Acetylcholine Receptors in Normal and Denervated Rat Diaphragm Muscle. II. Comparison of Junctional and Extrajunctional Receptors†

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ABSTRACT: Acetylcholine (ACh) receptors have been purified separately from normal rat diaphragm muscle (junctional receptors) and from extrajunctional regions of denervated diaphragm (extrajunctional receptors) in order to compare their properties. The toxin–receptor complexes of the two receptors were indistinguishable by gel filtration and by zone sedimentation in sucrose gradients, and showed identical precipitation curves with rabbit antiserum to the eel ACh receptor. Both toxin–receptor complexes bind concanavalin A and are therefore probably glycoproteins. Low concentrations of *d*-tubocurarine (dTC) were more effective in decreasing the rate of toxin binding to junctional than to extrajunctional receptors. The apparent dissociation constant for dTC binding to the junctional receptor was 4.5×10^{-8} *M*, whereas the value for the extrajunctional receptor was 5.5×10^{-7} *M*. When the complexes were analyzed by isoelectric focusing, the junctional complex focused at approximately 0.15 pH unit lower than the extrajunctional complex. This result was also found with crude preparations of receptor. We conclude that junctional and extrajunctional receptors are similar but distinct molecules. The properties of receptors present in neonatal diaphragm muscle were also examined and found to be similar to those of receptors in denervated muscle, as shown by dTC inhibition and isoelectric focusing.

The end plate membrane of a vertebrate skeletal muscle fiber contains a high density of receptors for the neurotransmitter acetylcholine (ACh).[1] In contrast, the extrajunctional membrane of such a fiber has few, if any, receptors. After denervation of the muscle, however, large numbers of receptors appear in the extrajunctional membrane, while the number at the end plate remains approximately constant. ACh receptors are also found in the extrajunctional membrane of muscles from neonatal animals and of myotubes formed in cell culture (see Harris, 1974, for review).

The physiological and pharmacological properties of the ACh receptors that appear in the extrajunctional membrane of a denervated muscle are very similar to those of end plate receptors in normal muscle. In both cases, the receptors mediate an increase in sodium and potassium permeabilities and show the pharmacological characteristics of a classical nicotinic receptor (Axelsson and Thesleff, 1959; Jenkinson and Nicholls, 1961; Beranek and Vyskocil, 1967). Nevertheless, quantitative differences in the sensitivity of the receptors to *d*-tubocurarine (dTC) and succinylcholine have been detected (Beranek and Vyskocil, 1967; Vyskocil and Beranek, 1970; Lapa et al., 1974; Chiu et al., 1974). The receptor density and membrane configuration in the two cases are different, however, and this complicates the interpretation of experiments on receptors in situ.

To make a less ambiguous comparison of the two receptors, we have purified junctional receptors from normal muscle and extrajunctional receptors from denervated muscle. The previous paper (Brockes and Hall, 1975) describes the purification of the receptors and the characteristics of their interaction with [^{125}I]-α-bungarotoxin ([^{125}I]-α-BuTx). In this paper we report a comparison of several pharmacological and physical properties of the two receptors. Our principal conclusion is that the molecules are closely related but distinct.

Experimental Section

Materials

Junctional ACh receptors (JR) from normal rat diaphragms, and extrajunctional ACh receptors (EJR) from diaphragms that had been denervated for 7–14 days were prepared as described previously (Brockes and Hall, 1975). Except where noted, in denervated muscle, regions lacking end plates were used to obtain only those receptors that appear after denervation. Neonatal receptors were prepared from the diaphragms of newborn Charles River rats which were kindly made available by Drs. E. J. Furshpan and D. D. Potter. In the neonatal muscles the nerve was not easily visible under the dissecting microscope, and regions without end plates were obtained by dissecting a narrow strip of muscle next to the ribs. The end plate region consisted of a broad strip dissected from the center of the muscle. The S2 fractions were obtained by Triton X-100 extraction of crude muscle membrane preparations; purified fractions were prepared by affinity chromatography of the S2 fraction using Sepharose beads to which cobra (*Naja naja siamensis*) toxin had been covalently attached. Further details of these procedures are given in the preceding paper (Brockes and Hall, 1975).

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[1] Abbreviations used are: ACh, acetylcholine; dTC, *d*-tubocurarine; [^{125}I]-α-BuTx, [^{125}I]-α-bungarotoxin; GAR, goat anti-rabbit immunoglobulin G antiserum; PBST, 0.9% NaCl, 1% Triton X-100, and 10 m*M* sodium phosphate (pH 7.1); JR, junctional acetylcholine receptors; EJR, extrajunctional acetylcholine receptors; TNT, 50 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.4), and 1% Triton X-100; Con A, concanavalin A.

Rabbit antiserum against purified acetylcholine receptor from *Electrophorus electricus* and goat anti-rabbit immunoglobulin G antiserum (GAR) (Patrick et al., 1973) were a generous gift from Dr. J. Patrick. The antireceptor serum (volume 2 ml) was passed three times over a 1-ml column of cobrotoxin-Sepharose to remove antitoxin activity (see Patrick et al., 1973).

Concanavalin A (Grade IV) and methyl α-D-mannopyranoside were obtained from Sigma. The concanavalin A was purified by adsorption to Sephadex G-75 and elution with 0.2 *M* sucrose according to Nicolson and Lacorbiere (1973), and was stored at 0° in 50 m*M* sodium phosphate (pH 6.5)–50 m*M* NaCl.

dTC was obtained from Mann Research Lab. A 5 *M* agarose was obtained from Calbiochem and β-galactosidase (8.4 mg/ml, 325 U/mg) from Worthington. [^{125}I]-α-BuTx was prepared and purified by isoelectric focusing as previously described (Brockes and Hall, 1975).

Other materials employed are described in the preceding paper (Brockes and Hall, 1975).

Methods

Antibody Precipitation Assay (modified from Patrick et al., 1973). The S2 fraction of each receptor (6.7×10^{-10} *M*) was reacted with [^{125}I]-α-BuTx (3.4×10^{-8} *M*) for 2 hr at 35° and the toxin–receptor complex formed was assayed by DEAE-cellulose filtration. The antibody reaction contained 4.2 fmol of receptor–[^{125}I]-α-BuTx complex, 0.2–0.3 pmol of [^{125}I]-α-BuTx, 5 μl of normal rabbit serum, different volumes of antireceptor serum at an appropriate dilution in 10 m*M* sodium phosphate (pH 7.1), 0.9% NaCl, and 1% Triton X-100 (PBST), and PBST to a final volume of 50 μl. After 12-hr incubation at room temperature, 25 μl of GAR was added and the reaction incubated for 12 hr at 4°. After addition of 1 ml of PBST, the immune precipitate was collected by sedimentation at 30,000*g* for 20 min and washed by two more cycles of resuspension in 1 ml of PBST followed by sedimentation. The final pellet was dissolved in 0.3 ml of 1% acetic acid and counted in a liquid scintillation counter after addition of 5 ml of Aquasol (New England Nuclear Corp.). The radioactivity trapped in the pellet by nonspecific precipitation was determined in parallel experiments performed with normal rabbit serum in place of antireceptor serum. The nonspecific precipitation, which was 10–15% of the plateau radioactivity (see Figure 1), was subtracted from the experimental values. Reactions performed with the two receptors were processed in parallel. The recovery of complex in both cases was 55–65%.

Zone Sedimentation and Gel Filtration. Zone centrifugation was performed in a Spinco Model L2 ultracentrifuge using an SW 50.1 rotor at 49,000 rpm for 5 hr at 20°. Samples were layered onto 4.8 ml of 5–20% sucrose gradients made up in 50 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.4), and 1% Triton X-100 (TNT). The recovery of radioactivity from gradients with purified receptors was 80–95%.

Gel filtration was performed at 4° on a column (29.5 × 0.5 cm) of A-5M agarose equilibrated with TNT. The flow rate was 4.8 ml/hr. β-Galactosidase (33 μg) was added to all samples as an internal marker. Fractions of 0.36 ml were collected into disposable plastic tubes and aliquots were assayed for β-galactosidase (Craven et al., 1965) and for radioactivity in a well type γ counter, or in a liquid scintillation counter after the addition of 5 ml of aquasol. The recovery of radioactivity after gel filtration was 70–90%.

Isoelectric Focusing. The receptor fractions were reacted with [^{125}I]-α-BuTx as detailed in the text, and assayed by DEAE-cellulose filtration. An aliquot of each reaction (generally 0.5–1 × 10^4 cpm of receptor–toxin complex in a volume of <50 μl) was mixed with 0.5 volume of 10% sucrose, 2% Triton X-100, 4% ampholine (pH 5–8), and 0.005% Evan's Blue. Cylindrical polyacrylamide gels (8 × 0.5 cm) were prepared in glass tubes using a reaction mixture of 4% acrylamide, 0.15% methylenebisacrylamide, 2% Triton X-100, 0.65% pH 4–6 ampholine, 1.3% pH 5–8 ampholine, and 0.06% ammonium persulfate. The tubes were sealed at the lower end with a dialysis membrane and the gels prerun for 30 min at 1 mA/gel. The upper reservoir buffer was 0.4% ethylenediamine and 2% Triton X-100 and the lower reservoir buffer was 0.2% H_2SO_4 and 2% Triton X-100. After layering the sample mixture on the gels, focusing was carried out for 2.5–3 hr at 4°. The voltage rose to a maximum value of 400 V by approximately 1.5 hr and then remained constant while the current fell from an initial value of 1 mA/gel to approximately 0.8 mA/gel at the end of the run. The gels were frozen and cut into 1-mm slices with a series of equally spaced razor blades. The slices were transferred to small glass tubes and counted at approximately 48% efficiency in a Packard well type γ counter. Water (2 ml) was added to selected tubes which were corked and left at room temperature for 24–48 hr. The pH value for each tube was determined at room temperature using a Radiometer electrode with an expanded scale. The recovery of receptor–toxin complex from the gels was generally 30–50%.

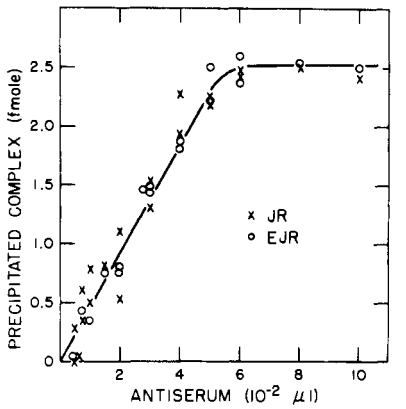

FIGURE 1: Precipitation of receptors by a rabbit antiserum. The reaction of receptors with rabbit antiserum to the purified receptor of *Electrophorus electricus* was investigated as described in the Experimental Section. (x) Junctional receptor; (○) extrajunctional receptor.

Results

Hydrodynamic Properties of the Two Receptors. The sedimentation behavior of the toxin–receptor complexes of purified junctional and extrajunctional receptors was investigated by zonal centrifugation on sucrose density gradients. In both cases the only peak of radioactivity recovered from the gradient that was distinct from free toxin occurred at approximately 9 S, the sedimentation constant of the toxin–receptor complex in crude extracts of muscle.

The complexes were also investigated by gel filtration on columns of 5 *M* agarose. Complexes formed by both junctional and extrajunctional receptors were detected as a broad peak of radioactivity that eluted close to β-galactosidase, an enzyme whose sedimentation constant is 16 S. There is thus a discrepancy in the apparent size of both complexes as estimated by the two techniques. This behav-

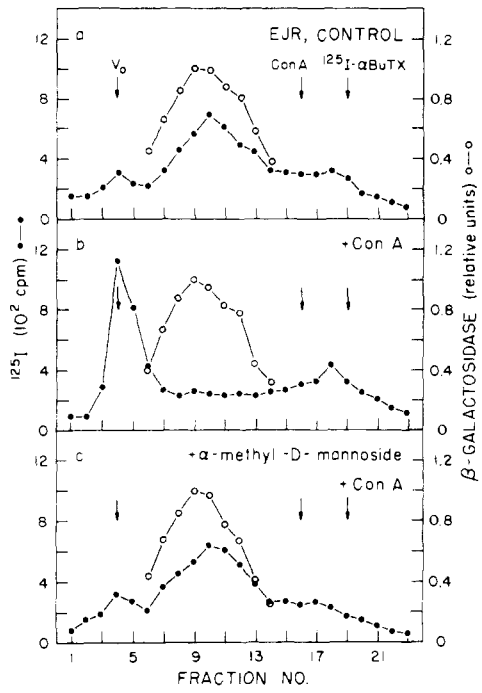

FIGURE 2: Analysis of Con A reactions by gel filtration on 5 *M* agarose. Preparation of complexes. The reaction (volume 155 μl) containing 1.1×10^{-9} *M* purified extrajunctional receptor in TNT, and 8.9×10^{-9} *M* [^{125}I]-α-BuTx was incubated for 1 hr at 35° and applied to a column (29 cm × 0.5 cm diameter) of Sephadex G-75. The column was eluted with TNT and fractions (0.25 ml) were collected and assayed by DEAE-cellulose filtration. The peak fractions, which eluted at the void volume, were pooled and stored at 0°. Reaction with Con A. Reactions (volume 175 μl) containing 9.5×10^{-11} *M* complex in TNT and, where appropriate, 4.3×10^{-6} *M* Con A and 47 m*M* methyl α-D-mannopyranoside were incubated for 30 min at 35°. After addition of β-galactosidase (33 μg), 150 μl of the reaction mixture was analyzed by gel filtration on 5 *M* agarose as described in the Experimental Section. When [^{125}I]-α-BuTx alone was incubated with 4.3×10^{-6} *M* Con A under these conditions, its elution profile was not significantly changed.

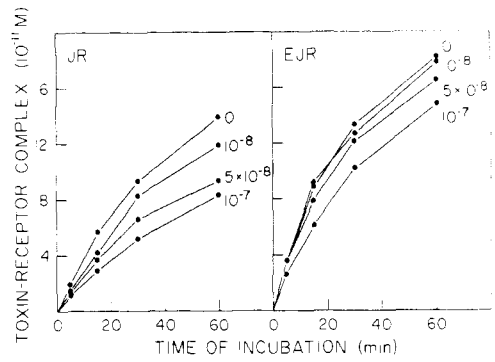

FIGURE 3: Inhibition of the rate of toxin binding by dTC. Reactions (volume 150 μl) containing 8.8×10^{-10} *M* affinity purified receptor in TNT, and the appropriate concentration of dTC, were incubated at 35° for 30 min. After addition of [^{125}I]-α-BuTx to a final concentration of 6.5×10^{-10} *M*, duplicate aliquots of 15 μl were taken at the times indicated and analyzed by filtration on DEAE-cellulose. Control experiments showed that 30 min was adequate for equilibration with dTC over the range of concentrations employed. dTC was diluted from a 10 m*M* stock solution just before use.

ior has also been noted with the receptor from *E. electricus* and shown to be at least partly due to the effect of bound detergent (Meunier et al., 1972).

We conclude that no differences in the size and shape of the purified receptors are detected by these methods.

Precipitation Reaction with Antiserum. Patrick and Lindstrom (1973) have shown that injection of purified receptor from *Electrophorus electricus* into rabbits produces a strong autoimmune paralysis due to the induction of cross-reacting antibodies in the serum. This serum also cross-reacts with the ACh receptors from rat muscle, and we have used it to compare the antigenic determinants present on the junctional and extrajunctional receptors of the diaphragm. The comparison was made on the S2 receptor fraction rather than on the purified receptor because of the possibility that toxin–receptor complex, as well as receptor, was eluted from the resin during affinity chromatography (Patrick et al., 1973), thus introducing an uncertainty into the comparison of the two receptors. When serum, raised against the eel receptor, was incubated with each of the two preparations of toxin–receptor complex, the precipitation curves obtained were identical (Figure 1), indicating that the antigenic determinants that react with this serum are common to both junctional and extrajunctional receptors.

Reaction with Concanavalin A. Concanavalin A (Con A), a lectin isolated from jackbeans, binds to oligosaccharides and glycoproteins possessing α-D-glucopyranosyl or α-D-mannopyranosyl residues (Goldstein et al., 1965; So and Goldstein, 1967). Purified preparations of receptor from *Electrophorus* interact with several lectins (Meunier et al., 1974). We have used gel filtration to investigate the interaction of Con A with the purified junctional and extrajunctional toxin–receptor complexes. Figure 2a shows the elution profile of a mixture of extrajunctional complex and toxin when they were chromatographed on a column of 5 *M* agarose. The principal peak of complex eluted with β-galactosidase, corresponding to an apparent molecular weight of approximately 5×10^5. After the complex had been reacted with Con A, however, the principal peak of radioactivity was excluded from the column (Figure 2b) indicating an apparent molecular weight of at least 5×10^6. The formation of this aggregate was completely inhibited (Figure 2c) by including in the reaction mixture methyl α-D-mannopyranoside, a potent inhibitor of the carbohydrate binding sites on the Con A molecule (Goldstein et al., 1965). Incubation of Con A with [^{125}I]-α-BuTx alone did not affect the elution profile of the toxin (see legend to Figure 2). Identical results with those in Figure 2 were obtained with the junctional receptor. These results suggest that Con A binds to α-D-glucopyranosyl or α-D-mannopyranosyl-like residues that are present on both purified receptors.

Inhibition of Toxin Binding by dTC. Physiological experiments have indicated that the sensitivity to dTC of extrajunctional receptors in denervated muscle may be different from that of receptors in normal muscle. Thus, it was of particular interest to compare the interaction of the purified receptor preparations with dTC. The limited amounts of receptor available did not permit a direct study of dTC binding, but the interaction could be followed by the inhibition of binding of [^{125}I]-toxin caused by dTC. The slow rate constants for the interaction of toxin with receptor made measurement of the equilibrium between dTC, receptor, and toxin impractical. Therefore we have studied the effect of various concentrations of dTC on the rate of the toxin–receptor binding reaction. Reactions were carried out after preincubation of dTC with purified junctional and extrajunctional receptor preparations. In each case, identical concentrations of receptor and [^{125}I]-α-BuTx were used. As shown in Figure 3, increasing concentrations of dTC caused a progressive decrease in the rate of the reaction for both

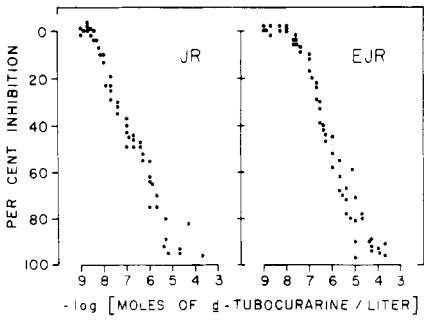

FIGURE 4: Inhibition of toxin binding to purified receptors as a function of concentration of dTC. Reactions were set up with the same concentrations as described in Figure 3. After 1 hr of incubation with toxin, duplicate aliquots were either filtered immediately or diluted into excess unlabeled toxin and then filtered. The data in this figure were obtained from two independent preparations of the junctional receptor and four independent preparations of the extrajunctional receptor.

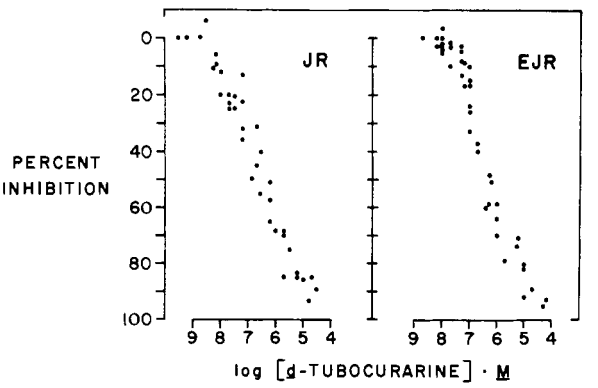

FIGURE 5: Inhibition of toxin binding to crude receptors as a function of concentration of dTC. Experiments with S2 receptor preparations were performed as in Figure 4. The data in this figure were obtained from two independent preparations of each receptor.

receptors. Although ideally the initial rate of the reaction would be measured, the reaction was strictly linear for only a short time, and we have approximated the initial rate by measuring the amount of complex formed after 1 hr under the standard conditions.

The result of a number of such determinations carried out with different preparations is shown in Figure 4. It is clear (see also Figure 3) that at low concentrations (5×10^{-9}-10^{-7} M) dTC is more effective in blocking the binding of toxin by purified junctional receptor than by the extrajunctional receptor. This difference in inhibition at low concentrations of dTC was also seen in identical experiments performed on crude preparations of both receptors (Figure 5) and therefore is not an artifact of the affinity purification. It should also be noted that more than 90% of the toxin binding could be competed by dTC in each case.

The data on dTC inhibition are conveniently analyzed by a plot of the reciprocal of the fractional inhibition vs. the dTC concentration. A plot for both purified receptors over the range $1\text{-}5 \times 10^{-8}$ M is shown in Figure 6. The points for the extrajunctional receptor fall on a straight line which extrapolates to give an "apparent" dissociation constant of 5.5×10^{-7} M. The data for the junctional receptor extrapolate to a value of 4.5×10^{-8} M. Thus, there is a clear difference between the apparent binding of low concentrations of dTC by the two receptors.

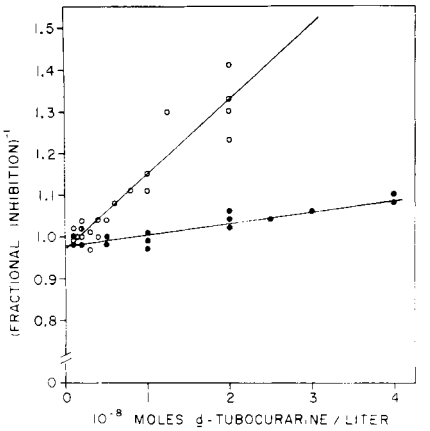

FIGURE 6: Analysis of dTC inhibition of toxin binding to purified receptors by fractional inhibition plot. The data of Figure 4 were analyzed as described in the text. The law of mass action predicts that preincubation with the reversible ligand dTC should lower the concentration of free receptor, and hence the initial rate of reaction with [^{125}I]-α-BuTx, by a factor of $1 + ([\text{dTC}]/K_D)$, where K_D is the dissociation constant for dTC. Thus a plot of (fractional inhibition)$^{-1}$ vs. [dTC] should have a slope equal to $1/K_D$ and a negative intercept of [dTC] = K_D. The lines were drawn by least-squares regression. (○) Junctional receptor; (●) extrajunctional receptor.

Isoelectric Focusing of the Two Receptors. The isoelectric point of the toxin-receptor complexes were investigated by focusing in polyacrylamide gels. When purified extrajunctional receptor was reacted with [^{125}I]-α-BuTx and subjected to focusing, a single major peak of radioactivity was detected at a pH value of approximately 5.3 (Figure 7a). The purified junctional receptor was analyzed on a parallel gel in the same way and displayed a peak at a pH value of approximately 5.1 (Figure 7b). The difference in isoelectric points is a small one and the pH values obtained vary in different experiments. Therefore the two complexes were mixed and run on a third parallel gel. When this was done, the radioactivity was recovered in two separate peaks (Figure 7c). This experiment shows that the purified receptors differ in the isoelectric points of their [^{125}I]-α-BuTx complexes. This distinction could be an artifact of purification and thus we have repeated these experiments on crude preparations of receptors. As shown in Figure 8a-c, the crude receptor-toxin complexes are also separated by the focusing procedure. In addition, the receptors present in end plate regions of denervated muscle were examined. The result (Figure 8d) shows a major peak with a minor component focusing at 0.12 pH unit lower. This is consistent with the relative amounts of extrajunctional and junctional receptors present in denervated muscle (Berg et al., 1972) and shows that the two species retain their characteristic isoelectric points in this tissue.

It is important to determine if the complexes reach their equilibrium pH value during the focusing regime of these experiments. This could be proved by applying the samples at opposite ends of the gel and showing that they reach the same position. Unfortunately, this was not possible because the complexes dissociated at the lower pH values. Therefore we have focused the gels for different times (generally 155 and 225 min) and verified that the complexes focus at identical pH values, although the form of the pH gradient shifts considerably over this interval. This is strong evidence that the pH values quoted are true isoelectric points.

We conclude from these experiments that the isoelectric points of complexes formed by the two receptors are distinct.

Receptors in Neonatal Muscle. It is known that the ex-

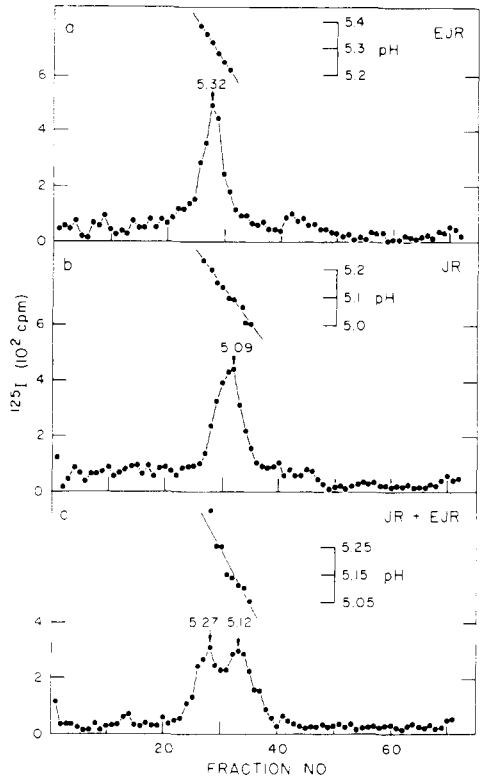

FIGURE 7: Isoelectric focusing of complexes formed by purified receptors. The reactions (volume 125 μl) contained in TNT 3.6 $\times$ 10^{-9} M [^{125}I]-α-BuTx and purified extrajunctional receptor (1.1 $\times$ 10^{-9} M) or purified junctional receptor (1.5 $\times$ 10^{-9} M). After incubating for 1 hr at 35°, 5 μl of 5 $\times$ 10^{-7} M α-BuTx was added and the incubation was continued for 15 min at 35°. The samples were applied to the gels as follows: (a) 11.8 fmol of extrajunctional complex; (b) 12.1 fmol of junctional complex; (c) 5.9 fmol of extrajunctional + 6.1 junctional complexes. A parallel gel was run with a reaction mixture from which receptor fractions had been omitted. No significant radioactivity was detected in the range of pH values from 4.5 to 5.5.

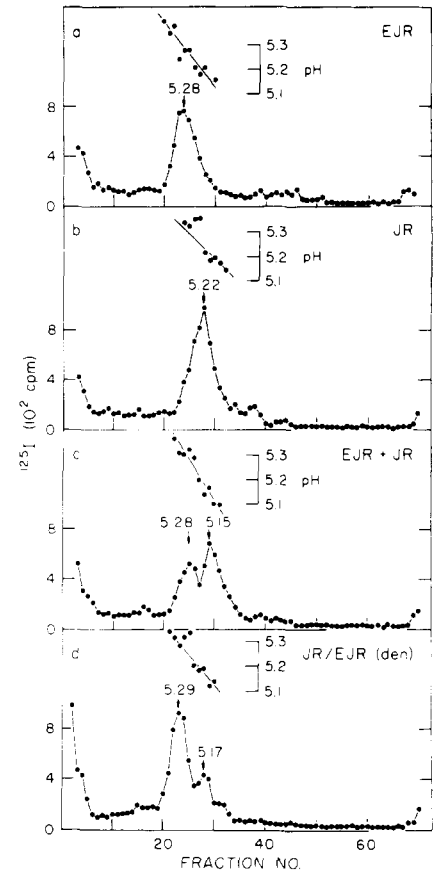

FIGURE 8: Isoelectric focusing of complexes formed by crude receptors. The reactions (volume 55 μl) contained in TNT, 5.5 $\times$ 10^{-9} M [^{125}I]-α-BuTx, and S2 fractions of junctional receptor (8.6 $\times$ 10^{-10} M, 302 μg), extrajunctional receptor (5.1 $\times$ 10^{-10} M, 35 μg), or receptors from end plate regions of denervated muscle (3.1 $\times$ 10^{-9} M, 175 μg). After incubating for 1 hr at 35°, 5 μl of 5 $\times$ 10^{-7} M α-BuTx was added and the incubation was continued for 10 min at 35°. The samples were applied to the gels as follows: (a) 14.7 fmol of extrajunctional complex; (b) 17.4 fmol of junctional complex; (c) 8.8 fmol of extrajunctional + 10.4 fmol of junctional complexes; (d) 43.7 fmol of complexes from end plate regions of denervated muscle. A parallel gel was run with a reaction mixture from which receptor fractions had been omitted. No significant radioactivity was detected in the range of pH values from 4.5 to 5.5.

trajunctional membrane of neonatal muscle fibers has a considerable number of ACh receptors (Diamond and Miledi, 1962; Berg et al., 1972), and we were interested in comparing these receptors with those present in denervated muscle. The low levels of extrajunctional receptor and the small amounts of neonatal diaphragm have made it difficult to do extensive studies. We have, however, purified the extrajunctional receptor from this source and analyzed the inhibition of toxin binding by dTC. As shown in Figure 9a, the dTC inhibition curve is similar to that of the extrajunctional receptor in denervated muscle (Figure 4). When these data were analyzed by the plot of Figure 6, they extrapolated to an "apparent" dissociation constant of 4.1 $\times$ 10^{-7} M (Figure 9b). This is close to the value of 5.5 $\times$ 10^{-7} M (Figure 6) obtained for the extrajunctional receptors of denervated muscle.

We have also analyzed the behavior on isoelectric focusing of crude preparations of neonatal receptors. As shown in Figure 10a, the extrajunctional preparation gave a single major peak at a pH value of 5.36. The junctional preparation gave a bimodal distribution (Figure 10b) which was similar to that obtained with a crude junctional preparation from adult denervated muscle (Figure 10c). Although it has not been possible to make extensive comparisons of the two sources, these data support the hypothesis that the species of receptor present in neonatal muscle are identical with those present in denervated muscle.

Discussion

Junctional and extrajunctional receptors are very similar by many of the criteria used in this study. Their behavior on gel filtration and sedimentation is anomalous, presumably because of bound detergent. Nevertheless, the hydrodynamic properties of the two receptors, as revealed by these techniques, appear to be identical. Both receptors also react with Con A, and thus are probably glycoproteins. Because high salt and detergent concentrations were used in the affinity purification, it is unlikely that the carbohydrate is attached to the receptors by noncovalent bonds. Finally, junctional and extrajunctional receptors have identical precipitation curves with an antiserum to the eel receptor.

dTC decreases the rate with which both receptors bind [^{125}I]-α-BuTx. Low concentrations of dTC, however, inhibited binding of toxin by the junctional receptor more effectively than binding by the extrajunctional receptor. The apparent dissociation constants for dTC were 5.5 $\times$ 10^{-7} M for the extrajunctional receptor and 4.5 $\times$ 10^{-8} M for the junctional receptor. These values should be regarded as tentative for several reasons. Under the conditions used, toxin

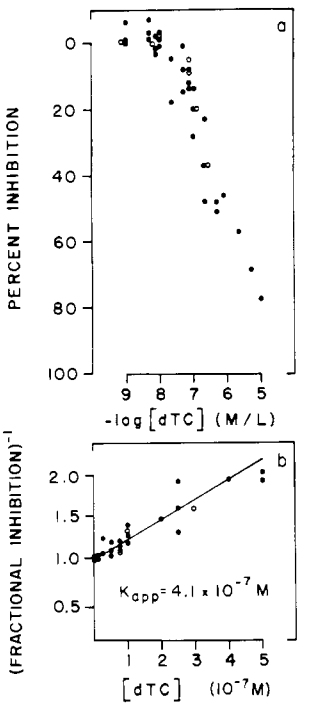

FIGURE 9: (a) Inhibition of toxin binding to extrajunctional neonatal receptor as a function of concentration of dTC. The reactions (volume 72 μl) contained 2.1 $\times$ 10^{-10} M purified or S2 fraction extrajunctional neonatal receptor, TNT, and the appropriate concentration of dTC, and were incubated at 35° for 30 min. After addition of [^{125}I]-α-BuTx to a final concentration of 5.0 $\times$ 10^{-10} M the reactions were incubated for 1 hr at 35°, when duplicate aliquots of 35 μl were analyzed by DEAE-cellulose filtration. (●) Affinity purified receptor; (○) S2 fraction receptor. (b) Analysis of dTC inhibition by fractional inhibition plot. The data of Figure 9a were analyzed as in Figure 6. The line was drawn by least-squares regression.

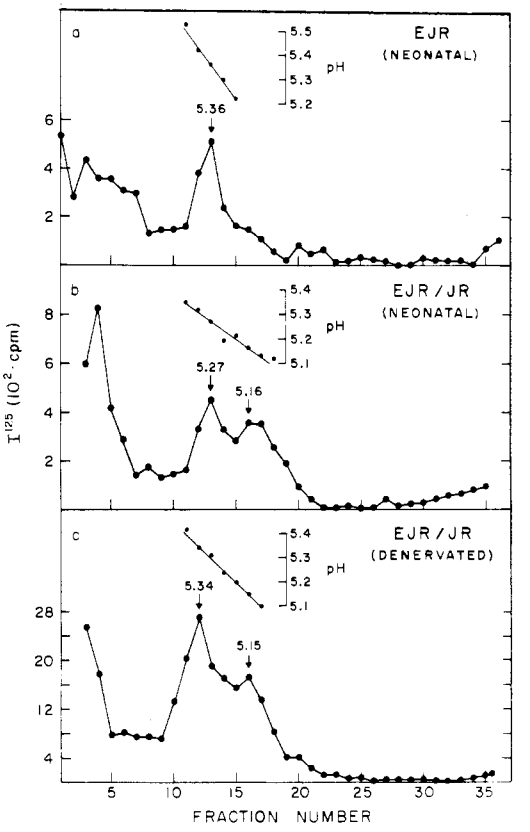

FIGURE 10: Isoelectric focusing of complexes formed by crude neonatal receptors. The reactions contained in a volume of 50 μl, 5.2 $\times$ 10^{-9} M [^{125}I]-α-BuTx and either 1.8 $\times$ 10^{-9} M extrajunctional S2 fraction (255 μg), 2.1 $\times$ 10^{-9} M junctional S2 fraction (220 μg), or 3.2 $\times$ 10^{-9} M S2 fraction of junctional regions of adult denervated muscle (210 μg). After incubating for 45 min at 35°, 5 μl of 5 $\times$ 10^{-7} M α-BuTx was added and the incubation was continued for 10 min at 35°. The samples were applied to the gels as follows: (a) 10.1 fmol of neonatal extrajunctional complex; (b) 12.1 fmol of neonatal junctional complex; (c) 38.4 fmol of junctional complexes of adult denervated muscle.

binding did not occur at a strictly linear rate. Further, the values obtained when inhibition was greater than 50% were not sufficiently precise to exclude the possibility of additional binding sites for dTC. If present, these would complicate the simple analysis that we have given. Finally, we have not investigated the relation of the dTC inhibition to the two sites for toxin binding described in the previous paper (Brockes and Hall, 1975).

In spite of these reservations, the values that we have obtained agree reasonably well with values derived from physiological experiments and from studies of toxin binding to muscle homogenates. Beranek and Vyskocil (1967) found that the concentration of dTC required to give 50% inhibition of ACh potentials from extrajunctional receptors in denervated rat diaphragms was approximately 2 $\times$ 10^{-7} M, while the corresponding value for the end plate potentials given by junctional receptors was 3 $\times$ 10^{-8} M. Colquhoun et al. (1974) analyzed their data for the inhibition by dTC of toxin binding to homogenates of denervated diaphragm in terms of a K_D of 4 $\times$ 10^{-7} M.

Recently, Lapa et al. (1974) and Chiu et al. (1974) have extensively studied the ability of dTC to block receptors in intact innervated and denervated muscles. As pointed out by Colquhoun et al. (1974), studies on intact tissue cannot be used to derive quantitative data about the rate of toxin interactions with the receptor, because diffusion of toxin into the tissue appears to be at least partially rate limiting. Nevertheless, the conclusion of the experiments on intact muscle (Lapa et al., 1974; Chiu et al., 1974) that receptors appearing after denervation bind dTC less tightly than normal receptors is qualitatively in agreement with the results presented here. The relatively poor inhibition of toxin binding in intact tissue by dTC has led to the suggestion that half of the toxin-binding sites may be insensitive to dTC (Miledi and Potter, 1971; Porter et al., 1973; Chiu et al., 1974). In experiments on receptor that has been solubilized (Figures 4 and 5) we find that the rate of toxin binding can be inhibited more than 90% by dTC (5 $\times$ 10^{-5} M) in both crude and purified preparations of junctional and extrajunctional receptors. Thus our experiments do not support the idea of a class of specific toxin-binding sites that are insensitive to dTC.

The binding of dTC to solubilized receptor has been investigated in two recent publications. Dolly and Barnard (1974) observed an apparent K_D of 3.3 $\times$ 10^{-7} M as determined with a partially purified preparation from denervated cat muscle. This is in reasonable agreement with the values reported here. Alper et al. (1974) have obtained an apparent K_D for dTC of 10^{-8} M for both end plate and extrajunctional receptors in solubilized extracts of rat diaphragm. The value derived for the extrajunctional receptor is different from that obtained by us and by others (Colquhoun et al., 1974; Dolly and Barnard, 1974). The reason for this difference is unclear, but may be related to the different conditions of ionic strength and receptor concentrations used by Alper et al. (1974).

In addition to the difference in binding of dTC, purified junctional and extrajunctional receptors form complexes with toxin that can be physically separated by isoelectric focusing. This separation is not a consequence of receptor purification since it is also seen when crude preparations of the receptors are used. Furthermore, junctional and extrajunctional complexes present in the end plate regions of denervated muscle can be separated (Figure 8d). This important result shows that the distinction between the two complexes is not an artifact of some other difference between innervated and denervated muscle, such as the level of protein degrading or modifying activities. Because the purified receptors are exposed to 1 *M* NaCl and 1% Triton X-100 during preparation it is unlikely that the difference between them arises from noncovalently bound lipid or other small molecules. Our results are most consistent with a structural difference between the major species present in junctional and extrajunctional receptor preparations. We cannot rule out the possibility that minor amounts of the extrajunctional receptor are present in the junctional receptor preparations and vice versa.

Several other differences, besides the ones reported here, have been observed between junctional and extrajunctional receptors. Studies of [^{125}I]-α-BuTx bound to receptors in intact muscle have shown that loss of toxin, which may be related to receptor turnover, occurs much more rapidly from extrajunctional than from junctional receptors (Berg and Hall, 1974). Also Katz and Miledi (1972) have found in frog muscle that the properties of the elementary event derived from analysis of ACh noise are different for receptors in normal muscle and those that appear after denervation. Feltz and Mallart (1971) have reported a difference in the ACh reversal potential for the junctional and extrajunctional receptors normally present in innervated frog muscle. This last phenomenon may be unrelated to the observations reported here since denervation changes the reversal potential of the junctional receptors to that seen for extrajunctional receptors (Feltz and Mallart, 1971; Mallart and Trautmann, 1973).

The physiological significance of the differences between junctional and extrajunctional receptors is unclear. The observation that the properties of extrajunctional receptors in neonatal muscle (Figures 9 and 10) resemble those seen in denervated muscle suggests that the difference could be important in development. If so, then the question arises as to how the two receptors are formed. The receptors could be the products of different genes; alternatively, extrajunctional receptors might serve as precursors for junctional receptors. In either case, it is of interest to know how the appearance of junctional receptors is related to synapse formation and what role the nerve might play in this process. A particularly interesting possibility (Langley, 1905) is that the nerve might mediate the formation of junctional receptors by introducing a covalent modification into the subsynaptic population of extrajunctional receptors, resulting in an altered isoelectric point and dTC sensitivity of the molecule. Since junctional receptors can now be identified, it should be possible to determine whether or not their appearance requires the nerve.

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Added in Proof

It has recently been called to our attention that Duguid and Raftery (1973) detected two peaks of toxin binding activity when analyzing membrane fragments of *Electrophorus* by density gradient centrifugation. These authors suggested that the two peaks, which displayed different affinities for dTC, might correspond to junctional and extrajunctional receptors.

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