Cholinergic Sites in Skeletal Muscle. I. Denervation Effects†

Richard R. Almon* and Stanley H. Appel

ABSTRACT: Cholinergic interactions in systems derived from rat skeletal mixed muscle are detailed. The isotherms of the binding of [^{125}I]diiodo-α-bungarotoxin over the range of 10^{-10}–10^{-5} M toxin have been separated into a "nonspecific" component exclusive to the toxin and a "specific" component that binds both the toxin and *d*-tubocurarine. The "specific" component appears to reflect two independent sets of binding sites. One of the sets has an affinity constant on the order of 10^9 M^{-1}. Following denervation, the number of sites in this high-affinity set begins to increase after 3 days, reaches a peak (28-fold higher than normal) on the 8th day, and begins to decline. Similar results are obtained when sensitivity of this set to an antibody derived from patients with myasthenia gravis is examined. This sensitivity is reflected by the inhibition of the α-bungarotoxin binding by the myasthenic IgG fraction. Following denervation, sensitivity first appears on day 3 and progresses coincidentally with the increase in new sites in the set. The characteristics of this set suggest that it represents the acetylcholine receptor and that the new sites appearing during the course of denervation are extrajunctional receptor sites. The interaction with the myasthenic IgG indicates an antigenic difference between junctional and extrajunctional receptors. The second set of specific binding sites has an affinity constant on the order of 10^5 M^{-1}. The number of sites in this set increases only fivefold as a result of denervation. The increase also begins between days 2 and 3. The definition of this low affinity set of sites is not presently clear.

The physiological properties of mammalian skeletal muscle vary considerably as a function of innervation. After denervation, the resting membrane potential declines (Albuquerque and McIssac, 1969), extrajunctional sensitivity to acetylcholine increases sharply (Albuquerque and McIssac, 1969), and changes occur in the characteristics of the action potential (Albuquerque and Thesleff, 1968; Colquhoun et al., 1974; Redfern and Thesleff, 1971). In recent years, considerable effort has been directed towards understanding the biochemical changes that underlie these physiological properties.

The level and distribution of cholinergic sensitivity is one aspect of the denervation process that has been studied extensively. As a result of the disruption of nerve–muscle contact, there is an increase in the sensitive area of the muscle membrane that is reflected by a 10–40-fold increase in the total number of measureable cholinergic sites (depending on the method of analysis and length of time following denervation) (Miledi and Potter, 1971; Fambrough, 1970; Berg et al., 1972; Almon et al., 1974a). The increase in the number of cholinergic sites can be inhibited by the early administration of inhibitors of RNA or protein synthesis (Fambrough, 1970). The majority of these studies on cholinergic sites in skeletal muscle have employed the elapid neurotoxins α-bungarotoxin and/or cobra α toxin. These homologous toxins are potent cholinergic antagonists directed specifically to the acetycholine (AcCh) receptor site (Chang and Lee, 1964; Lester, 1972a,b). Because of the relatively low number of cholinergic sites in skeletal muscle, as well as the variety of conditions, variables, and tissues studied, several questions require further clarification and elaboration. Among these are questions concerning the number of types of cholinergic sites in skeletal muscle and the possible lack of identity between junctional and extrajunctional AcCh receptors. Heterogeneity in the population of cholinergic sites as well as differences between normal junctional and denervated extrajunctional receptors have been indicated by a number of previous reports. Cholinergic sites derived from different sources, preparations, and locations on the cell surface have been reported to vary in turnover rate (Berg and Hall, 1974), sensitivity to cholinergic antagonists (Chiu et al., 1974; Lapa et al., 1974), α-bungarotoxin binding affinity (Almon et al., 1974a), antigenicity (Almon and Appel, 1975a), and isoelectric point (Brockes and Hall, 1975). Whether these data reflect differences in primary structure, conformation, or state of aggregation of the receptor has not yet been resolved. It is also not clear that all types of α-neurotoxin binding observed relate to the biological site responsible for muscle depolarization following agonist binding.

The present report analyzes equilibrium interactions between [^{125}I]diiodo-α-bungarotoxin and skeletal muscle preparations to determine sets of α-bungarotoxin binding sites with affinities between 10^5 and 10^{10} M^{-1}. Similar equilibrium interactions have been studied in denervated preparations; and the transition of the isotherms over a 10-day denervation period has been analyzed. This report also includes a study of the development of the antigenic differences between putative junctional and extrajunctional AcCh receptors (Almon and Appel, 1975a).

Materials and Methods

Materials. The venom of *Bungarus multicinctus* was obtained from the Miami Serpentarium. The component α-bungarotoxin was purified, labeled with ^{125}I, and characterized as described previously (Almon et al., 1974). [^{125}I]diiodo-α-bungarotoxin was employed in the binding studies (9–12 Ci/mmol). CM-Sephadex C-25, Sephadex G-200 and G-20 were obtained from Pharmacia; *d*-tubocurarine chloride (dTc)[1] was obtained from Sigma. All other chemicals were reagent grade.

Receptor Preparation. Adult female rats (200–250 g) were

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[1] Abbreviations used are: AcCh, acetylcholine; AcChE, acetylcholinesterase; CM, carboxymethyl; dTc, *d*-tubocurarine chloride.

unilaterally denervated by removal of a 2-cm segment of the sciatic nerve in the mid-thigh region (Almon et al., 1974a). The animals were sacrificed after a variable period of time (0–10 days). The receptor fraction was prepared from the gastrocnemius, anterior tibilias, and extensor digitorum longus muscles by extraction with the detergent Triton X-100 as described for the soleus and extensor in a previous report (Almon et al., 1974a). The receptor preparations were stored at −70 °C until use. The affinity shift noted previously in normal slow and fast muscle preparations also took place in this mixed muscle preparation as a result of either extended incubation with Triton X-100 or the freezing and thawing process associated with storage at −70 °C. As with the slow and fast muscle preparations characterized previously (Almon et al., 1974a), no change in the number of sites was noted as a result of the affinity transition.

Binding Assay. An aliquot of the Triton X-100 extracted muscle receptor fraction was incubated with an appropriate concentration of labeled toxin (10^{-10}–10^{-5} M) or the toxin plus 4×10^{-3} M dTc. Following the incubation (16 h, 4 °C), the bound and free toxin were assessed by rapid filtration on Sephadex G-200 as detailed in previous reports (Almon et al., 1974a,b; Almon and Appel, 1975a).

Analysis of Binding. The results were plotted as the amount bound ($\bar{\nu}$) vs. the $\log_{10}$ of the free toxin concentration (with and without the addition of a saturating concentration of dTc). The isotherm of the binding in the absence of dTc was analyzed as the sum of an unsaturable component and one or more saturable hyperbolic mass law functions. The unsaturable function was derived from the experimentally determined points in the presence of the saturating concentration of dTc. Since the residual toxin binding in the presence of dTc is to sites exclusive to the toxin, it is assumed to represent the so called nonspecific binding. Experiments employing a >500-fold excess in unlabeled α-Bgtx produced results that concurred with data obtained in the presence of 4×10^{-3} M dTc. (This method of isotope dilutions has been used in studying the insulin (Cuatrecasas, 1971), NGF (Banerjee et al., 1973), β-adrenergic (Mukherjee et al., 1975), and TTX (Colquhoun et al., 1974) receptors.) This residual binding (not blocked by dTc or abolished by isotope dilution) was unsaturable and empirically fit by the power function:

$$\bar{\nu} = a[\mathrm{C}]^b \qquad (1)$$

The saturable component representing binding that was blocked by dTc was defined by subtraction of the residual unsaturable component from the experimentally derived points in the absence of dTc. The corrected binding points were then fit to the mass law expression for the interaction of a homogeneous ligand population with one or more sets of identical independent sites by iterative curve fitting analysis:

$$\bar{\nu} = \frac{N_1K_1[\mathrm{C}]}{1 + K_1[\mathrm{C}]} + \ldots + \frac{N_nK_n[\mathrm{C}]}{1 + K_n[\mathrm{C}]} \qquad (2)$$

where $\bar{\nu}$ is the amount of bound ligand, K is the association constant; C is the free toxin concentration, and N is the number of available sites.

Inhibition of Toxin Binding by the Myasthenic IgG. In the present studies, IgG derived from patients (T.S.) and (J.F.) discussed in the previous reports was used to characterize an indicated antigenic transition in the cholinergic sites from normal to 10-day denervated muscle. The methods employed are described in those reports (Almon and Appel, 1975a,c). The maximum extent of inhibition of the [^{125}I]iodo-α-bungarotoxin binding by the myasthenic IgG was determined by adding increasing concentrations of a combined IgG pool from two patients.

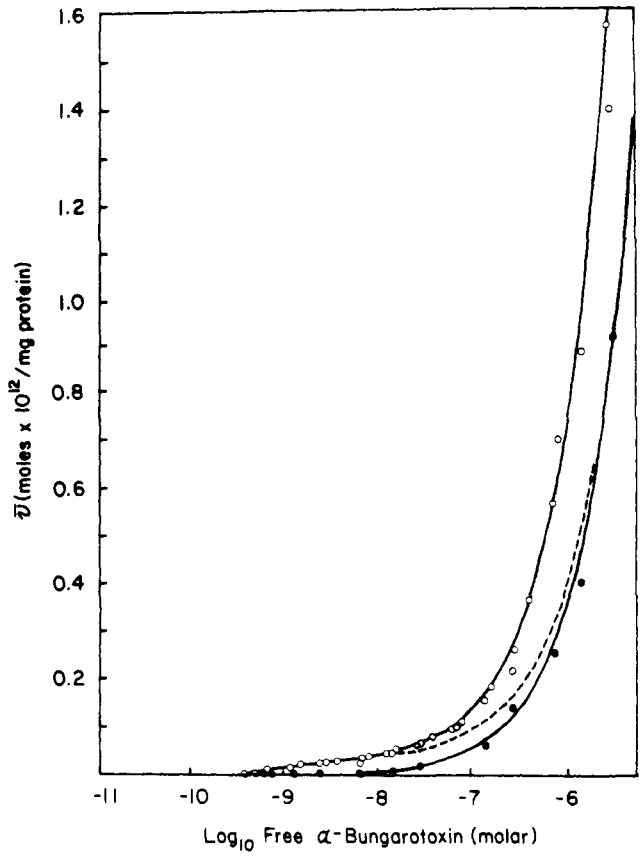

FIGURE 1: The binding of [^{125}I]iodo-α-bungarotoxin to the Triton X-100 extracted normal muscle preparation. The lower unbroken line represents the nonspecific binding fitted to the power function $a[\mathrm{C}]^b$. The closed circles from which the power function is derived are experimental binding points in the presence of 4×10^{-3} dTc. The open circles are experimental points in the absence of dTc. The center dashed line represents the sum of the power function plus the high-affinity set of binding sites.

$$\bar{\nu}' = a[\mathrm{C}]^b + \frac{N_1K_1[\mathrm{C}]}{1 + K_1[\mathrm{C}]}$$

The upper solid line represents the sum of the power function plus the high-affinity set of sites and the low-affinity set of sites.

$$\bar{\nu}' = a[\mathrm{C}]^b + \frac{N_1K_1[\mathrm{C}]}{1 + K_1[\mathrm{C}]} + \frac{N_2K_2[\mathrm{C}]}{1 + K_2[\mathrm{C}]}$$

Results

Figures 1 and 2 are the isotherms for the associations of toxin to muscle preparations derived from normal and 10-day denervated muscle, respectively. Two types of association were revealed by these studies.

Nonspecific Binding. One type represented by the bottom solid line in both figures is the power function derived from the experimental points in the presence of dTc (closed circles). Statistical analysis indicates that these points fit a power function (coefficients of determination: normal, 0.99; 10-day denervated, 0.96).

Specific Binding. The second type of association is that which is blocked by the addition of dTc. This binding is indicated by the difference between the upper open circles representing experimental points in the absence of dTc and the lower solid line fit to the closed circles. This binding closely fits an analysis based on the association of a homogeneous ligand (toxin) binding to two sets of identical, independent sites (eq

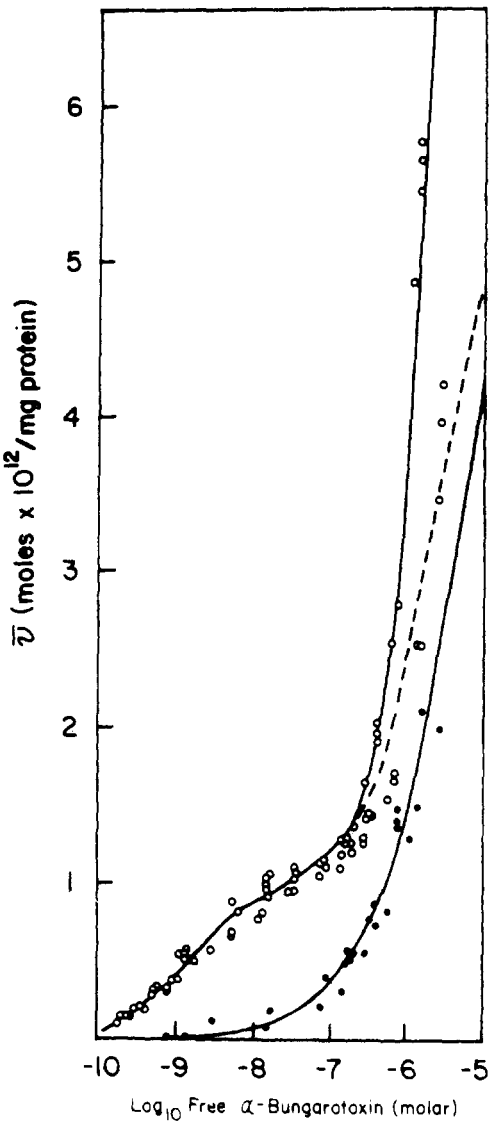

FIGURE 2: The binding of [^{125}I]iodo-α-bungarotoxin to the Triton X-100 extracted 10-day denervated muscle preparation. The lower unbroken line represents the nonspecific binding fitted to the power function $a[C]^b$. The closed circles from which the power function is derived are experimental binding points in the presence of 4×10^{-3} M dTc. The open circles are experimental points in the absence of dTc. The center dashed line represents the sum of the power function plus the high affinity set of binding sites.

$$\bar{\nu}' = a[C]^b + \frac{N_1K_1[C]}{1 + K_1[C]}$$

The upper solid line represents the sum of the power function plus the high-affinity set of sites and the low-affinity set of sites.

$$\bar{\nu} = a[C]^b + \frac{N_1K_1[C]}{1 + K_1[C]} + \frac{N_2K_2[C]}{1 + K_2[C]}$$

2). The upper solid lines in both Figures 1 and 2 are theoretical lines drawn based on a two-site analysis of the dTc-inhibited binding.

High Affinity Set. The first of the two sets of sites has a high affinity in the order of 10^9 M^{-1}. The sum of only the high affinity set of sites and the power function is represented by the dashed line in Figures 1 and 2. Figure 3A,B shows the high affinity set of sites and the corrected experimental points from normal and 10-day denervated muscle. The lines in these figures are theoretically drawn based on a derived N and K from a Scatchard analysis of the data. The contribution from the power function and the low affinity set have been subtracted

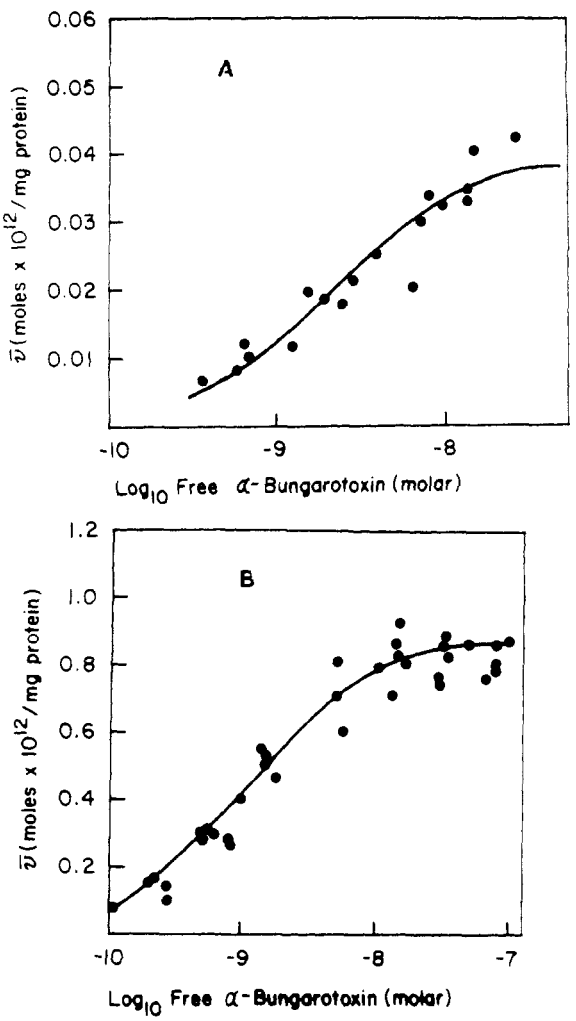

FIGURE 3: The binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of binding sites in the normal muscle preparations. The solid line represents the high-affinity set of sites minus the nonspecific binding and the low-affinity set of sites.

$$\frac{N_1K_1[C]}{1 + K_1[C]} = \bar{\nu} - \left[a[C]^b + \frac{N_2K_2[C]}{1 + K_2[C]}\right]$$

The closed circles represent the experimental binding points in Figure 1 minus the contributions from the nonspecific binding and the low-affinity set of sites. (A) Normal muscle preparation; (B) 10-day denervated muscle preparation.

from the points as discussed. Although the calculated affinity of this set of sites ($K_A \sim 10^9$ M^{-1}) remains relatively stable over the denervation period, the number of sites in the set is altered following denervation. Figure 4 shows the change in the number of sites in the high affinity set after denervation (closed circles). The increase in the number of sites begins at 3 days following denervation and progresses to a peak at 8 days (~28-fold), then begins to decline.

Low Affinity Set. The second of the two sets of sites is also blocked by dTc and has an affinity approximately three orders of magnitude lower than the high affinity set of sites ($K \simeq 1.6 \times 10^5$ M^{-1}). Figure 5 shows the graphical analysis of the low-affinity set of sites from normal muscle. The contributions of the high-affinity set of sites and the power function have been subtracted from the experimental points. Although only the lower 50% of the isotherm was obtainable experimentally, a straight line on a Scatchard plot and the close compliance to the theoretical line drawn on the log plot from the mass law equation (Figure 5) clearly indicate that these corrected points

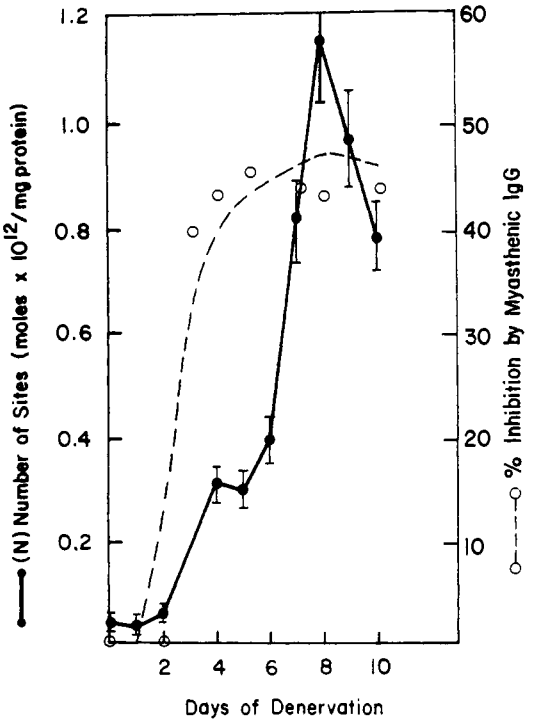

FIGURE 4: The change in the number of binding sites in the high-affinity set (N_1) over the first 10-day period following denervation (●). The points are derived from an analysis of the isotherms from preparations from each day following denervation. The change in maximal amount of inhibition of [^{125}I]iodo-α-bungarotoxin to the high-affinity set by IgG derived from the serum of patients T.S. and J.S. (○). The points are derived from a titration of the IgG effect in muscle preparation from each day. The line is calculated based on the IgG producing a maximum inhibition of 50% of all new sites in the set observed following denervation.

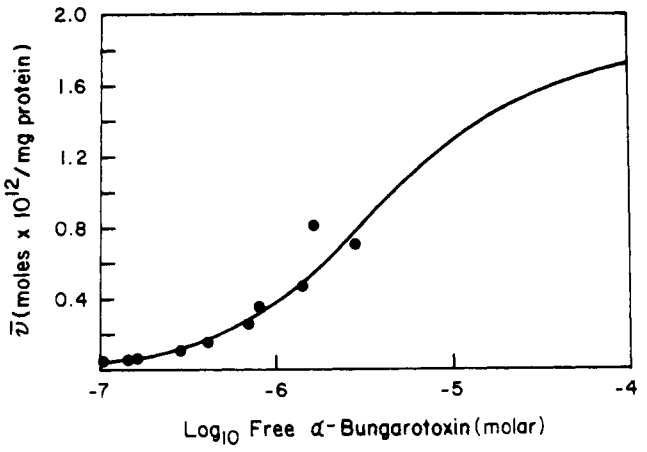

FIGURE 5: A log plot of the binding of [^{125}I]iodo-α-bungarotoxin to the low affinity set of sites in a normal muscle preparation. The data are those presented in Figure 3A. The extrapolation of the line is based on the Scatchard analysis. The data points represent the experimental points minus the contribution from the power function in the high-affinity set. The line is a plot of the difference between the upper solid line in Figure 1 minus the broken line.

reflect a single set of independent identical sites. Figure 6 is a graphical presentation of the change in the number of sites in the low-affinity set over the 10-day denervation period. These data demonstrate that changes in the low affinity set begin almost immediately following denervation but become most apparent at 3 days. The number of sites in this set increases four- to fivefold to a new level about day 4 and remains rather constant to day 10.

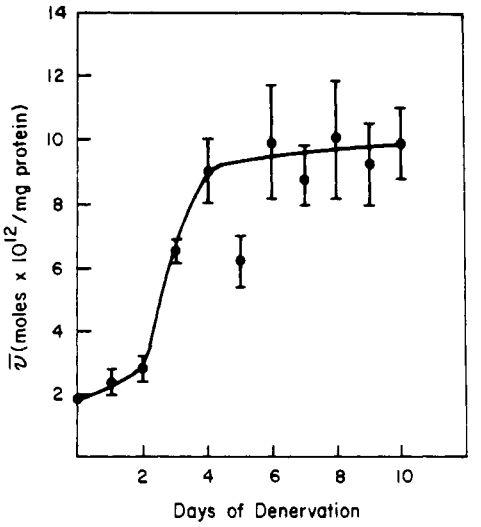

FIGURE 6: The change in the number of binding sites in the low-affinity set (N_2) over the first 10-day period following denervation. The points are derived from an analysis of the isotherms from preparations from each day following denervation.

TABLE I: Inhibition of [^{125}I]Iodo-α-bungarotoxin to High-Affinity Set during the Denervation Period.

Days of Denervation	N_1/N_c[a] Exptl	% Inhibition	N_1/N_c Predicted
0	1.01	0	1.00
1	–	–	–
2	1.05	0	0.87
3	0.609	40	0.68
4	0.566	43	0.6
5	0.54	46	0.57
6	–	–	–
7	0.56	44	0.525
8	0.57	43	0.519
9	–	–	–
10	0.56	44	0.48

[a] Represents the maximum amount of inhibition of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of sites derived by titrating inhibition with the myasthenic IgG (cf. Methods). Predicted ratio if 50% of the extrajunctional sites are blocked.

Antigenic Changes in the High Affinity Set. Previous reports from this laboratory described a serum IgG from patients with myasthenia gravis that interacted with the high affinity set of sites in a manner suggesting antigenic differences between the normal and denervated muscle. In the present study, we analyzed this apparent antigenic transition in the high-affinity set of sites over the 10-day denervation period. Table I presents the results of those experiments. Sensitivity to the myasthenic IgG appears at 3 days concurrently with the appearance of new sites in the high-affinity set. Figure 4 shows a graphical presentation of those data (open circles) compared with a plot of the expected results assuming a 50% block of all denervation-induced new sites in the high-affinity set (Almon and Appel, 1975c).

Discussion

In the present report, we have analyzed the binding isotherms of [^{125}I]diiodo-α-bungarotoxin over the range of 10^{-10}–10^{-5} M toxin. The binding isotherms can be separated into two components. The first component is an unsaturable component that cannot be blocked by dTc or by adding a

500-fold excess of unlabeled α-bungarotoxin. In other systems such binding has been variously denoted as "nonbiological" or "nonspecific binding". In the present report, as in other studies, the unsaturable component approximates linearity over a concentration range of one to two orders of magnitude. However, over the five orders of magnitude studied here, this component is best approximated by a power function in which the power (b) is less than unity. These unsaturable components probably represent a range of molecular associations mediated by several different noncovalent binding mechanisms.

The saturable component of the isotherm represents binding that can be blocked by dTc and is dramatically reduced by the addition of an excess of unlabeled toxin. This component consists of a discrete saturable number of sites. The progression of the isotherm over five orders of magnitude (10^{-10}–10^{-5} M toxin) can either indicate negative cooperativity or more than one set of sites. The former interpretation (cooperativity) would indicate that the binding of the toxin to each site decreases the probability of binding to each successive site. If the latter interpretation (two sets of sites) is employed, the data closely approximate the binding of the toxin to two sets of sites (the sites in each set being identical and independent). Although the present data cannot adequately differentiate between these two alternatives (ligand-induced heterogeneity or intrinsic heterogeneity), the changes in the isotherm over the 10-day denervation period favor the presence of two sets of sites. Following denervation, the higher affinity area of the isotherm is more affected than the lower affinity area. This apparent independence of the two areas of the isotherm suggests that the detergent-extracted preparation of the skeletal mixed muscle contain two sets of cholinergic sites. The first set has an affinity constant in the order of 10^9 M^{-1}. The number of sites in this set begins to increase between 2 and 3 days and continues for 8 days, at which time there is ~28-fold increase in number of sites. After the 8-day peak, the number of sites in this set begins to decline.

In previous reports from this laboratory, the set of high-affinity sites was characterized in the normal state and after 10 days of denervation using slow and fast muscle preparations (Almon et al., 1974a) and a mixed muscle preparation (Andrew et al., 1974). Those data demonstrated a 15-fold increase in the number of sites (per g of muscle) in fast muscle and a 20-fold increase in the number of sites (per g of muscle) in slow muscle after 10 days of denervation (Almon et al., 1974a). When we employed the toxin binding as a surface membrane marker in the fractionation of mixed muscle membranes before and after denervation, a 17-fold increase (per mg of protein) in the number of sites in the set was observed after 10 days of denervation (Andrew et al., 1974). The present data suggests that the level observed at 10 days (19-fold) actually represents a decline from a peak level (28-fold) observed at 8 days. These characteristics suggest that this high-affinity set represents the biological AcCh receptor.

The second set of sites has an affinity constant between 10^5 and 10^6 M^{-1}. Following denervation, the number of sites in this lower affinity set begins increasing almost immediately. A plateau is reached at 4 days, and this level is maintained to the end of the 10-day period. The new level is approximately fivefold higher than the normal muscle level. The biological definition of this set of sites is not clear. The AcChE has been shown to provide a second cholinergic site that interacts with α-bungarotoxin in the micromolar range. This interaction is also blocked by dTc (Stalc and Zupancic, 1972). However, AcChE decreases following denervation, whereas the low affinity set of sites increases fourfold after denervation. Another possible definition of the low affinity set of sites may be as a precursor to the high-affinity set. A more complete analysis of the molecular association of this site is in progress, following its partial purification. Such experiments may shed additional light on the biological role of this set of sites.

γ-Globulin isolated from patients with myasthenia gravis has been shown to provide a convenient means of monitoring certain antigenic properties of the AcCh receptor (Almon and Appel, 1975a). This IgG binds to the high-affinity set of sites in 10-day denervated muscle preparations but does not bind to the comparable complex in normal muscle preparations (Almon and Appel, 1975a). The myasthenic IgG appears to influence the number of available sites rather than the affinity of binding. In 10-day denervated muscle preparations, the maximal extent of this inhibition was between 44–48% of the total number of sites in the high-affinity set. This change in the antigenicity represents the only qualitative difference we have observed between junctional and extrajunctional receptors. The data in the present paper demonstrate that the sensitivity to myasthenic IgG appears concurrently with the increase in sites between 2 and 3 days. The fractional inhibition progresses as would be expected if 50% of the new sites in the high affinity set were blocked. More specifically, the extent of IgG inhibition appears to level off at a plateau of 40–50% inhibition as the number of residual normal muscle sites become relatively insignificant (3–5 days following denervation).

The change in antigenic properties of the high-affinity set during the period of denervation supports the observation of a difference between extrajunctional and junctional receptors suggested by studies of the turnover rate, sensitivity to acetylcholine, sensitivity to cholinergic antagonists, and isoelectric point previously discussed. Furthermore, the developmental disappearance of the high density of extrajunctional AcCh receptors following innervation and their reappearance following denervation suggest that they may play a role in intracellular nerve–muscle contact and synapse formation. Further investigations of the biochemical basis of the differences between junctional and extrajunctional receptors should prove important to understanding their potential role in synaptic formation as well as myasthenia gravis. Additional properties of these receptors are provided in the subsequent paper in this series.

Acknowledgments

We thank Ms. Millie Washington for technical assistance.

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Cholinergic Sites in Skeletal Muscle. II. Interaction of an Agonist and Two Antagonists with the Acetylcholine Site†

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ABSTRACT: The equilibrium interactions of α-bungarotoxin, *d*-tubocurarine, and carbamylcholine with junctional and extrajunctional skeletal muscle acetylcholine receptors were examined. *d*-Tubocurarine is a competitive inhibitor of the bindings of α-bungarotoxin to the acetylcholine receptor. No substantive difference was observed in the association of *d*-tubocurarine with the junctional and extrajunctional receptors. In contrast, the carbamylcholine inhibition of toxin binding is not competitive. The data indicate that either the single set of α-bungarotoxin and *d*-tubocurarine binding sites contains two subsets of carbamylcholine sites or that the carbamylcholine binds in a cooperative manner to a single set of sites. In addition, the affinity of carbamylcholine for extrajunctional receptors may be higher than the affinity for junctional receptors.

In the preceding paper of this issue, a set of high-affinity binding sites in normal and denervated skeletal mixed muscle preparations was described. It was concluded based on those data and the results of other investigations that this set represented the interaction of α-bungarotoxin with the cholinergic receptor site that mediates muscle depolarization. In normal muscle preparations, greater than 95% of the sites in the set represent junctional receptors, and in 10-day denervated muscle preparations, approximately 95% of the sites are extrajunctional receptors (Almon and Appel, 1975). A difference was also observed between junctional and extrajunctional receptors with respect to their interaction with an IgG derived from the serum of some patients with myasthenia gravis.

Fundamental to both the definition of this set of high-affinity binding sites as the AcCh receptor and understanding its role in the cholinergic effector mechanism is the analysis of its interaction with cholinergic agonists. The pharmacological behavior of cholinergic agonists and antagonists provides a framework within which the molecular interactions can be interpreted. Since the binding of a cholinergic agonist to the acetylcholine receptor results in membrane depolarization and the binding of a cholinergic antagonist does not, a fundamental difference in the binding of the two types of agents is indicated. In addition, the prolonged exposure of the muscle to cholinergic agonists results in the phenomenon known as desensitization (Katz and Thesleff, 1957). The characteristics of cholinergic desensitization suggest that it represents an agonist-mediated receptor inactivation (Rang and Ritter, 1969a,b). It is possible that the inactivation reflects a metastable conformational change in the receptor induced by the agonist but not requiring its presence for interim maintenance (Rang and Ritter, 1969a).

Although the analysis of thermodynamic characteristics does not allow the examination of dynamic biological events, it does allow the assessment of equilibrium variations which may help explain the functional properties. In the present re-

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