Acetylcholine Receptors in Normal and Denervated Rat Diaphragm Muscle. I. Purification and Interaction with [^{125}I]-α-Bungarotoxin†

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ABSTRACT: Acetylcholine receptors have been purified from junctional regions of normal rat diaphragm muscle and from extrajunctional regions of denervated diaphragm. The reaction of the purified receptors with [^{125}I]-α-bungarotoxin has been investigated by kinetic methods. The toxin-receptor complexes dissociated in a biphasic manner at 35° with a rapidly dissociating component ($t_{1/2}$ = 4 hr) and a slowly dissociating component ($t_{1/2} \geq 100$ hr). The association reaction between toxin and receptor did not obey simple second-order kinetics but could be analyzed in terms of two classes of binding sites corresponding to the two rates of dissociation. This treatment of the data allowed derivation of association rate constants for the two sites. Values obtained for the dissociation constants were 3.7×10^{-10} and $\leq 0.4 \times 10^{-10}$ M for the junctional receptor and 1.7×10^{-10} and $\leq 0.2 \times 10^{-10}$ M for the extrajunctional receptor. In each case it is the more tightly binding component that associates and dissociates more slowly. Receptors present in crude preparations were comparable to purified receptors in their reaction with [^{125}I]-α-bungarotoxin. The validity of the two site model is discussed in relation to the kinetic studies.

At the vertebrate neuromuscular junction, action potentials in the presynaptic nerve cause the release of acetylcholine (ACh)[1] from the nerve terminals. The transmitter diffuses across the synaptic cleft and interacts with a protein receptor in the end plate membrane of the muscle. The combination of ACh with its receptor causes an increase in the permeability of the end plate membrane to sodium and potassium ions, resulting in a local potential change across the muscle membrane (Katz, 1966).

The distribution of ACh receptors on muscle cells has been analyzed both by recording the membrane potential during localized iontophoretic application of ACh to the cell surface, and by examining the distribution of binding sites for radioactive α-bungarotoxin (α-BuTx), a small protein that binds tightly and specifically to the ACh receptor in muscle. These methods have shown that in adult vertebrate muscle, most ACh receptors occur in the membrane at the neuromuscular junction. Within several days after cutting the motor nerve to the muscle, however, receptors also appear in extrajunctional membrane (Axelsson and Thesleff, 1959; Miledi, 1960a; Miledi and Potter, 1971; Barnard et al., 1971; Berg et al., 1972; Fambrough and Hartzell, 1972). If the denervated muscle is then reinnervated, a reduction in the number of receptors outside the end plate occurs until the normal adult pattern is again achieved (Miledi, 1960b; McArdle and Albuquerque, 1973). A change in ACh receptor distribution also appears to occur during development. Individual myotubes formed in culture have ACh receptors over their entire surface (Fischbach and Cohen, 1973; Hartzell and Fambrough, 1972; Vogel et al., 1972; Steinbach et al., 1973), and muscle fibers in fetal or neonatal rats have substantial sensitivity to ACh outside the end plate (Diamond and Miledi, 1962).

Although their functional significance is unknown, the changes in ACh receptor distribution that occur after denervation and during development have attracted considerable interest. The striking correlation between the presence of extrajunctional receptors and the ability of muscles to establish new synapses (Janssen et al., 1973) has suggested that extrajunctional receptors may be important for synapse formation. The recent demonstration that extrajunctional ACh sensitivity of frog parasympathetic neurons is increased after denervation (Kuffler et al., 1971) indicates that the variations in extrajunctional ACh sensitivity that occur in muscle may be of general interest in the nervous system.

To gain further insight into the changes that occur after denervation, we have extensively purified the end plate ACh receptors from normal rat diaphragm muscle and the ACh receptors that appear in extrajunctional regions of muscle after denervation and have compared the properties of the two receptor preparations. The comparison is made possible by the availability of small protein toxins that bind tightly and specifically to the ACh receptor (Chang and Lee, 1963). These toxins have been useful in the purification and study of the ACh receptor from the electric organs of eel and marine ray (Karlin, 1974), but are absolutely essential for study of the ACh receptor in muscle where the concentration of receptor is very low. Because of its tight binding to the ACh receptor and the availability of toxin at high specific radioactivity, we have used [^{125}I]-α-BuTx in investigations of the isolated ACh receptors. In this paper we report the purification of junctional and extrajunctional ACh receptors from rat diaphragm muscle and the characteris-

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[1] Abbreviations used are: ACh, acetylcholine; TN, 50 mM NaCl-50 mM Tris-HCl (pH 7.4); TNT, 50 mM NaCl-50 mM Tris-HCl (pH 7.4)-1% Triton X-100; α-BuTx, α-bungarotoxin; JR, junctional acetylcholine receptor; EJR, extrajunctional acetylcholine receptor; SDS, sodium dodecyl sulfate.

tics of their reaction with [^{125}I]-α-BuTx. In the succeeding paper, the biochemical and pharmacological properties of the two receptors are further compared.

Experimental Section

Materials

White rats (Sprague-Dawley) were obtained from Gofmoor Farms. Denervation was performed by transection of the left phrenic nerve in the thorax.

Ampholine was obtained from LKB-Produkter AB, Triton X-100 (scintillation grade) was from Packard Instrument Co., Inc., carbamylcholine chloride was from Sigma, and DEAE-cellulose discs from Reeve-Angel.

The venom of *Bungarus multicinctus* was obtained from Ross Allen Reptile Institute and α-BuTx purified according to Berg et al. (1972). Cobrotoxin (also referred to as Siamensis 3 toxin by Karlsson et al., 1971) was purified from the venom of *Naja naja siamensis* (obtained from Miami Serpentarium) according to Cooper and Reich (1972), except that the final chromatography step was on CM-Sephadex instead of CM-cellulose. When 27 μg of the purified cobrotoxin was analyzed by SDS gel electrophoresis (Weber and Osborn, 1969), a single component was observed with a mobility corresponding to a molecular weight of approximately 8×10^3. The purified toxin at a concentration of 4 μg/ml blocked the response of the isolated diaphragm preparation to iontophoretically applied acetylcholine within 20 min.

Methods

Preparation of Affinity Resin. Cobrotoxin-Sepharose was prepared according to the method of Cuatrecasas (1970). Sepharose 4B (15 g wet weight) was suspended in 33 ml of distilled water, and activated by addition of 625 mg of cyanogen bromide. The activated Sepharose was washed with cold 0.2 *M* sodium carbonate (pH 9.4) and reacted with 11.2 mg of purified cobrotoxin in 25 ml of the same buffer. After 20 hr at 4°, the resin was washed with 1 l. of 1 *M* NaCl–0.2 *M* sodium carbonate (pH 9.4), 1 l. of 1 *M* NaCl–0.2 *M* sodium acetate (pH 4.5), and 1 l. of 0.01 *M* Tris-HCl (pH 7.4), and stored at 4° in 0.01 *M* Tris-HCl (pH 7.4) and 0.02% sodium azide. Less than 10% of the cobrotoxin was recovered in the wash indicating a content of 0.35–0.40 mg of toxin/ml of packed Sepharose beads.

DEAE-Cellulose Filter Binding Assay. Binding of [^{125}I]-α-BuTx to the receptor was measured by a DEAE-cellulose filter assay similar to that described by Klett et al. (1973) and by Schmidt and Raftery (1973). The receptor sample in 50 m*M* Tris-HCl (pH 7.4)–50 m*M* NaCl–1% Triton X-100 (TNT) was incubated at 35° with [^{125}I]-α-BuTx either in a small siliconized glass tube or, for incubations longer than several hours (see Figures 2 and 4), in a 4.8×0.3 cm stoppered plastic tube. Losses due to evaporation from the stoppered tube were less than 2% per day, as evaluated by counting aliquots during the incubations. At the end of the incubation, aliquots of less than 50 μl were applied in duplicate to a double thickness of DEAE-81-cellulose paper discs (diameter 24 mm), previously washed with 50 m*M* Tris-HCl (pH 7.4)–50 m*M* NaCl (TN). The DEAE discs were washed with 15 ml of TN on a Millipore filtration apparatus, dried under a heat lamp, and counted for radioactivity (at approximately 32% efficiency) after the addition of 5 ml of fluor (0.2% 2,5-diphenyloxazole and 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene). Nonspecific binding was always determined in parallel by assaying reactions performed without receptor or with a 200-fold excess of α-BuTx. Nonspecific binding was 1–3% of the total input radioactivity, depending on the batch of DE discs and the toxin preparation. The assay was linear with respect to volume of extract over the range used here and the retention of receptor–toxin complex on the discs was quantitative as determined by gel filtration of reaction mixtures on 0.5 *M* agarose columns. All points presented in this paper are the average of duplicate assays.

Iodination of α-BuTx. α-BuTx was iodinated according to the general method of Greenwood et al. (1963). A reaction containing 35 μl of carrier free Na^{125}I (9.4 mCi) in 0.1 *M* NaOH, 150 μl of 1 *M* sodium phosphate (pH 7.5), and 5 μl of α-BuTx (65 μg) was initiated at 0° by addition of 5 μl of 25 mg/ml of chloramine T and terminated 5 sec later with 50 μl of 50 mg/ml of sodium metabisulfite. The reaction mixture was applied to a column of Bio-Gel P2 in a siliconized Pasteur pipet (void volume 1.9 ml) previously washed with 1 ml of 5 mg/ml of bovine serum albumin and 30 ml of 0.01 *M* sodium phosphate (pH 7.4). The column was eluted with 0.01 *M* sodium phosphate (pH 7.4) and fractions (0.18 ml) were collected in sterile disposable plastic tubes. The excluded fractions, which contained approximately 50% of the initial radioactivity, were pooled.

The iodinated toxin was further purified by isoelectric focusing. [^{125}I]-α-BuTx was mixed with 0.5 volume of 10% sucrose, 2% ampholine (pH 7–10), and 1% ampholine (pH 9–11) and applied to an 8×0.5 cm polyacrylamide gel [5% acrylamide, 0.19% methylenebisacrylamide, 0.85% ampholine (pH 9–11), and 0.25% ampholine (pH 7–10)], that had been polymerized in a siliconized glass tube, and prerun for 30 min at 1 mA/gel with an upper reservoir buffer of 0.1 *M* NaOH and a lower buffer of 0.2% H_2SO_4. α-BuTx (20 μg) was run on a parallel gel as a marker. After focusing for 1.5 hr at 1 mA/gel, the radioactive gel was carefully extruded and frozen, while the α-BuTx gel was stained for 1 hr according to Malik and Berrie (1972). The alkaline half of the radioactive gel was sliced into 1- or 2-mm segments which were counted directly in a Packard well type γ counter. Radioactive slices were pooled, and the toxin was extracted by shaking for 24 hr at 4° in sterile 0.5 *M* Tris-HCl (pH 7.4), 0.1 *M* NaCl, and 1 mg/ml of bovine serum albumin. The supernatant was decanted and stored frozen at −20°. Since the monoiodotoxin has a slightly more acidic isoelectric point, this procedure results in enrichment for the monoiodo species. The toxin was analyzed by digestion with Viokase (Vogel et al., 1972), and paper chromatography in 1-butanol [120]–H_2O[50]–glacial acetic acid [30] with markers of monoiodotyrosine and diiodotyrosine. Part of the radioactivity (15–20%) migrated within 10 cm of the origin, presumably representing products of incomplete digestion, while 80–85% migrated with monoiodotyrosine and background amounts (i.e., less than 2%) with diiodotyrosine. The range of specific activity of various preparations used in this work was 200–1500 Ci/mmol. Parallel experiments performed with the two receptors always used the same preparation of [^{125}I]-α-BuTx and identical reagents as far as possible.

Purification of Receptors. Protein in subcellular fractions was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The precipitate that formed when the phenol reagent was mixed with samples or standards containing Triton X-100 was removed by sedimentation. Protein in affinity purified fractions was determined by the fluorometric assay of Böhlen et al. (1973) with bovine serum albumin as standard. Standards were

Table I: Purification of Acetylcholine Receptors.[a]

Fraction	Protein (mg)	Binding Capacity (pmol of [^{125}I]-α-BuTx)	Specific Activity (pmol of [^{125}I]-α-BuTx/mg of Protein)	Purification	Recovery of Binding Capacity (%)
		A. Junctional Receptor			
1. Crude homogenate	602	18.9	0.031	1	100
2. S2 supernatant	100	12.8	0.12	3.9	68
3. Cobrotoxin-Sepharose	~0.02[b]	3.8	~190	~6100[c]	20
		B. Extrajunctional Receptor			
1. Crude homogenate	312	50.8	0.17	1	100
2. S2 supernatant	33.6	34.0	1.0	5.9	67
3. Cobrotoxin-Sepharose	~0.02[b]	9.7	~530	~3100	19

[a] Summary of representative purifications of each receptor. The binding capacity was estimated by incubating samples of each fraction with 3×10^{-8} *M* [^{125}I]-α-BuTx at 35° for 1 hr, followed by filtration on DEAE-cellulose discs. For toxin binding concentrations of $0.5-1 \times 10^{-9}$ *M* this results in >90% saturation of binding capacity. [b] Provisional value obtained by reaction with fluorescamine. [c] Degree of purification is dependent on original dissection, viz., amount of extrajunctional tissue cut out in strips of muscle containing end plates.

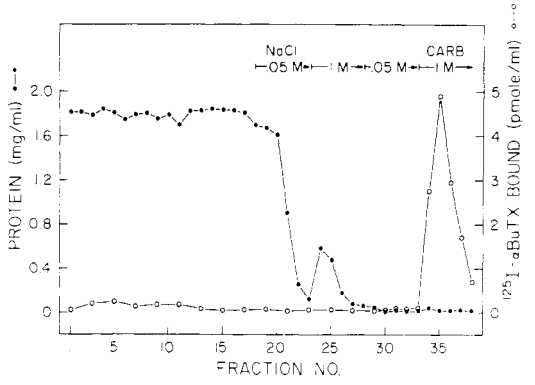

FIGURE 1: Affinity chromatography of extrajunctional receptor on a column of cobrotoxin-Sepharose. Fraction S2 (33.6 mg of protein and 34.0 pmol of [^{125}I]-α-BuTx bound) (19.8 ml) was chromatographed on a column of cobrotoxin-Sepharose as described in the Experimental Section. The fraction size was approximatey 1 ml.

made up in TNT buffer and, together with samples, dialyzed exhaustively against 0.05 *M* sodium phosphate (pH 8.0), 0.05 *M* NaCl, and 1% Triton X-100 before reaction with fluorescamine. The toxin binding capacity of the receptor was monitored throughout the purification with the DEAE-cellulose filtration assay.

For preparation of the junctional receptor (JR), the end plate regions of the diaphragm were dissected from 60 to 100 normal rats. To obtain the extrajunctional receptor (EJR) appearing after denervation, regions without end plates were dissected from 40 to 50 hemidiaphragms that had been denervated 7–21 days previously (Berg et al., 1972). During the dissection, which lasted 4–7 hr, the strips were stored at 0° in Krebs–Ringer bicarbonate (pH 7.3). The strips were weighed and were then homogenized, at a concentration of 50 mg/ml, at 0° in TN in a Waring Blendor or "Virtis" apparatus. The homogenate was centrifuged at 15,000*g* for 30 min at 0°, and the pellets were resuspended in TNT (0.1–0.3 of the original homogenate volume) with a Teflon pestle. After extraction at 0° for 30 min, the suspension was centrifuged at 100,000*g* for 1 hr at 0°. The supernatant (S2 fraction) contained 60–70% of the receptor originally present. Recent experiments (C. Weinberg, unpublished results) indicate that more than 90% of the receptor can be recovered in the S2 fraction if the original homogenate is centrifuged at 100,000*g* for 1 hr. For investigation of receptor properties in crude extracts, the S2 fractions were sterilized by Millipore filtration (Swinnex-13 filter unit) and stored at 0°.

For further purification, the S2 fraction was applied at room temperature to a plastic syringe (0.5 × 5 cm) containing 1 ml of cobrotoxin-Sepharose equilibrated with TNT. The flow rate was approximately 10 ml/hr. The S2 fraction was passed through the column twice, resulting in retention of approximately 90% of the toxin binding capacity. The column was then washed successively with 5 ml of TNT, 5 ml of a solution containing 50 m*M* Tris-HCl (pH 7.4), 1 *M* NaCl, 1% Triton X-100, and 5 ml of TNT and was eluted with 5 ml of 50 m*M* Tris-HCl (pH 7.4), 1 *M* carbamylcholine chloride, and 1% Trixton X-100. Fractions of 1 ml were collected and dialyzed exhaustively against TNT at 4°. The peak fractions were pooled and stored in a sterile plastic tube at 0°. A typical elution profile from such a column is shown in Figure 1. Preparations of both receptors lost approximately 70% of their activity over 10–12 months.

The purification of both receptors is summarized in Table I.

Determination of the Proportion of the Two Complexes as a Function of the Time of Incubation. The binding reaction contained in a volume of 200 μl, 3×10^{-9} *M* purified extrajunctional receptor, 3×10^{-8} *M* [^{125}I]-α-BuTx, 0.1% NaN_3, 0.1% Triton X-100, 50 m*M* NaCl, and 50 m*M* Tris-HCl (pH 7.4). After incubating in a stoppered plastic tube for 1 hr at 35°, duplicate aliquots of 3 μl were assayed by DEAE-cellulose filtration, and 75 μl was passed through a column (29.5 × 0.5 cm diameter) of Sephadex G-75 equilibrated and run in 0.1% Triton X-100, 50 m*M* NaCl, and 50 m*M* Tris-HCl (pH 7.4). The radioactivity in the fractions (volume 760 μl) eluting at the void volume was more than 95% retained on DEAE-cellulose. The fractions were pooled, made 3×10^{-7} *M* in α-BuTx and 0.03% in NaN_3, and assayed by DEAE-cellulose filtration. After 24-hr incubation at 35°, the mixture was assayed again and the proportion of the two forms was determined as described previously.

The original binding reaction as also incubated for 24 hr, when the analysis was repeated as described above. The amount of complex formed after 24-hr incubation was within 5% of that formed after 1 hr. An aliquot of the G-75 purified complex was incubated for 24 hr at 35° without unlabeled toxin. The degree of dissociation was within 10% of that observed with excess cold toxin in the reaction. This shows that negative cooperativity effects of the kind recently described by DeMeyts et al. (1973) and Frazier et al. (1974) are not significant in this case.

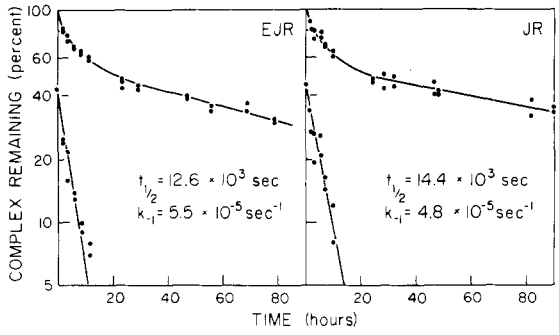

FIGURE 2: Time course of the dissociation of complexes containing purified receptor and [^{125}I]-α-BuTx. The reaction for the extrajunctional receptor (volume 204 μl, conditions identical with those in Figure 4) was incubated at 35° for 2 hr, when 75 μl was diluted into 200 μl of 6.10^{-7} M α-BuTx in 1 mg/ml of bovine serum albumin, TNT, and 0.1% NaN_3. At various times of incubation at 35°, duplicate aliquots of 5 μl were removed and assayed by DEAE-cellulose filtration. The reaction for the junctional receptor (conditions identical with those in Figure 4) was incubated at 35° for 2 hr, when 190 μl was diluted into 110 μl of 1×10^{-6} M α-BuTx solution and analyzed as above. Comparable results have been obtained with two independent preparations of each receptor.

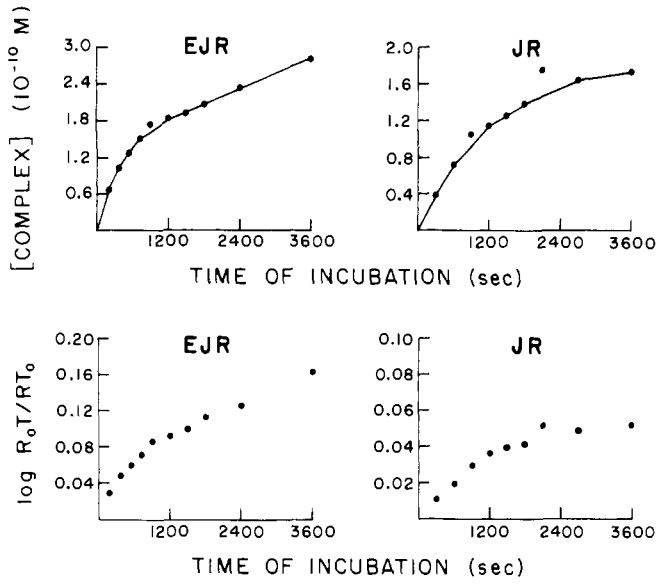

FIGURE 3: Time course of binding of [^{125}I]-α-BuTx to purified receptors. The reaction for the extrajunctional receptor contained, in a volume of 200 μl, 1.38×10^{-9} M [^{125}I]-α-BuTx and 0.62×10^{-9} M purified receptor in TNT. After incubation at 35° for various times a 15-μl aliquot was diluted into 35 μl of 2 μM α-BuTx, from which duplicate aliquots of 20 μl were assayed by DEAE-cellulose filtration. The reaction for the junctional receptor contained, in a volume of 200 μl, 1.38×10^{-9} M [^{125}I]-α-BuTx and 0.77×10^{-9} M purified receptor in TNT. After incubation at 35° the reaction was analyzed as above. The data for each reaction were plotted according to the integrated second-order rate equation: $\log [R]_0[T]/[R][T]_0 = ([T]_0 - [R]_0)kt/2.303$, where $[R]_0$ = initial receptor concentration; $[T]_0$ = initial toxin concentration; [R] and [T] = concentration of receptor and toxin, respectively, at time t; and k is the second-order rate constant. Comparable results have been obtained with three independent preparations of each receptor.

Results

Purification of Receptors. ACh receptors from normal rat diaphragm muscles (junctional receptors) and from regions of denervated muscles without end plates (extrajunctional receptors) were purified separately. The purification consisted of detergent extraction of crude membrane preparation followed by adsorption of the receptor to an affinity column of Sepharose beads derivatized with toxin from *Naja naja siamensis* (see Methods) and elution of the receptor with carbamylcholine (Karlsson, et al., 1972). The purification was monitored by measuring the binding of [^{125}I]-α-BuTx to the solubilized receptor. Because of the low levels of receptor in skeletal muscle and the small amounts of tissue available, [^{125}I]toxin of very high specific activity (>300 Ci/mmol) was required.

The results of typical purification procedures for the two receptors are shown in Table I. No obvious differences were noted in the behavior of the two receptors during purification, as, for example, in their elution from the affinity resin. In four different preparations of the junctional receptor (JR) and five preparations of the extrajunctional receptor (EJR), final yields ranged from 13 to 29%. The very small amounts of purified receptor did not permit a physical examination of purity. The specific activity of the purified extrajunctional receptor (0.5 μmol of toxin/g of protein) is approximately 10% of values obtained for purified receptors from electric fish (quoted in Meunier et al., 1974).

Reaction with [^{125}I]-α-BuTx. The reaction of the purified receptors with [^{125}I]-α-BuTx was studied by using a DEAE filter assay. In this assay, toxin–receptor complex is retained by the filter while free toxin passes through. In initial experiments, we attempted to study the characteristics of the toxin-binding reaction at equilibrium. Although the times required to achieve equilibrium at nonsaturating concentrations of toxin and receptor were too long to be practicable, Scatchard plots derived from data close to equilibrium showed two intersecting straight lines, suggesting the existence of two classes of binding sites (data not presented).

The characteristics of the reaction were further analyzed by kinetic methods. The complex nature of the interaction between toxin and receptor indicated by the Scatchard plots was confirmed by analysis of the rate of dissociation of the toxin–receptor complexes. Reaction mixtures containing radioactive toxin–receptor complex were diluted into a large excess of unlabeled toxin and the amount of radioactive complex remaining after various times was determined. In the case of each receptor, a semilogarithmic plot of the percent original complex vs. time indicated that dissociation of the complex occurs with at least two different rate constants (Figure 2). The slower reaction has a half-time of about 100 hr in each case. This value should be taken as a lower limit, since it is possible that denaturation or proteolysis increases the apparent dissociation of complex during such long incubations. After correction for the contribution of the slower component, the data for the faster component in each case fitted a straight line in the semilogarithmic plot (Figure 2). The values for the half-time of dissociation for the fast reaction were 3.5 and 4 hr for extrajunctional and junctional complexes, respectively. By extrapolating the slow reaction back to zero time, the original proportion of slowly dissociating toxin–receptor complex formed could be estimated, and was about 55% in each case. It should be noted that quantitative conversion of receptor to receptor–toxin complex did not occur under these conditions (see below).

The rate of formation of toxin–receptor complex was also analyzed. The time course of the binding reaction during incubation at 35° is shown for each receptor in Figure 3. When these data were analyzed by assuming that a single class of receptor sites binds toxin in a simple second-order reaction, and were plotted according to the integrated form

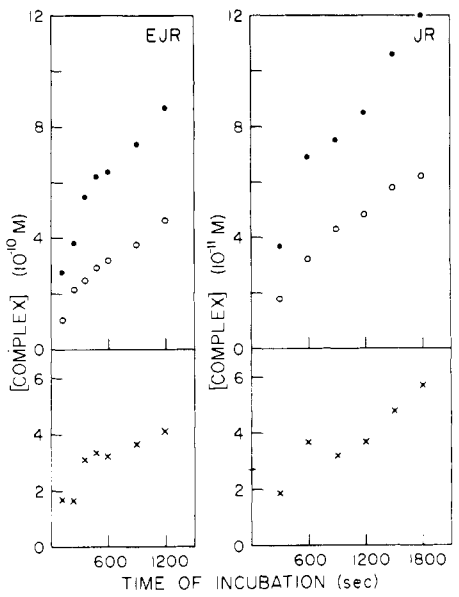

FIGURE 4: Time course of binding of [^{125}I]-α-BuTx to purified receptors. The reaction for the extrajunctional receptor contained, in a volume of 102 μl, $3.44.10^{-9}$ M [^{125}I]-BuTx and $2.64.10^{-9}$ M receptor in TNT. After incubation at 35° for various times an 8-μl aliquot was diluted into 72 μl of 4.10^{-7} M α-BuTx in 1 mg/ml of bovine serum albumin, TNT, and 0.1% NaN_3, from which duplicate aliquots of 10 μl were assayed by DEAE-cellulose filtration. The remainder was incubated for 20 hr at 35° after which duplicate aliquots of 20 μl were assayed to determine the amount of slow component remaining (see text). The amount of the fast component was derived by subtraction. The reaction for the junctional receptor contained, in a volume of 400 μl, $1.38.10^{-9}$ M [^{125}I]-α-BuTx and 1.00×10^{-9} M receptor in TNT. After incubation at 35° an aliquot of 30 μl was diluted into 42 μl of α-BuTx solution (as above) and duplicate aliquots of 12 μl were filtered. After incubation as above, aliquots of 20 μl were filtered. Comparable results have been obtained with two independent preparations of the extrajunctional receptor and one preparation of the junctional receptor. (●) Total complex formed; (○) slow dissociating complex; (x) fast dissociating complex.

Table II: Iterative Estimates of the Association Rate Constants for the Two Components.[a]

A. Junctional Receptor				B. Extrajunctional Receptor			
$k_1 \times 10^5$ (M^{-1} sec^{-1})							
Δt (min)	Fast	Δt	Slow	Δt (min)	Fast	Δt	Slow
0–5	1.6	0–5	0.6	0–2	5.9	0–2	1.5
5–15	0.7	5–10	0.5	2–6	3.4	2–4	1.7
15–20	0.5	10–15	0.4	6–8	1.4	4–6	0.4
20–25	1.2	15–20	0.2	8–15	0.9	6–8	0.9
25–30	1.2	20–25	0.4	15–20	1.5	8–10	0.5
		25–30	0.2			10–15	0.5
						15–20	0.8
Mean	1.1		0.4		2.6		0.9

[a] The association rate constants were calculated for various time intervals (Δt) as described in the text. Data are taken from Figure 4.

Table III: Estimates of Dissociation Constants for the Two Components.[a]

		Value at 35°	
Kinetic Constant		Junctional Receptor	Extrajunctional Receptor
k_{-1} (sec^{-1})	Fast	4.8×10^{-5}	5.5×10^{-5}
	Slow	$\leq 1.9 \times 10^{-6}$	$\leq 1.9 \times 10^{-6}$
k_1 (M^{-1} sec^{-1})	Fast 1.	1.1×10^5	1. 2.6×10^5
	2.	1.5×10^5	2. 3.8×10^5
	Slow 1.	0.4×10^5	1. 0.9×10^5
	2.	0.6×10^5	2. 0.9×10^5
K_D (M) = k_{-1}/k_1	Fast	3.7×10^{-10}	1.7×10^{-10}
	Slow	$\leq 0.4 \times 10^{-10}$	$\leq 0.2 \times 10^{-10}$

[a] The k_{-1} values are from Figure 2; the k_1 values are given for two experiments analyzed as in Table II. The K_D's were calculated using mean values of k_1.

of the second-order rate equation, in neither case did the data fit a straight line (Figure 3). Thus this simple formulation of the binding reaction is incorrect.

The characteristics of the dissociation reaction suggested that it would be appropriate to analyze the rate of formation of complex in terms of two independent binding reactions. Although such a situation is most conveniently analyzed under the pseudo-first-order conditions of toxin excess, the high background of the filter assay did not allow the use of these conditions. Fortunately, the large difference in dissociation rates for the two complexes permitted us to separate their contributions to the total binding reaction.

To determine separately the amount of each complex, samples were removed at intervals during the binding reaction into a large excess of unlabeled toxin. The amount of radioactive toxin–receptor complex was determined by immediate assay and by assay after 20 hr of incubation to allow dissociation of the fast component. A small correction was applied (assuming a $t_{1/2}$ of 100 hr) for dissociation of the slow component during the incubation in unlabeled toxin. Thus, at each time point in the association reaction, the amount of total complex and the amount of slowly dissociating complex was determined. After calculation, by difference, of the amount of rapidly dissociating complex, the total binding could be expressed as the sum of two separate reactions as shown in Figure 4.

In order to calculate the forward rate constants for each reaction it was necessary to determine the initial concentrations of the two toxin-binding sites. Receptor preparations were incubated with excess [^{125}I]-α-BuTx and freed from most of the unreacted toxin by rapid gel filtration. The total amount of toxin–receptor complex was determined by filtration assay and the amount of slowly dissociating component by assay after incubation for 20 hr in excess unlabeled toxin. This value was corrected as above to give the initial concentration of the slowly dissociating complex. The purified preparations of junctional and extrajunctional receptor used in these experiments each contained about 70% of the slowly dissociating component.

The association rate constants for the two components were then estimated from the data in Figure 4 by considering the reaction as a series of intervals between points on the curves. For such an interval, the initial and final receptor concentrations and initial toxin concentration are known, and the final toxin concentration can be estimated to be that resulting from reaction only with the appropriate component. (This treatment ignores the change due to reaction with the other component, which introduces a small error.) Therefore, the second-order rate constant can be derived for each interval. The results of such analysis, using the data in Figure 4, are shown in Table II. For each reaction, the rate constants calculated for all the intervals agree within a factor of 5. The rate constants derived from this and other experiments are shown in Table III.

Dissociation constants for the two components in each receptor preparation were calculated from the kinetic con-

stants. Average values were 3.7×10^{-10} and $\leq 0.4 \times 10^{-10}$ M for the two sites associated with the junctional receptor, and 1.7×10^{-10} and $\leq 0.2 \times 10^{-10}$ M for the extrajunctional sites. In each case it is the more tightly binding component that associates and dissociates more slowly.

The course of the reactions was simulated by programming a computer with the experimental values for receptor and toxin concentrations and rate constants. The agreement (Figure 5) is reasonable for both purified receptors, indicating that the two site model is sufficient to explain these data. Other possibilities are considered in the discussion.

We have assumed during analysis of these data that the two toxin-receptor complexes are derived from two toxin-binding components in the receptor preparations. An alternative hypothesis is that there are two classes of labeled toxin molecule, one (T_1) that both reacts and dissociates faster than the other (T_2). In order to test this possibility, we have incubated purified receptor with a saturating concentration of toxin and assayed the proportion of the two classes of complex after 1 and 24 hr of incubation. If there are two classes of receptor, the proportion should be independent of the time of incubation. If there are two classes of toxin, then for short incubation times the proportion of the two classes of complex will reflect their relative association rates. Over longer incubation times, the proportion of the two will "relax" to their equilibrium values. This occurs because the slowly dissociating form increases as RT_1 dissociates and free receptor reacts with T_2, i.e.

$$\begin{aligned} &R + T_1 \rightleftharpoons RT_1 \\ &+ \\ &T_2 \rightleftharpoons RT_2 \end{aligned}$$

The proportion of the two forms at 1 and 24 hr was measured in an experiment in which extrajunctional receptor was incubated with a tenfold excess of toxin (see Methods). The proportion of the slow form was 60–62% of the total in two separate determinations at each time and therefore did not change during this period; thus the heterogeneity is a property of the receptor preparation.[2] In addition we have verified that this proportion is independent of the ratio of toxin/receptor at saturating toxin concentrations (data not shown). If the two complexes arise from heterogeneity in the receptor preparation, then the proportion of the more rapidly formed complex should increase as the toxin concentration increases.

Are the two components present as sites on distinct molecules, or on the same molecule, perhaps on different subunits? When aliquots of the reactions from Figure 4 were sedimented on sucrose density gradients, all the radioactivity was recovered either in a position characteristic of free toxin or at approximately 9 S, the sedimentation constant of the solubilized toxin-receptor complex (Berg et al., 1972). We conclude that if the sites are on distinct molecules, these molecules are not readily separated by zonal centrifugation. In the absence of extensive chemical information about the purified receptors, we cannot give a detailed answer to this question.

Reaction of Crude Receptor Fractions with [^{125}I]-α-BuTx. Since it is possible that the affinity chromatography procedure has either selected out a particular population of receptors or altered their properties, we have performed the above analyses on less pure preparations of both receptors.

[2] We are grateful to Dr. Frank Solomon for suggesting this test of toxin heterogeneity.

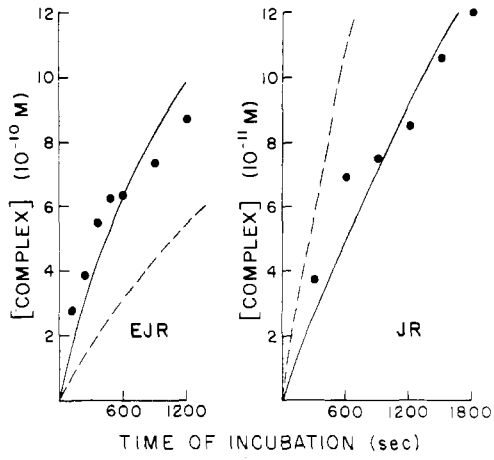

FIGURE 5: Computer simulation of the toxin binding reactions. The course of the reactions of Figure 4 was simulated with an iterative computation, using the experimental concentrations of receptor and toxin and the average k_{+1} and k_{-1} values of Table III. The contribution of k_{-1} slow was ignored. The interval of computation was 30 sec of reaction and the result was printed out every minute. The resulting points were joined to give the continuous lines. The experimental points are those of Figure 4. (—) Computed with k values for the appropriate receptor; (- - -) computed with k values for the other receptor.

Table IV: Kinetic Properties of Crude Receptors.[a]

Kinetic Constant		Value at 35°	
		Junctional Receptor	Extrajunctional Receptor
$t_{1/2}$ off (sec)	Fast	1.1×10^4	1.1×10^4
	Slow	$\geq 3.6 \times 10^5$	$\geq 3.6 \times 10^5$
k_{-1} (sec^{-1})	Fast	6.3×10^{-5}	6.3×10^{-5}
	Slow	$\leq 1.9 \times 10^{-6}$	$\leq 1.9 \times 10^{-6}$
k_1 (M^{-1} sec^{-1})	Fast	1.0×10^5	3.1×10^5
	Slow	0.3×10^5	1.2×10^5
$K_D = k_{-1}/k_1$ (M)	Fast	6.3×10^{-10}	2.0×10^{-10}
	Slow	$\leq 0.6 \times 10^{-10}$	$\leq 0.2 \times 10^{-10}$
Ratio of two components (fast/slow) at saturating toxin concentrations		1:4	1:3

[a] The kinetic properties of S2 fractions (see Methods) of the two receptors were investigated as detailed for the purified receptors. These data were obtained for one preparation of each receptor.

As shown in Table IV the values for the various kinetic constants and ratio of the two components are comparable to those obtained for the purified receptors (Table III). We conclude that the toxin-binding properties of purified receptors are representative of receptors present in less pure preparations.

Discussion

We have used affinity chromatography to purify junctional receptors from normal rat diaphragm muscle and extrajunctional receptors from regions of denervated muscle lacking end plates. Although both receptors were purified several thousand fold (Table I), the amount of protein in the purified preparations was too little for physical characterization by conventional techniques. The properties of the purified receptors were not significantly different from those of crude extracts of muscle; thus the affinity chromatography procedure does not seem to have selected a nonrepresentative population of receptors.

We have analyzed in some detail the interaction of the receptors with monoiodinated [^{125}I]-α-BuTx. The most

noteworthy feature of the reaction for both receptor preparations is the apparent existence of two classes of binding sites. This is indicated most clearly in the biphasic nature of the dissociation reaction where about 30% of the toxin bound to receptor dissociates with a half-time of 3.5–4 hr and the remainder with a half-time of 100 hr or more. In addition, Scatchard plots of data derived from reactions close to equilibrium show clear evidence of two classes of binding sites. These observations led us to analyze the association reactions in terms of two classes of binding sites, since both the muscle receptors consistently showed deviation from linearity in the integrated second-order plots (Figure 3). The assumption of two independent sites accounts satisfactorily for the kinetics of the toxin–receptor interaction (Figure 5) and gives a reasonably consistent set of values for the two association rate constants. The considerable variation in the association rate constants observed within each experiment (Table II) is likely to be exaggerated because, in the iterative estimate, the position of each point affects the rate constant for two neighboring intervals. The variability between average estimates for individual experiments, including those with crude preparations (Tables III and IV), is much less than that within an experiment.

We have chosen to analyze the kinetic properties of the toxin–receptor interaction in terms of two independent binding sites because of the simplicity of the model and its ability to account for the data. Other interpretations, however, are possible. Thus the data could also be consistent with negative cooperativity between identical sites, or with distinct sites that are in conformational equilibrium. These possibilities could, in principle, be distinguished by rapid reaction techniques, but the minute amounts of receptor in muscle prohibit such analyses. In any case, models based on two classes of toxin molecules are ruled out by the observation that the proportion of the two complexes is not dependent on the time of incubation. We have verified by computer simulation that this experiment is a clear test of the question.[3]

A number of laboratories have described the interaction of radioactively labeled neurotoxins with the ACh receptor from *Electrophorus* or *Torpedo* as well as muscle and their results can be compared with those obtained here. Multiple species that bind α-bungarotoxin have been detected in crude extracts of *Torpedo* (Raftery et al., 1972) and of mouse muscle (Chiu et al., 1973) by gel filtration or sucrose gradient sedimentation. The two binding sites detected in our experiments are not separated by these techniques. It is possible that the two sites are present on the same molecule, but further experiments will be required to determine this. In several preparations of junctional and extrajunctional receptor, the percentage of the slow site has varied between 60 and 80% of the total toxin-binding activity. Thus it is unclear whether the two sites have a strict stoichiometric relation.

The complex nature of the association reaction seen in our experiments has not been observed in studies on receptors from *Electrophorus* or *Torpedo*. The α-neurotoxins from both *Naja naja siamensis* and *Naja nigricollis* appear to bind to the eel receptor with a single second-order rate constant (Klett et al., 1973; Weber and Changeux, 1974; Lindstrom and Patrick, 1974). The reaction between α-bungarotoxin and the receptor from *Torpedo* also appears to be a simple bimolecular reaction (Franklin and Potter, 1972). In all cases, the second-order rate constants obtained are in approximately the same range ($0.5–5 \times 10^5\ M^{-1}$ sec^{-1}, see Weber and Changeux, 1974) as those obtained here.

A much wider range of values has been obtained for the rate constants of the dissociation reaction; in addition, complex kinetics have been observed in several cases. Klett et al. (1973) detected two classes of dissociation rates with complexes formed by purified receptor from *Electrophorus* and a pyridoxal derivative of α-toxin from *Naja naja siamensis*. They observed that the rapidly dissociating complex was converted to the slow form on prolonged incubation. Such a conversion was not found with the receptors from muscle. Weber and Changeux (1974) have also described a complex dissociation of *Naja nigricollis* α-toxin from membrane fragments and crude receptor preparations of *Electrophorus*.

Because the association and dissociation reactions between α-bungarotoxin and the muscle receptors occur so slowly, and the binding is so tight, it was not possible to study the toxin–receptor reaction at equilibrium. The dissociation constants for the two sites have been derived, however, from the kinetic data. In the case of both junctional and extrajunctional receptors, the lower dissociation constant is associated with the site whose rate of association and dissociation are slower. The value of this dissociation constant is close to that observed by Weber and Changeux (1974) for *Naja nigricollis* toxin and eel receptor, but is considerably lower than has been seen in the other systems. As pointed out above, this difference arises mostly from the very slow rate of dissociation.

Because of the complication of diffusion of toxin into the intact tissue, we have not explored the relation between the characteristics of the binding reaction observed in solution and the binding of α-bungarotoxin by the receptor in intact muscle. The existence of two classes of specific binding sites, however, present in comparable amounts, has been noted with several other receptor proteins. Thus the insulin receptor on lymphocytes (Gavin et al., 1973), the purified galactose binding protein of *Escherichia coli* (Boos et al., 1972), and the purified asialoglycoprotein receptor of hepatic plasma membranes (Hudgin et al., 1974) all show binding properties similar to those presented here. The physiological significance of these properties in the function of the acetylcholine receptor in situ is unclear.

Finally, these experiments show that the association rates for the toxin–receptor interaction may be significantly slower for the junctional receptor than for the extrajunctional receptor (Table III, Figure 5), although the variability in individual estimates of the rate constants (Table II) makes this conclusion a tentative one. More convincing evidence for the distinct nature of junctional and extrajunctional receptors is presented in the accompanying paper (Brockes and Hall, 1975).

[3] From other experiments, it was estimated that if the toxin were heterogeneous, the rapidly associating component must constitute between 16 and 66% of the total. Using these limits, and assuming a rate constant of 3×10^5 for the rapid component and $3–9 \times 10^4$ for the slow component, the time course of the binding reaction was computed for a variety of conditions. The range for the difference in proportion of the two forms at 1 and 24 hr was 18–36% of the total under these conditions.

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