework of established pharmacological and biological data. Since previous experiments did not assess the free energy of the ligand receptor interaction, the assessment of the affinity of toxin for the ACH receptor may both permit a comparison of junctional and extra-junctional receptor states and reflect possible modulating interactions of the receptor with its environment.

In the present experiments the thermodynamic properties of α-bungarotoxin binding to detergent extracted muscle fractions were used to assess the receptor derived from the normal and denervated states of slow and fast mammalian skeletal muscle. Receptor solubilized in this fashion from both muscle (Berg *et al.*, 1972) and electric tissue (Raftery *et al.*, 1972) exists in a complex particle with an $s_{20,w}$ of 9.0–9.5. We have employed this particle to study the receptor and its environment. Our data confirmed the increased number of receptors (N) in both denervated slow and fast muscle. Furthermore, the affinity of binding (K) was different in the preparations of normal muscles and in their denervated counterparts. The data suggest that the increase in ACH sensitivity following denervation may reside not only in a change in the number of receptors, but also in a change in other physical properties of the receptor sites.

Experimental Section

Preparation of [^{125}I]*Bungarotoxin.* Crude *Bungarus multincinctus* venom was fractionated by the method of Mebs *et al.* (1971). When applied to the frog sartorious neuromuscular preparation at a concentration of 100 nM, the purified α-bungarotoxin blocked end-plate potentials, MEPP's,[1] and the response to exogenous acetylcholine. The toxin had no influence on direct electrical stimulation of muscle.

The α-bungarotoxin (toxin) was labeled with ^{125}I by a modification of the method of Mickey *et al.* (1971). Free [^{125}I]iodine was separated from labeled protein by multiple passages through Dowex-1. After Dowex chromatography, the labeled toxin was lyophilized and chromatographed on Sephadex G-50 (2.5 × 60) in 50 mM Tris-HCl (pH 7.4)–100 mM NaCl. The polymeric form of the toxin which is excluded was discarded and the monomeric form which is included was employed in these experiments. The specific activity of the labeled toxin (3 Ci/mol) was controlled by the ratio of ^{125}I to ^{127}I of the KI employed in the procedure. Since this labeling method involved the addition of large amounts of oxidized iodine to a stirred protein solution, the reaction was virtually instantaneous and yielded a very homogeneously substituted ligand population. Based on the ratio of ^{125}I to ^{127}I, the molecular weight of the toxin, and a Lowry protein determination, it was calculated that the toxin molecules contained 2 atoms of iodine. The homogeneity of the ligand population was further substantiated by gel electrophoresis.

The biological activity of the labeled toxin was also tested against frog sartorious neuromuscular preparations. At a toxin concentration of 100 nM, the labeled toxin was as potent a blocker of end-plate potentials, MEPP's, and the response to exogenous acetylcholine as the unlabeled toxin.

Denervation. Adult female rats (150–200 g) were unilaterally denervated by removal of a 2-cm segment of the sciatic nerve in the mid-thigh region.

Preparation of Soluble Extracts from Muscle. The rats were sacrificed by decapitation. The extensor digitorum longus (fast) and the soleus (slow) muscles were removed and placed in iced buffer (50 mM Tris-HCl (pH 7.4)–100 mM NaCl). The muscles were weighed, suspended in four volumes of buffer (w/w), and homogenized at 4° with a Brinkmann polytron homogenizer (Model Pt IOST) for 90 sec at setting 9. The muscle homogenates were allowed to equilibrate with room temperature. After equilibration, 10% Triton X-100 in 50 mM Tris-HCl (pH 7.4)–100 mM NaCl was added to a final concentration of 1% Triton (v/v). The solutions were agitated at room temperature for 90 min, cooled to 4°, and centrifuged at 28,000g for 15 min. The supernatant (approximately 4 mg of protein/ml) was employed in the binding experiments.

Binding Assay. The supernatant was divided into 0.5-ml aliquots, and 1–100 μl of solutions containing various concentrations of labeled toxin in 0.05 M Tris-HCl (pH 7.4)–0.1 M NaCl–1% Triton X-100 was added to each fraction. The volume of the assay varied from 500 to 600 μl. The precise concentration of toxin in each incubation was derived from the final volume and the amount of radioactivity measured in the tube. Since the 500–600-μl range in volume represented an insignificant fraction of the column assay volume (0.001), no variation was introduced into the result. After incubation for the designated experimental period (4°), the incubations were applied to a Sephadex G-200 column (1.6 × 45 cm) and developed with 50 mM Tris-HCl (pH 7.4)–100 mM NaCl–1% Triton X-100. The toxin bound to the receptor eluted in the excluded volume after a period of 30–45 min. The free toxin emerged at 90% of the column volume. Based on the Lowry method, there was complete recovery of protein from the column. In addition, the possible adsorption of bound toxin to the column was investigated by the reapplication of part of the excluded bound toxin peak to another identical G-200 column. In these experiments there was a complete recovery of the applied bound toxin in the excluded front of the second column. No radioactivity appeared in the excluded peak when control incubations without extracted receptor were applied to the columns. These data agree with previously described cochromatograms on G-200 in Triton of free toxin and toxin receptor complex (Miledi *et al.*, 1971).

In order to assess the homogeneity of material appearing in the excluded peak of Sephadex G-200, the material in the peak was chromatographed on Sepharose 6B and Sepharose 4B. Over 90% of the bound toxin eluted from the columns in a single peak with chromatographic properties indicative of a particle larger than 7 S (IgG) but significantly smaller than Dextran Blue. The characteristics of the bound toxin were similar when assessed by sucrose density gradient centrifugation (5–20% sucrose, 50 mM Tris–100 mM NaCl (pH 7.4)–1% Triton). The results were compatible with observations made by previous investigators localizing the receptor in a 9.0–9.5S particle (Berg *et al.*, 1972; Raftery *et al.*, 1972).

The binding of the toxin to the receptor fraction was initially investigated over the range of 10^{-10}–10^{-4} M toxin. Above 10^{-7} M, the binding increased monotonically suggesting a multiplicity of inseparable sites, none of which were influenced by cholinergic ligands. Below 10^{-7} M toxin the binding was discrete. The majority of experiments were,

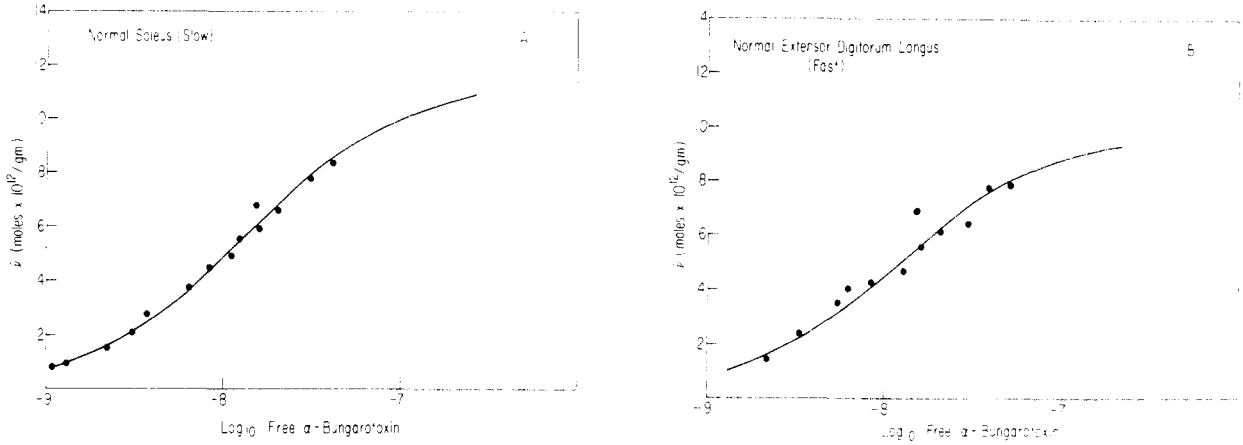

FIGURE 1: (A) A representative experiment of the binding isotherm of labeled α-bungarotoxin to a Triton X-100 extracted receptor fraction from normal soleus (slow). The fraction was incubated with 1% (v/v) Triton X-100 for 1 day. The reaction was performed in 50 mM Tris, 100 mM NaCl, and 1% Triton (v/v) (pH 7.4). The amount of bound toxin was determined by gel permeation chromatography on Sephadex G-200. The K_A for this experiment was 0.07×10^9 l./mol; the number of sites (N) is 11.8 pmol/g of muscle. (B) A representative experiment of the binding isotherm of labeled toxin to a Triton X-100 extracted receptor fraction from normal EDL (fast). The points were experimentally derived and the isotherm is the theoretical line derived by introducing the quantities (K) and (N) from a Scatchard analysis of these data into the mass law equation for the interaction of a homogeneous ligand population with a single set of identical, independent sites. The conditions are the same as above. The K_A for this experiment is 0.087×10^9 l./mol; the number of sites (N) is 9.85 pmol/g of muscle.

therefore, conducted over the range of 10^{-10}–10^{-7} M toxin; 20 separate muscle preparations from 400 animals were employed in these experiments.

In order to test for equilibrium of the reaction between the toxin and its binding site, experimental points were derived in both directions on the binding isotherm. No hysteresis was observed in these experiments, indicating the system was at equilibrium.

Data Analysis. The first step in the analysis of these experiments was to plot the data by the method of Scatchard (Scatchard, 1949). By the method of linear regression, K and N were calculated from the Scatchard plot. The values for K and N were introduced into the mass law expression

$$C = \left(\frac{\bar{\nu}}{n - \bar{\nu}}\right)\frac{1}{K} \qquad (1)$$

Using expression 1 a mass law curve was drawn through the experimentally derived points on the standardly used plot of $\bar{\nu}$ against log C. This plot and the correlation coefficient of the linear regression of the points in the Scatchard analysis gave an estimate of how well the data fit the model of a homogeneous ligand population interacting with a single set of identical noninteracting sites.

In order to analyze K, the quantity N was standardized to 1.0 for each experiment by a linear transformation. This standardization permitted unweighted calculation of K using experimental data derived from a large number of tissue preparations. The correlation coefficient and the test of significance of the correlation coefficient from the Scatchard analysis of the combined data were used as an index of how well the data fit the law of mass action for the model of a homogeneous ligand population interacting with a single set of identical, independent sites. The quantity (N) was calculated as the mean ± the standard deviation for the several experiments.

Inhibition Experiments. In experiments to test the inhibitory effect of various cholinergic ligands on the binding of labeled α-bungarotoxin to the Triton extracted muscle fractions, 0.5-ml aliquots were incubated for 30 min at 22° with increasing concentrations of the cholinergic ligands. After the initial incubation period labeled α-bungarotoxin was added to the aliquots to a final free concentration corresponding to the K_D of the set of binding sites being examined and the reaction was incubated at least 16 hr at 4° before being assayed by the method described above.

General. White rats were obtained from West Jersey Biological. Crude *Bungarus multicinctus* venom was purchased from the Miami Serpentarium. $K^{125}I$ was purchased from International Chemical and Nuclear Co.

Results

When concentrations of toxin between 10^{-10} and 10^{-7} M were employed, a single set of binding sites was observed in preparations of normal soleus and extensor digitorum longus (Figure 1A and B). By Scatchard analysis data derived from the binding isotherm for both types of muscle fit a straight regression line characteristic of identical noninteracting sites interacting with a homogeneous ligand population (soleus $r = 0.84$, $p = 0.001$; EDL $r = 0.81$, $p = 0.001$). Significant bias in the analysis can be ruled out by observing the point distribution compared with the calculated mass law isotherms in Figure 1A and B.

The number of sites (N) available for toxin binding was comparable in preparations from both slow and fast muscle (8.97 ± 1.24 pmol/g in soleus and 8.36 ± 2.09 pmol/g in extensor). Slow and fast muscle were also comparable with respect to the free energy of binding (Table I). The concentration at which half the binding sites (N) were occupied for soleus muscle was 0.94×10^{-8} mol/l. and the concentration of half-saturation for extensor was 1.26×10^{-8} mol/l. Statistical analysis indicates that these values were not different.

When similar toxin concentrations were employed with receptor preparations derived from soleus and extensor muscles denervated for 10 days, changes in both the number of binding sites (N) and the affinity of binding (K) were observed. There was approximately an order of magnitude increase in the affinity constant after denervation in both types of muscle (Table I). The concentration at which half of the binding sites (N) were occupied was therefore decreased comparably (denervated soleus 1.4×10^{-9} mol/l; denervated extensor 1.25×10^{-9} mol/l.) The binding of toxin to receptor derived from denervated muscle also was consistent with the mass law equation for a single set of in-

TABLE I: Receptor Activity in a 1% Triton X-100 Extraction of Normal and Denervated Slow and Fast Skeletal Muscle 1 Day after Preparation.[a]

Muscle	No. of Observations	Association Constant K_A (10^9 mol/l.)	Dissociation Constant K_D (10^{-9} mol/l.)	r	Correlation Coefficient p	No. of Sites (N) (pmol/g of Muscle) $\bar{x} \pm$ SD
Normal EDL	51	0.08	12.60	−0.81	0.001	8.36 ± 2.09
Normal soleus	24	0.11	9.43	−0.84	0.001	8.97 ± 1.24
Denervated EDL	52	0.80	1.25	−0.76	0.001	128 ± 32
Denervated soleus	54	0.71	1.42	−0.86	0.001	99 ± 32

[a] The number of isotherms used to determine the number of binding sites (N) was: normal EDL, 5; normal soleus, 2; denervated extensor, 5; denervated soleus, 5. The correlation coefficient (r) was derived from the linear regression of the standardized Scatchard analysis of (*cf.* Method of Analysis). Number of sites (N): (N. EDL = D. EDL, $p \neq 0.005$) (N. soleus $\neq$ D. soleus, $p = 0.005$).

dependent sites interacting with a homogeneous ligand population (Table I and Figure 2A and B). Following denervation, the number of binding sites (N) increased more than tenfold in both types of muscle (denervated soleus 99.3 ± 32 pmol/g; denervated extensor 128 ± 32 pmol/g). The increase in (N) is compatible with previous observations on denervated and normal mammalian skeletal muscle (Miledi *et al.*, 1971; Berg *et al.*, 1972).

Since the affinity of binding is a function of the interacting molecules and their environment, the differences in binding affinities of toxin to innervated and denervated muscle receptors suggest the presence of either different primary receptors or a single receptor modified by different molecular interactions in its environment. As an approach to the problem, preparations of receptor from control innervated muscle were incubated with 1% (v/v) Triton X-100 for 8–10 days (4°) prior to assay. The assumption was that if the hydrophobic environment of the receptor were an important determinant of the different affinities, the prolonged exposure of the receptor particle (Raftery *et al.*, 1972; Greene *et al.*, 1973; Klett *et al.*, 1973) to an amphiphile (Triton) might alter the hydrophobic interactions of the receptor and consequently affect the toxin binding affinity.

The results show that prolonged exposure of the receptor to detergent does affect some of the characteristics of the toxin interaction with receptor from normal muscle. After incubation of the receptor particle with Triton X-100 for 8–10 days, a single set of binding sites could be demonstrated in preparations derived from both types of normal muscle (Figure 3A and B). The association constants (K) for the binding of toxin to the detergent incubated normal muscle receptor preparations were almost an order of magnitude higher than the constants for the nonincubated preparations, and were similar to association constants (K) obtained for preparations derived from the corresponding denervated muscles (Table II). In contrast to these results obtained with normal muscle, prolonged exposure of denervated muscle to Triton X-100 did not affect the binding affinity (K) or the number of sites (N) (Table II). The values for binding observed in the detergent incubated preparations were compatible with the previously described model for the interaction of a single set of noninteracting sites with a homogeneous ligand population. It is additionally significant

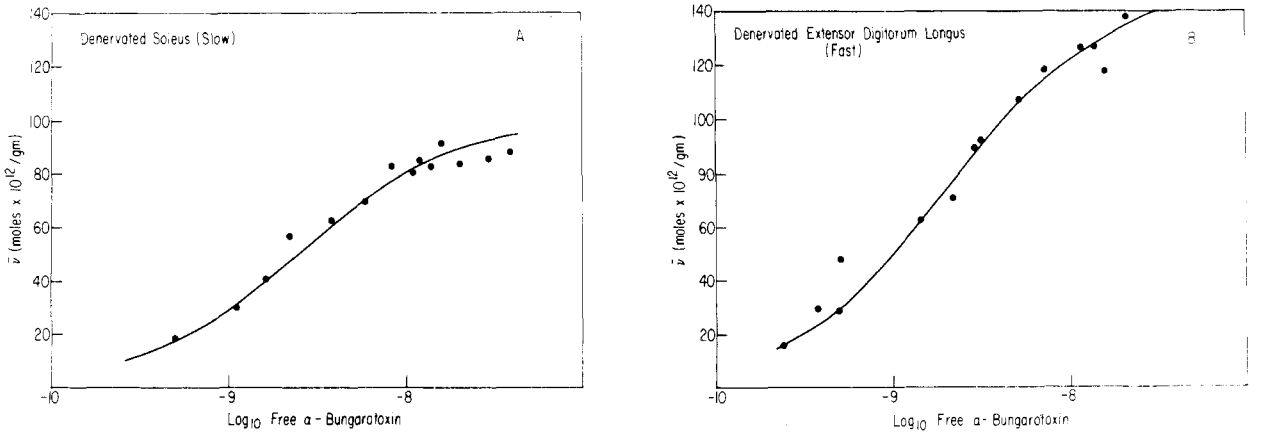

FIGURE 2: (A) A representative experiment of the binding isotherm of labeled α-bungarotoxin to a Triton X-100 extracted receptor fraction from denervated soleus (slow). The fraction was incubated with 1% (v/v) Triton X-100 for 1 day. The reaction was performed in 50 mM Tris–100 mM NaCl–1% Triton (v/v) (pH 7.4). The amount bound was determined by gel permeation chromatography in Sephadex G-200. The K_A for this experiment is 0.43×10^9 l./mol. The number of sites (N) is 99 pmol/g of muscle. (B) A representative experiment of the binding isotherm of labeled toxin to a Triton X-100 extracted receptor fraction from denervated EDL (fast). The conditions are the same as above. The K_A for this experiment is 5×10^9 l./mol; the number of sites (N) is 148 pmol/g.

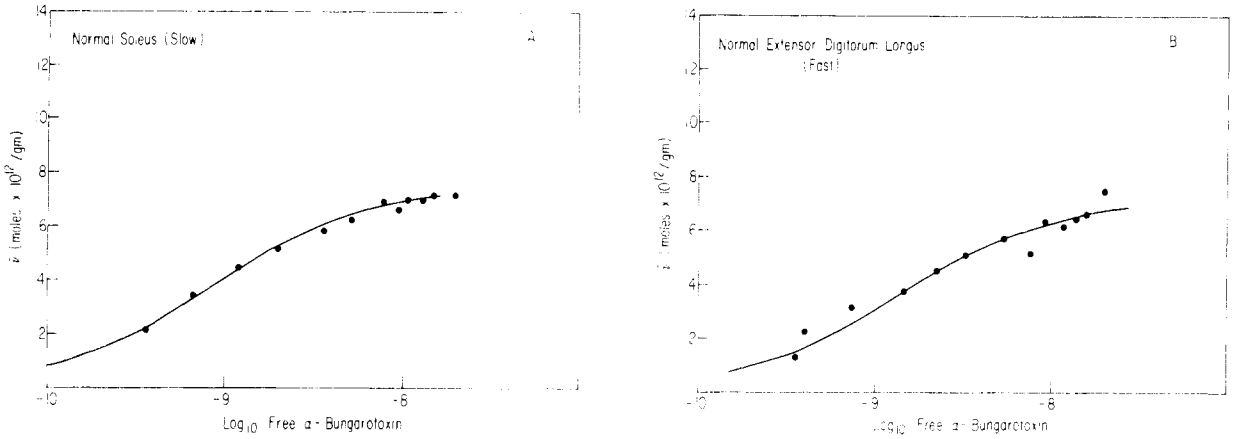

FIGURE 3: (A) A representative experiment of the binding isotherm of labeled α-bungarotoxin to a Triton X-100 extracted receptor fraction from normal soleus (slow). The fraction was incubated with 1% Triton and toxin for 10 days prior to assay. The reaction was performed in 50 mM Tris-100 mM NaCl-1% Triton (v/v) (pH 7.4). The amount of bound toxin was determined by gel permeation chromatography on Sephadex G-200. The K_A for this experiment is 0.73×10^9 l./mol; the number of sites (N) is 7.17×10^9 pmol/g of muscle. (B) A representative experiment of the binding isotherm of labeled toxin to a Triton X-100 extracted receptor fraction from normal EDL. The conditions are the same as above. The K_A for this experiment is 1.21×10^9 l./mol; the number of binding sites (N) is 7.43 pmol/g of muscle.

that although the affinity (K) for normal muscle was influenced by detergent incubation, the number of sites (N) was unchanged.

Binding affinities were also investigated at an intermediate period of incubation with detergent. The results indicate that the binding goes through a transition period. Figure 4A-C shows the Scatchard analysis of representative experiments with receptor extracted from normal extensor after 1 day (4A), 4 days (4B), and 10 days of incubation (4C) with Triton X-100. Whereas the binding in the initial times and long period of time fit the model for a single set of identical noninteracting sites (Figure 4A and C), the binding at intermediate times (Figure 4B) can be best explained by two intrinsic association constants or by cooperative effects (Scatchard, 1949). Analysis of the data as two sites yields association constants similar to the initial and final period ($K_{A1} = 1.19 \times 10^9 \pm 0.39$ l./mol; $K_{A2} = 1.34 \times 10^8 \pm 0.30$ l./mol). Alternatively, the data from the intermediate period can be analyzed as a cooperative effect. If the curvature is due to cooperative effects, additional terms must be added to expression $\bar{\nu}/c = K(N - \bar{\nu})$. Although it is not possible to distinguish between the two alternative interpretations, these data do indicate that the characteristics of the binding sites are in a state of transition during the intermediate period.

The biological specificity of the α-bungarotoxin binding was further evaluated by measuring the inhibitory effect of the cholinergic ligands decamethonium, d-tubocurarine, and carbamylcholine. These three ligands produced substantial inhibition of toxin binding (Figure 5). In these experiments we employed a concentration of toxin which half-saturated the binding sites being investigated. These ligands almost completely inhibit the binding of bungarotoxin to the sets of sites described in both normal and denervated muscle. These data suggest that the incomplete inhibition of toxin binding by cholinergic ligands observed by previous investigators (Miledi and Potter, 1971; Berg *et al.*, 1972; Chiu *et al.*, 1974) may be due to the inclusion of a significant amount of nonbiological absorption of toxin.

Discussion

The biological specificity of α-bungarotoxin as a cholin-

TABLE II: Receptor Activity in a 1% Triton X-100 Extraction of Normal and Denervated Slow and Fast Skeletal Muscle after 8–10 Days of Incubation in 1% Triton X-100.[a]

Muscle	No. of Observations	Association Constant K_A (10^9 mol/l.)	Dissociation Constant K_D (10^{-9} mol/l.)	r	Correlation Coefficient p	No. of Sites (N) (pmol/g of muscle) $\bar{x} \pm$ SD
Normal EDL	26	0.98	1.02	−0.91	0.001	8.64 ± 1.71
Normal soleus	24	0.47	2.14	−0.90	0.001	7.39 ± 2.15
10-day denervated EDL	31	1.46	0.73	−0.86	0.001	122 ± 50
10-day denervated soleus	26	0.77	1.30	−0.84	0.001	150 ± 33

[a] The number of isotherms used to determine the number of binding sites (N) was: normal EDL, 3; normal soleus, 2; denervated EDL, 3; denervated soleus, 3. The correlation coefficient (r) was derived from the linear regression of the standard Scatchard analysis (*cf.* Methods of Analysis). Number of sites (N): (N. EDL $\neq$ D. EDL, $p = 0.005$) (N. soleus $\neq$ D. soleus, $p = 0.005$).

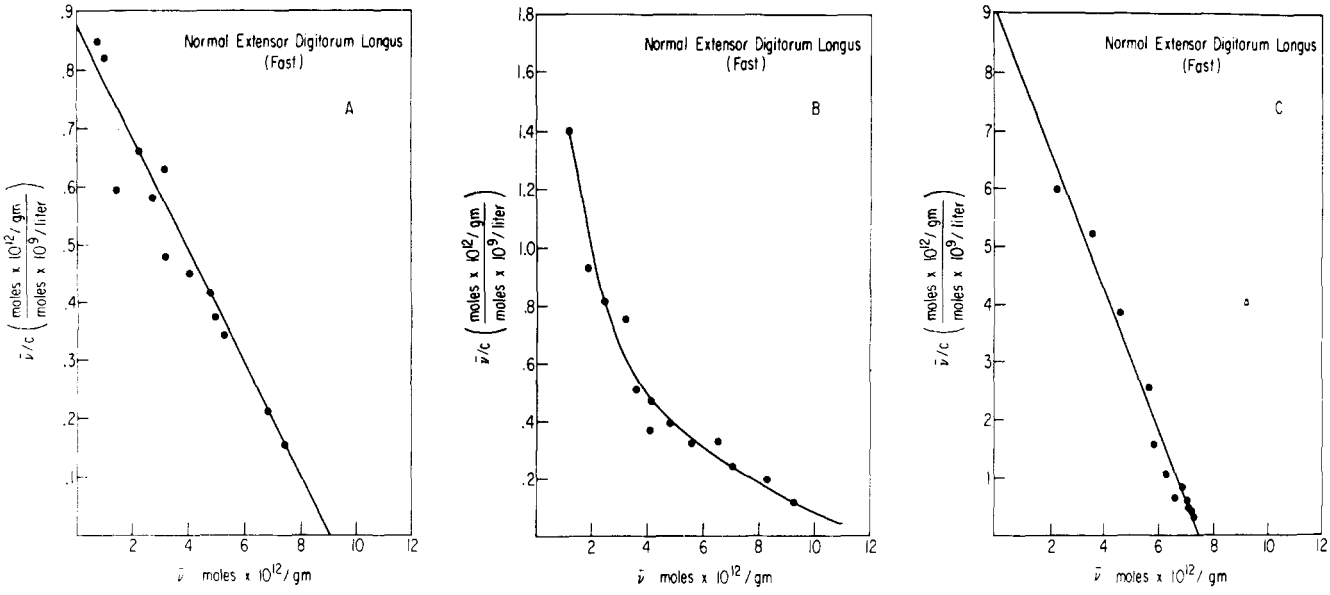

FIGURE 4: The standardized Scatchard analysis of three representative binding isotherms of toxin to Triton X-100 extracted receptor fraction. The reaction was performed in 50 mM Tris–100 mM NaCl–1% Triton (v/v) (pH 7.4). (A) Assay performed after 1 day of incubation in Triton, K_A, 0.1 $\times 10^9$ l./mol. (B) Assay performed after 4 days of incubation in Triton (transitional). (C) Assay performed after 10 days of incubation in Triton, K_A, 1.2 $\times 10^9$ l./mol.

ergic antagonist which directly blocks the acetylcholine receptor site is well established (Chang and Lee, 1964). However, the biochemical specificity of the toxin as a ligand which binds only to the ACH receptor site is not established. On the contrary, the toxin is a basic polypeptide of 8000 with the propensity to adsorb to a wide variety of substances. The advantage of examining multiple equilibria of binding between a ligand and a complex biological preparation over a wide range of ligand concentration is that it allows the separation and characterization of the interactions of the ligand within the specified concentration range. In this context, the term "nonspecific binding" becomes meaningless and all ligand interactions can be examined with respect to a particular biological relationship.

The binding to receptor preparations at concentrations of α-bungarotoxin from 10^{-7} to 10^{-4} M increased monotonically suggesting the presence of a multiplicity of inseparable sites and/or highly cooperative interactions. Binding at these toxin concentrations was not appreciably altered by denervation and was not inhibited by cholinergic agonists or antagonists. These data clearly suggest that the binding above 10^{-7} M was not related to interactions with the ACH receptor.

In contrast, the binding at toxin concentrations below 10^{-7} M appeared related to interaction with ACH receptor. In preparations of both normal and denervated muscle a single saturable set of binding sites was observed. In both slow and fast normal muscle half the sites are occupied at approximately 10^{-8} M toxin, whereas in denervated muscle half the sites are occupied at approximately 10^{-9} M toxin. The binding isotherm in both cases closely approximated the mass law equation for the interaction of 1 mol of homogeneous ligand with 1 mol of identical independent sites. The number of binding sites (N) in this range increased more than tenfold following denervation. These results are in accord with demonstrated changes in ACH sensitivity following denervation (Albuquerque and Thesleff, 1968; Albuquerque and McIsaac, 1970; Miledi and Potter, 1971; Fambrough, 1970; Berg *et al.*, 1972). Furthermore, the binding in this range to both normal and denervated preparations has characteristics compatible with the pharmacological specificities of the ACH receptor site. The evidence therefore indicates that the lower affinity set of binding sites characteristic of normal muscle preparations and the higher affinity set of sites characteristic of denervated muscle preparations both represent toxin interaction with an ACH receptor site.

The difference in the concentration at which half the receptor sites are occupied indicates a difference in the free energy of binding of the toxin to the receptor derived from normal and denervated muscle. Since over 90% of the receptors in denervated muscle are extra-junctional and over 90% of the receptors in normal muscle are junctional, the observed difference in free energy clearly represents a distinction between junctionally and extrajunctionally derived receptors (Berg *et al.*, 1972). Recent observations suggest-

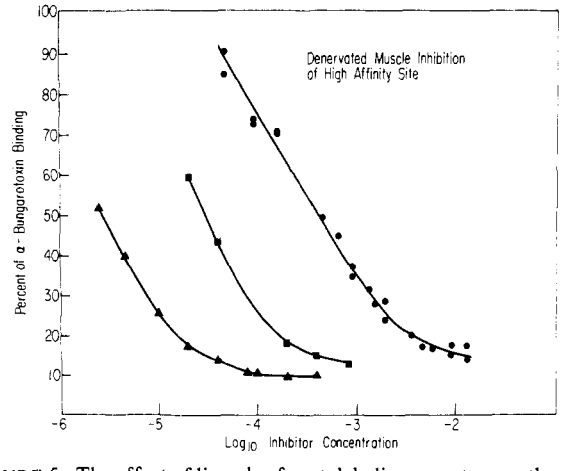

FIGURE 5: The effect of ligands of acetylcholine receptors on the binding of [^{125}I]-α-bungarotoxin to a Triton X-100 extracted fraction from denervated soleus muscle. Aliquots (0.5 ml) were incubated for 30 min at 22° with increasing concentrations of the ligand. After the initial incubation period [^{125}I]-α-bungarotoxin was added to the aliquots to a final free concentration of 1 nM in the uninhibited fraction. The amount of bound toxin was determined by gel permeation chromatography on Sephadex G-200. (●) decamethonium chloride; (■) carbamylcholine; (▲) *d*-tubocurarine.

ing a difference in the rate of turnover provide evidence for a distinction between junctional and extra-junctional receptors (Berg and Hall, 1974). Additional data suggesting a distinction have been reported (Beranek and Vyskocil, 1967; Feltz and Mallart, 1971; Axelsson and Thesleff, 1959).

The difference in free energy of binding of toxin to receptors derived from normal and denervated muscle must reflect either a difference in the primary structure of the receptor proteins or a difference in the molecular interactions of the receptor with the constituents of its environment. Although these alternative explanations cannot be resolved with the present data, it is of interest that prolonged exposure of normal muscle to a nonionic amphiphile (Triton X-100) at a concentration above its critical micelle concentration, significantly altered the free energy of binding of toxin to the normal muscle receptors and resulted in a binding affinity quite similar to the affinity of toxin for receptor derived from denervated muscle. These results suggest that the different binding affinities may reflect different interactions of the receptor with constituents of its environment.

Data from several sources show that the composition of the hydrophobic environment of membrane proteins may have a significant effect on the functional properties of such membrane proteins (Tsukagoshi and Fox, 1973; Wilson and Fox, 1971). Since the ACH receptor is extracted from the muscle membrane in a complex protein, lipid, detergent particle, some of the significant biological associations of the receptor with other membrane consituents may remain intact. The most probable structure for the receptor particle is a mixed micelle (Robinson *et al.*, 1974) consisting of the detergent and extracted membrane amphophiles (proteins and phospholipids). The particle would therefore represent the hydrophobic environment of the receptor previously assumed by the cell membrane. Recent experiments have shown that proteins which naturally associate with lipids interact with large amounts of detergents with low critical micelle concentrations (Triton, sodium deoxycholate) while hydrophilic proteins apparently interact with little or no such detergents (Helenius and Simons, 1972). Since it is not uncommon for detergent mediated events to take place over rather long periods of time (Pitt-Rivers and Impionbato, 1968), it is possible that the difference between receptors derived from normal and denervated muscle reflects a difference in the composition of a mixed micelle in which the receptor is included after extraction. The elimination of this difference with prolonged exposure to the detergent would then represent a Triton replacement or modification in the micelle composition with time.

An alternative explanation of our data would suggest that proteolytic activity may have occurred with prolonged incubation of normal receptor in Triton X-100. Thus by analogy the difference in the free energy of toxin binding to receptors from normal and denervated muscle may reflect differences in the primary structure of the receptor protein. Although we cannot presently rule out this possibility, the constancy of the total number of binding sites (N) during the incubation makes a covalent structural change less likely.

In any case, whether the variation in the free energy of binding of the toxin to the receptor reflects differences in the primary structure of the receptor protein or differences in molecular interactions of the receptor with its environment, the variation does represent a clear difference between normal junctionally derived receptors and extra-junctional receptors extracted from denervated muscle. The results from the present experiments and the observations reported by previous investigators (Berg and Hall, 1974; Beranek and Vyskocil, 1967; Feltz and Mallart, 1971; Axelsson and Thesleff, 1959) clearly indicate that the ACH receptor is not a homogeneous population with respect to all characteristics. The genesis of these differences and their biological significance are important topics for future investigation. It is also clear that the physical properties of the interaction of the receptor with other molecules may be both important indices and determinants of the functional state of the ACH receptor.

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