Subunit Structure and Peptide Mapping of Junctional and Extrajunctional Acetylcholine Receptors from Rat Muscle†

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ABSTRACT: We have purified the junctional acetylcholine receptor from normal rat skeletal muscle and compared its structure with that of the extrajunctional receptor from denervated muscle. The two receptors from leg muscle were distinguished by isoelectric focusing and by reaction with sera from patients with myasthenia gravis. The junctional form of the acetylcholine receptor was purified from normal leg muscle by affinity chromatography on concanavalin A/Sepharose and cobrotoxin/Sepharose followed by sucrose gradient centrifugation. Analysis of radioiodinated receptor by polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that the subunit structure of the junctional receptor was similar to that previously determined for the extrajunctional form (Froehner, S. C., et al. (1977) *J. Biol. Chem. 252*, 8589–8596), with two major polypeptides, whose apparent molecular weights in 9% polyacrylamide gels were 45 000 and 51 000. In addition, several minor polypeptides were found. When the two receptors were labeled with different isotopes of iodine and run together on a sodium dodecyl sulfate gel, the subunits of one receptor could not be resolved from those of the other. As seen earlier with the extrajunctional form, the affinity alkylating reagent [^{3}H]MBTA labeled the 45 000- and 49 000-dalton polypeptides of the junctional receptor. Peptide mapping showed that the two MBTA binding subunits are structurally related, although they are unrelated to the other polypeptides, and that the 45 000- and 51 000-dalton polypeptides of the junctional receptor were indistinguishable from those of the extrajunctional receptor. In addition, peptide mapping of the four subunits of acetylcholine receptor isolated from *Torpedo californica* electric organ showed that these four polypeptides appear to be structurally unrelated.

Two kinds of acetylcholine receptor are found in vertebrate skeletal muscle cells. Junctional receptors occur in the specialized postsynaptic muscle membrane at the neuromuscular junction and are the predominant form of receptor in normal adult muscle (Fertuck & Salpeter, 1974). In developing muscle (Diamond & Miledi, 1962) and in denervated adult muscle (Axelsson & Thesleff, 1959; Miledi, 1960), ACh[1] receptors are also found in extrajunctional muscle membrane. The two types of receptor, junctional and extrajunctional, differ in a number of their properties. In situ, the two types of receptors have different rates of turnover in the muscle membrane (Berg & Hall, 1975; Chang & Huang, 1975) and the elementary permeability event associated with each receptor is different (Katz & Miledi, 1972; Dreyer et al., 1976). Junctional and extrajunctional receptors also differ in their affinities for *d*-tubocurarine, both in situ (Beranck & Vsykocil, 1967) and after solubilization (Brockes & Hall, 1975). Differences in isoelectric points (Brockes & Hall, 1975) and in immunological properties (Almon & Appel, 1975; Weinberg & Hall, 1979) indicate that the two receptors may be structurally different.

This structural difference may be important for the maturation of the neuromuscular junction. We and others have suggested that the extrajunctional receptor may be a precursor of the junctional receptor and that, during formation of a mature end plate, extrajunctional receptors may be covalently modified to form junctional receptors (Brockes & Hall, 1975; Changeux & Danchin, 1976). The recent demonstration by Anderson & Cohen (1977) of the accumulation of extrajunctional receptors at sites of nerve–muscle contact in culture is consistent with this idea. It is thus important to define the structural differences between the two types of receptor, and to relate these to differences in their functional properties.

We have previously reported the purification of the extrajunctional ACh receptor from denervated muscle using sequential affinity chromatography on Con A/Sepharose and cobrotoxin/Sepharose followed by sucrose gradient sedimentation. The final preparation had a specific activity of 7–11 nmol of α-[^{125}I]BuTx bound/mg of protein, comparable to that found for ACh receptors purified from *Torpedo* or *Electrophorus*. $NaDodSO_4$ gel electrophoresis of the purified receptor preparation revealed two major polypeptide chains with apparent molecular weights of 45 000 and 51 000, with minor components of 49 000, 56 000, and 62 000. Two of the polypeptides, the 45 000 and 49 000 dalton components, bound the affinity reagent, [^{3}H]MBTA, and thus contain at least part of the acetylcholine binding site (Froehner et al., 1977a,b).

Because the receptor appeared to have a complex subunit structure, it was difficult to determine the purity of the final preparation and to identify individual polypeptides as belonging to the receptor. Our approach to this problem was to prepare a control from which the receptor was specifically eliminated. This was done by following the same purification protocol except that α-BuTx was added to the preparation prior to the cobrotoxin/Sepharose step. Comparison of the protein content and polypeptide composition of control and receptor preparations showed that the receptor was greater than 90% pure and that all of the polypeptides were associated with the receptor. To develop methods that could be used with much smaller amounts of receptor, we iodinated the extrajunctional receptor and showed that each of the chains was labeled (Froehner et al., 1977b).

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[1] Abbreviations used: ACh, acetylcholine; α-BuTx, α-bungarotoxin; Con A, concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate; [^{3}H]MBTA, 4-(*N*-maleimido)benzyltri[^{3}H]methylammonium iodide.

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In the present paper, we report on the application of these techniques to the study of junctional receptor and compare its subunit structure with that of the extrajunctional receptor. In addition, we describe a convenient method for peptide mappings of oligomeric proteins and compare the peptide maps of the major subunits of junctional and extrajunctional muscle receptors. We also use this method to analyze the peptide maps of the four subunits of the acetylcholine receptor purified from *Torpedo californica* electric organ.

Experimental Section

Materials

White, male Sprague-Dawley rats (200–300 g) were obtained from Simonsen. Sodium dodecyl sulfate was obtained from BDH, Ltd. Elastase, benzethonium chloride, and pepstatin A were obtained from Sigma Chemical Co., *S. aureus* protease was from Miles Laboratories, Inc., and α-chymotrypsin from Worthington Biochemicals. Concanavalin A/Sepharose was either made as previously described (2 mg of protein/mL of resin) (Froehner et al., 1977b) or purchased from Sigma Chemical Co. (6–10 mg of protein/mL of resin). ACh receptor from *Torpedo californica* was purified by affinity chromatography on cobrotoxin/Sepharose (Forehner et al., 1977b). Detergent-free receptor was obtained by eluting the column with a carbachol solution lacking Triton. $Na^{125}I$, $Na^{131}I$, and [^{14}C]formaldehyde were obtained from New England Nuclear Corp. All other materials were as previously described (Froehner et al., 1977a).

Methods

ACh Receptor Binding Assay. Receptor activity was determined with a DEAE filter assay (Froehner et al., 1977b). One picomole of ACh receptor is defined as the amount of receptor that binds 1 pmol of α-[^{125}I]BuTx under saturating conditions.

Purification of Rat Muscle ACh Receptor. Junctional and extrajunctional receptors were purified by modification of the previously described procedure (Froehner et al., 1977b) by using affinity chromatography on Con A/Sepharose and cobrotoxin/Sepharose followed by sucrose gradient sedimentation. Extrajunctional receptor was prepared from lower leg muscles (150–200 g containing 2000–2500 pmol of ACh receptor from 30–35 rats) that had been denervated by transection of sciatic nerve 1.5–2 weeks earlier. Junctional receptor was prepared from upper and lower normal leg muscles, excluding the soleus (700–800 g containing 400–500 pmol of ACh receptor from 30–35 rats). Receptor and "nonreceptor" control preparations were made as described earlier (Froehner et al., 1977b) and in the text. After solubilization of the receptor, 0.1 mM benzethonium chloride was added to all buffers as an additional protease inhibitor. Elution from cobrotoxin/Sepharose was made in 1% Triton X-100 (instead of deoxycholate) and, after dialysis, 300-μL samples were applied to a 5–20% linear sucrose gradient (4.6 mL) containing 10 mM $NaPO_4$, pH 7.4, 0.1% deoxycholate. After centrifugation at 33 000 rpm in a SW 50.1 rotor for 15 h, fractions were collected and assayed for receptor activity. Receptor sedimenting at 9 S and the corresponding fractions from the "nonreceptor" control gradient were dialyzed against 10 mM $NaPO_4$, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA and stored in sterile plastic tubes at 0 °C. For both preparations, recoveries of receptor ranged from 25% to 50% for the Con A/Sepharose column, 10% to 25% for the cobrotoxin/Sepharose column, and 40% to 75% for sucrose gradient centrifugation. Two to ten micrograms of highly purified extrajunctional receptor could be obtained from 150 g of normal muscle, while 0.1–0.8 μg of junctional receptor could be obtained from 700 g of normal muscle.

Antibody Precipitation Assay. Precipitation of antibody–receptor complexes was performed as described by Weinberg & Hall (1979), by using the myasthenia serum designated there as serum V and 2 fmol of muscle ACh receptor per assay.

Isoelectric focusing of receptor-α-[^{125}I]BuTx complexes was performed as previously described (Brockes & Hall, 1975) with the following changes. Receptor–α-[^{125}I]BuTx complexes were formed by incubation for 12 h on ice, followed by the addition of unlabeled α-BuTx (0.2–1.0 nmol) and incubation for an additional hour at 0 °C. Gels (0.2 × 13 cm) were prerun for 30 min at 0.25 mA/gel (constant current); their temperature was maintained at 0 °C with a circulating water–ice bath. After layering the samples onto the gels, focusing was carried out for an additional 4.5–6 h. The voltage rose to 950 V by 30–60 min and was maintained at this level as the current decreased to approximately 0.1 mA/gel by the end of the run. Visible amounts of ferritin and myoglobin were added to the sample buffer as standards to ensure that the focusing was satisfactory. Gels were cut to 4-mm slices and counted in a Beckman Biogamma II counter.

Radioiodination of Purified Receptor. Radioiodination of receptor under native conditions was performed essentially as previously described (Froehner et al., 1977b). For each reaction, 5 μmol of sodium phosphate, pH 7.4, 100 μCi carrier-free $Na^{125}I$, and 2 μg of Chloramine-T were added to both the samples (240–500 fmol, 100–200 μL) and to a corresponding volume of "nonreceptor" control. After 20 min on ice, the reaction was stopped by the addition of 100 nmol of dithiothreitol. Five minutes later, 12.5 nmol of L-tyrosine and 100 μg of NaI were added. After an additional 20 min, 200–300 μL of 50 mM Tris-Cl, pH 7.4, 0.25 mM tyrosine was added, and the samples were immediately subjected to $NaDodSO_4$ gel electrophoresis. Cl_3CCOOH-precipitable radioactivity of duplicate 5-μL samples was determined by the method of Hubbard & Cohn (1975).

For iodination in $NaDodSO_4$, samples were heated in 2% $NaDodSO_4$ for 15 min at 37 °C, $Na^{125}I$ (200 μCi) and Chloramine-T (4 μg) were then added, and the incubation was continued for 20 min at room temperature. The reaction was stopped and the Cl_3CCOOH-precipitable radioactivity determined as described above.

$NaDodSO_4$ gel electrophoresis was modified from the procedure of Laemmli (1970) as described (Froehner et al., 1977a). The following proteins were used as molecular weight standards: bovine serum albumin (68 000), pyruvate kinase (57 000), fumarase (49 000), aldolase (40 000), and glyceraldehyde-6-phosphate dehydrogenase (36 000). In general, 9% acrylamide was used in the separation gel. Slab gels were 0.75 mm in thickness with 2–3-cm stacking gel and a 16-cm separation gel. When cylindrical gels were made, tubes (0.5-cm i.d.) were acid washed and siliconized before use, and filled with a 14-cm separation gel and 2-cm stacking gel. Cylindrical gels were fixed and washed in 25% isopropyl alcohol, 10% acetic acid to remove free iodine, cut with a Mickle gel slicer and counted.

Affinity Alkylation. Purified junctional, extrajunctional, and *Torpedo* receptors were labeled with [^{3}H]MBTA by reduction of the receptor for 10 min at 23 °C, followed by alkylation for 1 min at 23 °C, as described by Froehner et al. (1977a).

Peptide Mapping. We modified the method of Cleveland et al. (1977), in which peptide mapping is performed by partial

enzymatic proteolysis and analysis of the cleavage products by $NaDodSO_4$ gel electrophoresis, for use in a two-dimensional system. Samples (20 μL) of iodinated receptor (containing 5–20 fmol of receptor) were subjected to $NaDodSO_4$ tube gel electrophoresis as described above except that the tubes had 0.25-cm i.d. The concentration of acrylamide was usually 9%. When 15% acrylamide was used, the duration of electrophoresis was 2.5 times the time required for the dye to run off the bottom of the gel. After completion of electrophoresis, gels were extruded from the tubes and stored frozen. For peptide mapping of the subunits, the thawed gels were shaken for 15 min with 10 mL of 0.125 M Tris-Cl, pH 6.8, 0.1% $NaDodSO_4$, and the stacking gel as well as the portion of the separation gel below and including the dye front were removed. The protease to be used was dissolved at 40 °C in a small amount (100–200 μL) of 1% agarose in $NaDodSO_4$ sample buffer and layered on an $NaDodSO_4$ slab gel consisting of a 15% acrylamide separation gel (13-cm long) and a 5% stacking gel (5-cm long). The tube gel was then laid on top of the slab gel and secured with an additional 200–300 μL of agarose, as described by O'Farrell (1975). The final concentrations of protease in the agarose were: *S. aureus* protease, 200 μg/mL; chymotrypsin, 1 mg/mL; and elastase, 5 μg/mL. After addition of 0.75 mL of 0.05% bromophenol blue to the upper electrode reservoir, electrophoresis was performed in the normal manner, except that the current was turned off for 30 min when the dye was 0.5 cm from the bottom of the stacking gel to allow digestion of the receptor subunits. The current was then turned on, and electrophoresis of the newly generated peptides was completed.

Labeling of Torpedo Receptor by Reductive Methylation with [^{14}C]Formaldehyde. Detergent-free *Torpedo* receptor was labeled by the method of Means & Feeny (1968). [^{14}C]Formaldehyde (120 nmol, 45 mCi/mmol) was added to 67 pmol of detergent-free receptor on ice, followed by five aliquots of $NaBH_4$ for a total of 60 nmol. The procedure was repeated once and the solution then dialyzed against 10 mM Tris-Cl, pH 7.4, 1% Triton X-100, 0.1 mM EDTA. Approximately 2.5 mol of ^{14}C was incorporated per mol of receptor.

Ferguson Plots. $NaDodSO_4$ slab gel electrophoresis was performed as described above, except that the acrylamide concentrations of the various separation gels were 8%, 10%, 11%, and 13%. The positions of the subunits which are not well resolved on gels (such as α_2) were taken as the positions of the corresponding shoulders appearing on the main subunit peaks. Retardation coefficients, defined as the slopes of the plots of the log R_f vs. percent gel concentration, were determined for the standards and for the receptor subunits. The extrapolated molecular weights of the receptor subunits were determined from the plots of log retardation coefficient vs. log molecular weight for the standard proteins (Frank & Rodbard, 1975).

Results

Junctional and Extrajunctional ACh Receptors in Rat Leg Muscle. Earlier experiments compared the properties of isolated junctional and extrajunctional receptors by using end-plate-rich regions of normal rat diaphragm muscles and end-plate-free regions of denervated diaphragms (Brockes & Hall, 1975). In the present experiments, we used normal and denervated rat hind leg muscle as the source of receptor to obtain larger amounts of starting material. Because the muscles were not divided into junctional and extrajunctional regions, it was necessary to establish that the predominant species present in each case had the expected properties.

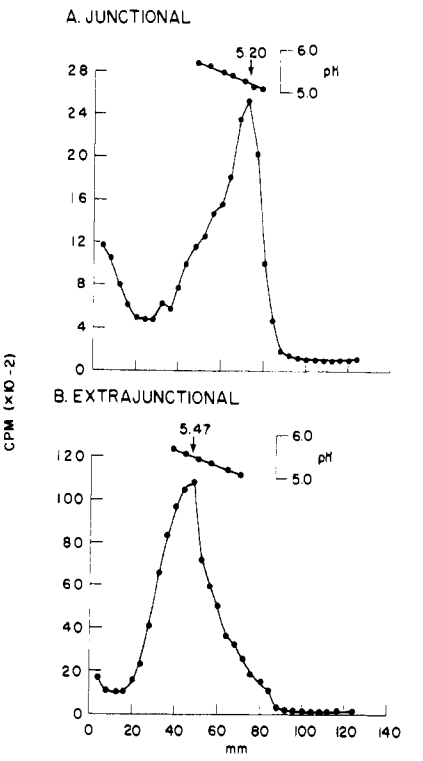

FIGURE 1: Isoelectric focusing of partially purified acetylcholine receptor–α-[^{125}I]BuTx complexes. Complexes of toxin with receptor from normal muscle (containing junctional receptor) (A) and denervated muscle (containing extrajunctional receptor) (B) were formed and submitted to isoelectric focusing as described under Methods. The relative amounts of each receptor were estimated graphically by assuming that each peak was symmetrical.

Previously, we reported that junctional and extrajunctional receptors from rat diaphragm have different isoelectric points (Brockes & Hall, 1975). Attempts to confirm this difference by using crude extracts containing receptor from leg muscle did not give reproducible results, in part because the receptor often aggregated and failed to enter the isoelectric focusing gels. After partial purification on cobrotoxin/Sepharose, however, receptors from normal and denervated muscles could be compared. Toxin–receptor complex derived from normal muscle consistently focused at a pH that was 0.2–0.3 unit more acidic than the complex derived from denervated muscle (Figure 1), confirming the previous observation made with junctional and extrajunctional receptors derived from rat diaphragm (Brockes & Hall, 1975). Based on the relative areas under the shoulders and main peaks on the gels shown in Figure 1, we estimate that the preparations from normal and denervated muscle contain at least 75% junctional and extrajunctional receptors, respectively.

The receptor preparations can also be distinguished by immunological criteria. Sera from many patients with myasthenia gravis contain antibodies that react only with extrajunctional receptor, as well as antibodies that react with both receptor types. These sera thus bind more extrajunctional than junctional receptor, and can be used to identify the predominant species in a receptor preparation (Almon & Appel, 1973; Weinberg & Hall, 1979). We have used one such serum to compare the reactivity of junctional and extrajunctional receptors in crude extracts of rat diaphragm with the reactivity of receptor in extracts of normal and denervated leg muscle. This serum was tested against equivalent concentrations of each receptor in diaphragm extracts, and precipitated 2.2-fold more extrajunctional than junctional receptor. When receptor preparations from denervated and

Table I: Iodination of Junctional and Extrajunctional ACh Receptors[a]

	acid-precipitable radioact. 10^6 cpm	total radioact. minus control 10^6 cpm	radioact. % in AChR
A. native receptor			
junctional receptor	2.24	0.87	39
junctional control	1.37		
extrajunctional receptor	5.52	5.53	97
extrajunctional control	0.19		
B. receptor in $NaDodSO_4$			
junctional receptor	7.42	4.54	61
junctional control	2.88		
extrajunctional receptor	5.50	5.42	98
extrajunctional control	0.08		

[a] Radioiodination of receptor (240 fmol) or a corresponding volume (200 μL) of control solution was carried out in the absence (A) or presence (B) of 2% $NaDodSO_4$ as described under Materials and Methods. Buffer blank of 0.95×10^6 cpm in the absence of $NaDodSO_4$ and 1.66×10^6 cpm in the presence of $NaDodSO_4$ was subtracted from the original values to give the numbers above.

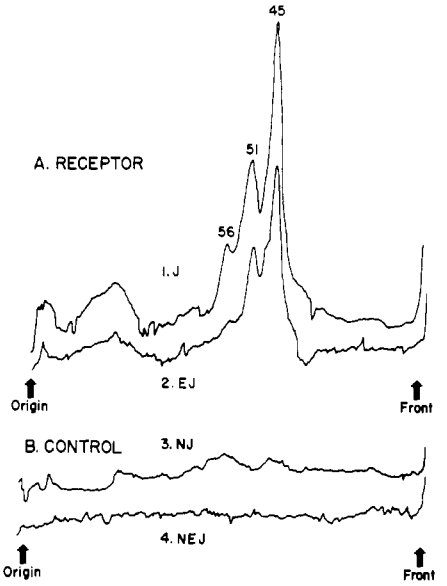

FIGURE 2: Densitometric tracings of an autoradiograph of $NaDodSO_4$–polyacrylamide gels of ^{125}I-labeled purified receptor (A) and "nonreceptor" control (B) preparations from rat muscle as described under Methods. (1) Junctional receptor; (2) extrajunctional receptor; (3) junctional "nonreceptor" control preparation; (4) extrajunctional "nonreceptor" control preparation.

normal leg muscle were tested at the same concentration used with diaphragm, a ratio of 2.4 was obtained. Thus, both isoelectric focusing and immunological reactivity show that the differences observed between junctional and extrajunctional receptors from diaphragm are also found in receptors from leg muscle.

To ensure that minor components were not enriched by the purification procedure (see below), we also used the same myasthenic serum to examine junctional and extrajunctional receptors that had been highly purified from leg muscle. The value obtained for the ratio of purified extrajunctional to junctional receptors precipitated was 2.4, identical with that obtained with crude extracts. Competition experiments and the inability of saturating amounts of purified junctional receptors to bind as much antibody as extrajunctional receptor have led us previously to conclude that purified junctional receptor preparations contain less than 10% contamination by extrajuntional receptor (Weinberg & Hall, 1979).

Purification and Radioiodination of Receptors. Both junctional and extrajunctional receptors were purified from hind leg muscles of rats by sequential affinity chromatography on Con A/Sepharose and cobrotoxin/Sepharose followed by sucrose gradient sedimentation. Control preparations were made as previously described (Froehner et al., 1977b) for each receptor by incubating half of the Con A/Sepharose eluate with excess unlabeled α-BuTx. This specifically prevented adsorption of the receptor in the subsequent cobrotoxin/Sepharose step. Receptor and control preparations were then carried identically through cobrotoxin/Sepharose and sucrose gradient purification steps.

For further analysis, identical amounts of junctional and extrajunctional receptor and the corresponding control preparations were radioiodinated by a Chloramine-T procedure in the presence or absence of $NaDodSO_4$ (Table I). For the extrajunctional receptor, incorporation of ^{125}I was the same under both conditions. Comparison with the control preparation indicated that over 90% of radioactive protein in this extrajunction preparation corresponded to receptor. This value is similar to the degree of purity estimated in a previous preparation by direct protein determination (Froehner et al., 1977b).

Incorporation of ^{125}I into the junctional receptor, however, was much higher in the presence, than in the absence, of $NaDodSO_4$. In $NaDodSO_4$, the two receptors, after correction for the control, were iodinated to almost the same extent on a molar basis, while under native conditions the extent of iodination of the junctional receptor was only 15% that of the extrajunctional. This difference may reflect an altered conformation or greater aggregation of the junctional receptor which renders it less susceptible to iodination unless it has been denatured in detergent.

Protein determinations were not made because of the very small amount of receptor available (see Methods). Comparison of the junctional receptor and control preparations after iodination in $NaDodSO_4$ indicated that about 60% of the radioactive protein was receptor.

Comparison of Subunit Structures of Junctional and Extrajunctional Receptors. The extrajunctional receptor in muscle has previously been shown to have a complex structure with subunits of apparent molecular weights of 45 000, 49 000, 51 000, 56 000, and 62 000 (Froehner et al., 1977b). We will refer to these subunits as α_1, α_2, β, γ, and δ, respectively. This terminology is similar to one used recently for *Torpedo* acetylcholine receptor (Hucho et al., 1976). The same letter is used for the 45 000- and 49 000-dalton polypeptides, because both of these polypeptides bind MBTA (Forehner et al., 1977a) and because their peptide maps are similar (see below).

We have previously shown (Froehner et al., 1977b) that essentially the same subunit structure is seen for the extrajunctional receptor after direct visualization by protein staining with Coomassie Blue or radioiodination and autoradiography. Here we compare the subunit composition of junctional and extrajunctional receptors by $NaDodSO_4$ slab gel electrophoresis after iodination in 2% $NaDodSO_4$. Iodination in $NaDodSO_4$ results in broader bands than those seen after iodination in the absence of detergent, but was required to achieve equivalent labeling of the two receptors. Because of this broadening, the minor bands were not often well resolved in these experiments. Nevertheless, the subunit patterns seen for the two receptors were very similar (Figure 2). The

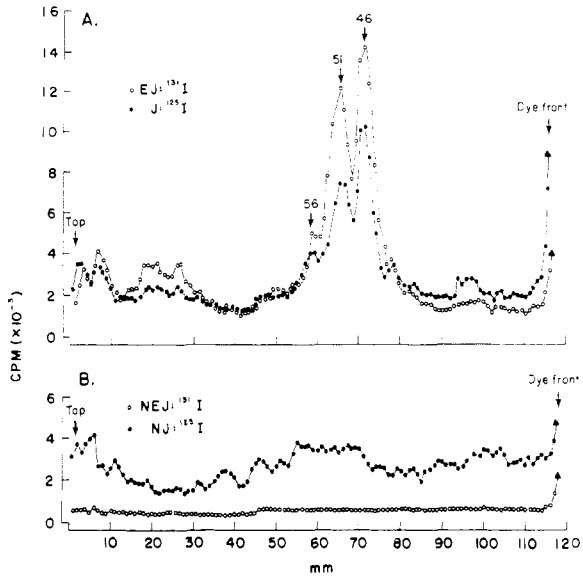

FIGURE 3: (A) $NaDodSO_4$ tube gel electrophoresis of extrajunctional ACh receptor labeled in $NaDodSO_4$ with ^{131}I (open circles) and junctional ACh receptor labeled in $NaDodSO_4$ with ^{125}I (closed circles). Approximately 46 fmol of extrajunctional receptor and 31 fmol of junctional receptor were applied to the gel. The gel was fixed, cut, and counted as described under Methods. (B) $NaDodSO_4$ tube gel electrophoresis of "nonreceptor" control preparations for extrajunctional receptor labeled with ^{131}I (open circles) and junctional receptor labeled with ^{125}I (closed circles).

apparent molecular weights of the two main subunits, α_1 and β, were identical for both receptors. In addition, no difference was seen between the γ subunits or between the α_2 subunits which appear in Figure 2 as shoulders on the α_1 peaks. The δ subunit is not readily apparent in the densitometric tracing shown in Figure 2; however, this peak was revealed by a longer autoradiographic exposure of the gel and was found to have the same apparent molecular weight (66 000) in both cases. Both gels also contained radioactive material migrating in a broad band of approximately 100 000 daltons. As has been suggested previously (Forehner et al., 1977b), this material may arise through cross-linking of subunits, and we have not investigated it further.

A more sensitive comparison of the subunit structure of the two types of receptors was made by labeling extrajunctional receptor and its control with ^{131}I and junctional receptor and its control with ^{125}I. The two receptors were mixed and run on the same gel, as were the respective controls. The gels were then sliced and counted for each isotope (Figure 3). The peaks of the two main subunits, α_1 (which in this experiment had a molecular weight of 46 000) and β, were coincident, and, although the α_2 subunit was not well-resolved, no significant difference in the $^{125}I/^{131}I$ ratio (less than 20% variation) was found over the region of the gel corresponding to 40 000–55 000. The gel profiles of the control preparations show no major contaminating polypeptides in either case (Figure 2 and 3), suggesting that the polypeptides seen in the receptor preparations are in fact associated with the ACh receptor.

When iodinated junctional and extrajunctional receptors were run on $NaDodSO_4$ gels of varying polyacrylamide concentration, a strong dependence of the apparent molecular weights of the α_1, α_2, and β subunits on the acrylamide concentration was found (Table II). In contrast, the apparent molecular weights of γ and δ subunits were relatively independent of the gel concentration. Molecular weights determined by Ferguson plots for the five subunits of muscle ACh receptor were 57 000, 64 000, 67 000, 60 000, and 69 000 for

Table II: Dependence of Apparent Molecular Weight of Muscle ACh Receptor Subunits on Acrylamide Concentration[a]

	acrylamide concn					extrapolated mol wt
	8%	9%	10%	11%	13%	
α_1	45 000	45 000	46 000	47 000	52 000	57 000
α_2	49 000	49 000	50 000	51 000	52 000	64 000
β	51 000	51 000	52 000	54 000	57 000	67 000
γ	56 000	56 000	56 000	56 000	57 000	60 000
δ	68 000	66 000	66 000	65 000	66 000	69 000

[a] Samples of radioiodinated junctional and extrajunctional ACh receptors were submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis, at several concentrations of acrylamide, and extrapolated molecular weights (±2000) determined as described under Methods.

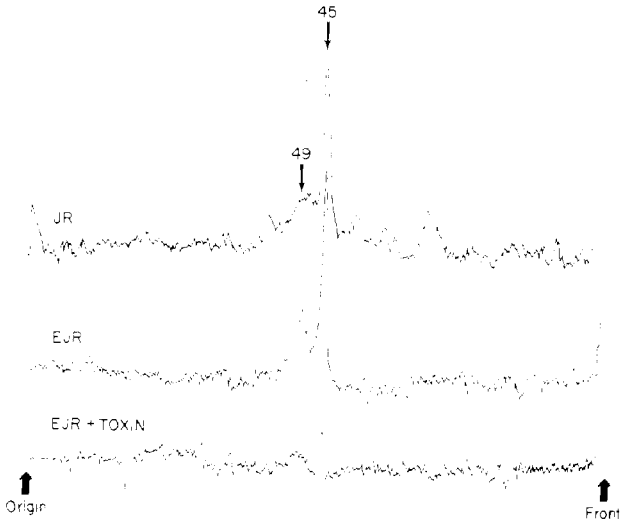

FIGURE 4: Densitometric tracing of fluorograms of an $NaDodSO_4$–polyacrylamide gel containing ACh receptor labeled with [^{3}H]MBTA. (Upper) Muscle junctional receptor; (middle) muscle extrajunctional receptor; (lower) muscle extrajunctional receptor labeled in presence of α-BuTx.

the α_1, α_2, β, γ, and δ subunits, respectively (Table II). No differences were seen in the behavior of homologous subunits from junctional and extrajunctional receptors.

Affinity Alkylation. The affinity reagent [^{3}H]MBTA specifically alkylates a sulfhydryl group within 1 nm of the ACh binding site in the *Electrophorus* receptor (Karlin et al., 1971). For acetylcholine receptors from both *Torpedo* and eel, one subunit is labeled with MBTA and is thus identified as possessing at least part of the ACh binding site (Reiter et al., 1972; Karlin & Cowburn, 1973; Weill et al., 1974; Damle et al., 1978). In contrast, it has been previously shown that [^{3}H]MBTA labels two subunits of the extrajunctional muscle receptor. Comparison on the same $NaDodSO_4$ gel of the positions of the subunits stained by Coomassie Blue with the positions of the polypeptides labeled with [^{3}H]MBTA and detected by fluorography clearly showed that the α_1 and α_2 subunits were labeled, while the β subunit was not (Froehner et al., 1977a). In the present experiments, we have compared the subunits of purified junctional (Figure 4a) and extrajunctional (Figure 4b) receptors that are labeled by [^{3}H]-MBTA. After reduction and alkylation, followed by $NaDodSO_4$ gel electrophoresis fluorography, two labeled bands of apparent molecular weight 45 000 and 49 000 are seen in each case. There is also a small amount of lower molecular weight material labeled in the junctional receptor, which presumably results from proteolysis (see below). Alkylation of both subunits of the muscle receptor was virtually eliminated

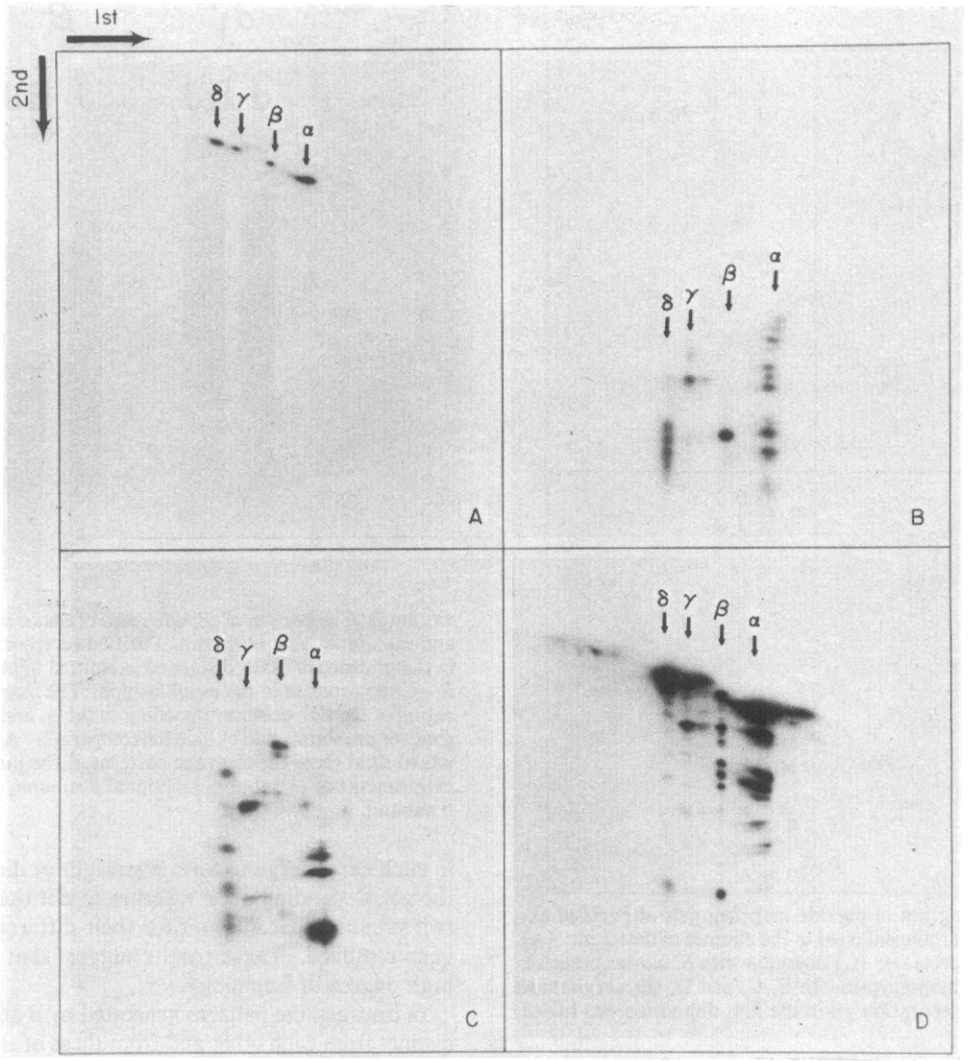

FIGURE 5: Autoradiogram of peptide mapping gels of purified radioiodinated *Torpedo* ACh receptor. Purified receptor was iodinated in the absence of detergent, submitted to $NaDodSO_4$–polyacrylamide tube gel electrophoresis (first dimension) to separate subunits, and then electrophoresed in the second dimension in the presence of various proteases as described under Methods. (A) No protease; (B) digestion with elastase; (C) digestion with *S. aureus* protease; (D) digestion with chymotrypsin.

(80–90%) by α-BuTx (Figure 4c). To prevent proteolysis (Froehner et al., 1977a), the labeling reaction with [^{3}H]MBTA was not carried to completion, and thus the difference in amount of label incorporated into the two subunits probably reflects a difference in the rates of reaction for each subunit and not a difference in their stoichiometries.

Peptide Mapping of the Torpedo Receptor. In order to compare the various subunits of each receptor, we have modified a procedure for peptide mapping described by Cleveland et al. (1977) in which proteins are partially digested by protease and the cleavage products analyzed by polyacrylamide gel electrophoresis. These authors showed that the method yields highly reproducible cleavage patterns that are characteristic of the polypeptide substrate and of the protease used. Because the molecular weights of α_1, α_2, and β subunits of the ACh receptor from muscle are very similar and the subunits frequently not well resolved, we modified the original procedure for use as a two-dimensional technique. The subunits of purified iodinated receptor were separated in the first dimension on a cylindrical gel, which was then attached to a slab gel, by using agarose in which a protease had been dissolved. Protease and iodinated subunits were then electrophoresed into the stacking gel of the slab gel where proteolytic digestion occurred. Finally, the cleavage products were resolved in the separation gel and visualized by autoradiography. Thus peptide maps of all receptor subunits are generated on a single slab gel.

Figure 5 shows the technique applied to purified iodinated *Torpedo californica* receptor. In Figure 5A, the four undigested subunits can be seen with molecular weights of 44 000, 53 000, 60 000, and 65 000. In addition, there is a fifth subunit of apparent molecular weight of 57 000, which was occasionally seen. This fifth subunit lay above the hyperbola which passed through the other four subunits. The anomalous migratory behavior in $NaDodSO_4$ gels of different acrylamide concentrations, termed "off-diagonal" behavior by Wang & Richards (1974), suggests that this polypeptide may be highly glycosylated. Figures 5B, 5C, and 5D show the cleavage patterns of *Torpedo* receptor digested with elastase, *S. aureus* protease, and chymotrypsin. As is readily apparent, each of the four subunits yields distinctive cleavage patterns with each protease, suggesting that these polypeptides have very little amino acid sequence homology. In contrast, the fifth polypeptide of apparent mol wt 57 000 has many peptides in common with the γ subunit of apparent molecular weight 60 000 (Figure 5B and 5D). This fifth polypeptide is thus very closely related to the γ subunit and may either be more glycosylated, as suggested by its "off-diagonal" behavior, or proteolytically derived from the γ subunit during or after purification of the receptor.

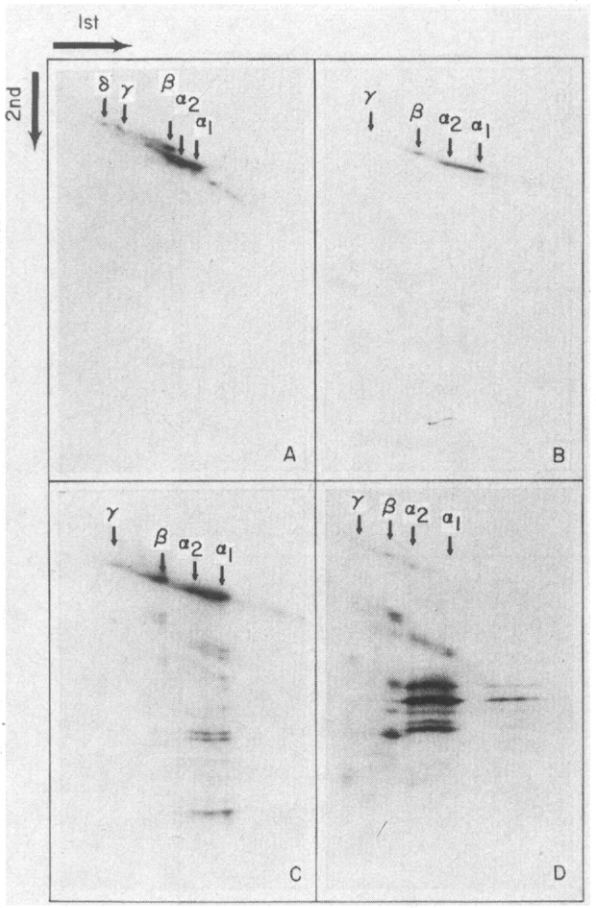

FIGURE 6: Autoradiogram of peptide mapping gels of purified extrajunctional receptor, radioiodinated in the absence of detergent. (A) No protease; (B) no protease; (C) digestion with *S. aureus* protease; (D) digestion with chymotrypsin. In B, C, and D, the acrylamide concentration of the separation gel in the first dimension was raised from 9% to 15%.

To ensure that the tyrosine-containing iodinated peptides generated by proteolytic digestion of radioiodinated receptor were not a special class of all possible peptides produced, *Torpedo* receptor was labeled by reductive methylation of lysines with [^{14}C]formaldehyde as described under Methods. Peptide mapping and autoradiography of ^{14}C-labeled receptor by proteolytic digestion with *S. aureus* protease, chymotrypsin, and elastase also demonstrated that the four subunits of the *Torpedo* receptor are unrelated (N. M. Nathanson and Z. W. Hall, unpublished data). In addition, over 70% of the peptides produced by digestion of the ^{14}C-labeled receptor had mobilities identical with those of peptides produced by digestion of radioiodinated receptor (N. M. Nathanson and Z. W. Hall, unpublished data), suggesting that the peptides seen after digestion of radioiodinated receptor are not a special subset of all peptides.

Peptide Mapping of Extrajunctional Receptor. Peptide mapping was peformed on extrajunctional receptor that had been iodinated in the absence of detergent to permit maximum resolution of the subunits. Even so, the α_1, α_2, and β bands were not well resolved in the first dimension (Figure 6A). The separation between the β subunit and the α_1 and α_2 subunits was therefore increased by taking advantage of the anomalous dependence of migration on acrylamide concentration. When 15% gels were substituted for 9% gels in the first dimension, satisfactory resolution was achieved (Figure 6B). After proteolytic digestion (Figure 6C and 6D), the pattern of peptides seen for α_1 and α_2 appear to be related, while that for β is quite distinct. The peptides generated by α_1 and α_2 in each case merge to form a straight or diagonal line across the gel, depending upon whether or not the portion(s) of the two subunits that account for their different mobilities have been removed. These results suggest that α_1 and α_2 have a high degree of homology.

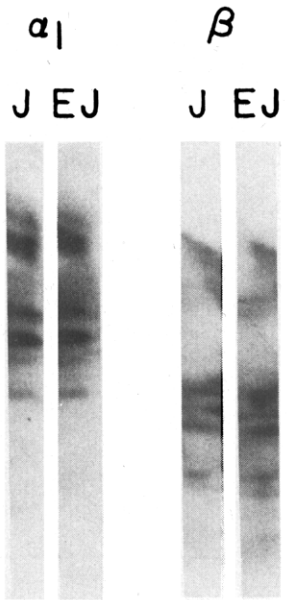

FIGURE 7: Comparison of peptide maps of major subunits of junctional and extrajunctional receptors. Purified receptor was radioiodinated in the presence of NaDodSO$_4$ and submitted to peptide mapping with *S. aureus* protease in the usual fashion. The cleavage patterns in the region of the slab gels corresponding to the α_1 and β subunits for each receptor are shown side by side for comparison. Autoradiograms from left to right show the cleavage patterns of the junctional α_1 subunit, extrajunctional α_1 subunit, junctional β subunit, and extrajunctional β subunit.

In contrast, the patterns generated by β and γ subunits were distinct from each other and from those of α_1 and α_2. Because of the small amount of radioiodinated δ subunit present, it was not possible to determine whether or not it was related to the other subunits. There are also polypeptides with molecular weights lower than α_1 (viz. Figure 3). These generate peptide maps which are very similar to that of the α subunits (Figure 6C and 6D) and thus presumably represent proteolysis products of the α subunits.

Comparison of Peptide Maps of Junctional and Extrajunctional Receptor. Peptide maps of junctional and extrajunctional receptor were compared after iodination in NaDodSO$_4$. As noted above, this causes broadening of bands and decreased resolution on NaDodSO$_4$ gels and, under the conditions used, obscures visualization of peptide maps for the two minor subunits γ and δ. However, as seen in Figure 7, the peptide maps of the α_1 and β subunits produced after digestion with *S. aureus* protease were identical for junctional and extrajunctional receptors. Thus, the major subunits of the junctional muscle receptor have a high degree of sequence homology with the corresponding subunits of extrajunctional receptors.

Discussion

Purification of Junctional and Extrajunctional Receptors from Rat Leg Muscle. We have purified acetylcholine receptor from normally innervated rat leg muscle using procedures described in an earlier study of extrajunctional receptor from denervated muscle. By the criteria of isoelectric focusing and reactivity toward serum from patients with myasthenia gravis, this receptor has the properties of junctional receptor. Because

of the small amounts of material available, determination of the specific activity of the purified junctional receptor was not made. All of our experiments were carried out, however, with radioiodinated receptor, which we estimate to comprise about 60% of the radioactive protein in the preparation. This probably gives a rough estimate of the biochemical purity of the preparation since, in earlier studies on extrajunctional receptor (Froehner et al., 1977b), determination of purity by protein assay gave results identical with estimates obtained from radioiodination of receptor and control preparations. $NaDodSO_4$ gel electrophoresis of radioiodinated control preparations showed minor amounts of contaminating polypeptides that appeared to be distributed across a broad molecular weight range with no major individual contaminants.

In order to compare the structures of junctional and extrajunctional receptors, it is clearly important that the extent of radiolabeling be comparable in the two cases. This condition was only achieved when the radioiodination was carried out in $NaDodSO_4$. In 2% $NaDodSO_4$, the extent of incorporation of radioactivity was the same in both cases and represented approximately 4.6 mol of iodine per toxin-binding site. Iodination in the absence of detergent-reduced labeling of the junctional receptor sixfold, but had little effect on the labeling of extrajunctional receptor. The reason for this difference is unknown but may reflect a difference in aggregation state or in conformation of the receptors under these conditions, that renders particular tyrosine residues inaccessible.

Subunit Composition of Junctional and Extrajunctional Receptors. Junctional and extrajunctional receptors appear to have the same subunit composition. Each has two major subunits, α_1 and β, and at least three minor subunits, α_2, γ, and δ. In addition, a variable amount of higher molecular weight material, presumably due to cross-linking of lower molecular weight subunits, was seen in both cases. For the extrajunctional receptor, the identification of each of these polypeptides as part of the receptor rests upon two observations (Froehner et al., 1977b). First, the binding of α-bungarotoxin to the receptor prior to the cobrotoxin/Sepharose purification step specifically eliminates these polypeptides from the final preparation. Since over 90% of the α-bungarotoxin binding activity in denervated muscle has the pharmacological properties of the nicotinic receptor, this criterion is highly specific. Secondly, sucrose gradient sedimentation analysis of the radioiodinated receptors shows that each of these polypeptides is associated with both monomeric (9S) and dimeric (13S) forms of the receptor. These two observations suggest that, at least after solubilization, all of these polypeptides occur as part of a single complex that has binding activity for α-BuTx and other cholinergic ligands. In the case of the junctional receptor, α_1, β, and γ subunits are clearly not present in the control preparation produced by incubation with α-BuTx during preparation. The evidence for α_2 and δ is less complete: α_2, because it is poorly resolved in the gels of material radioiodinated in $NaDodSO_4$, and δ, because the extent of radioiodination in junctional and extrajunctional preparations was low, and it was sometimes difficult to detect above the high background found in junctional control preparations. Thus, it was not possible always to identify these bands unequivocably and their inclusion as part of the junctional receptor is made tentatively. Analysis of the radioiodinated junctional receptor by sucrose gradient sedimentation was not possible because complete labeling could not be achieved under native conditions. It is important to stress, however, that each of the subunits that we had previously found to be part of the extrajunctional receptor were also found in junctional receptor preparations.

Analysis of the subunits in gels of different acrylamide concentrations showed that α_1, α_2, and β have anomalous behavior, suggesting that they may be highly glycosylated. Their actual molecular weights are therefore uncertain, but, in any case, the behavior of homologous subunits from junctional and extrajunctional receptors was identical. The anomalous behavior may account for slight variability in the positions of the subunits that were sometimes observed to occur in different experiments. None of the four subunits of *Torpedo* showed such anomalous behavior (N. M. Nathanson and Z. W. Hall, unpublished; Rafto & Froehner, 1979). An additional source of difficulty in estimating an exact molecular weight for α_2 is that, in some preparations, α_2 appears as a doublet band (see Figures 6C and 6D). This apparent heterogeneity is not an artifact of iodination, because it can also be detected by Coomassie Blue staining (Froehner et al., 1977b).

In an effort to detect small differences in the molecular weights of corresponding subunits, the two receptors were labeled with different isotopes and electrophoresed together. For the major subunits, α_1 and β, both isotopes had identical peaks; further, the ratio of the two isotopes was relatively constant throughout the range of 40 000–55 000 daltons. Although differences in the minor subunits could easily have been missed, we estimate that a difference of as little as 1000 daltons (corresponding to a difference in the electrophoretic mobility of 2 mm for the experiment in Figure 3) would have been detected for α_1 and β subunits. As previously discussed (Froehner et al., 1977b), there is no information on the role of most of the subunits, although for both junctional and extrajunctional receptors the two subunits, α_1 and α_2, are labeled with MBTA and thus apparently contain ACh-binding sites.

Acetylcholine receptor, presumably corresponding to extrajunctional receptor, has recently been purified from a nonfusing muscle cell line and has a subunit structure similar to that reported here for rat muscle receptor (Boulter & Patrick, 1977). In contrast, extrajunctional receptor purified from primary cultures of embryonic calf muscle consists of only two subunits, a main one of 40 000 daltons and a minor one of 46 000 daltons (Merlie et al., 1978). Extrajunctional receptor purified from denervated cat muscle has also been reported to consist of a single main subunit of 41 000 daltons and several minor subunits of higher molecular weight (Shorr et al., 1978). Similar discrepancies have been reported in the number of subunits of the acetylcholine receptor purified from the electric organ of *Torpedo* (Karlin et al., 1976; Raftery et al., 1976; Sobel et al., 1977; Lindstrom et al., 1978; Weitzmann & Raftery, 1978). Despite these apparent differences in subunit structures, all the purified receptors have approximately the same specific activities (10 nmol/mg) and the same sedimentation coefficients (9S). As has been discussed (Merlie et al., 1978), differences in the subunit structure of ACh receptor isolated by different laboratories may be due to (a) differences in purification procedures resulting in the loss of loosely associated subunits or the retention of tightly bound contaminating polypeptides, (b) species or organ-related differences due to differences in the starting source of ACh receptor, or (c) differences due to alteration of the apparent pattern of the subunits by proteolysis during the course of purification. Further experimentation for both *Torpedo* and muscle receptors will be required to resolve these differences.

Peptide Mapping of the Receptor Subunits. We have further investigated the relation of the various subunits of the

muscle ACh receptor to each other by peptide mapping. There were two reasons for these experiments. First, the large number of subunits and the different results obtained in different laboratories raised the possibility that some subunits were derived from others, either by proteolysis or by post-translational modification. In particular, we wished to compare the two subunits, α_1 and α_2, that bind MBTA. Second, we wished to make a more sensitive comparison between the corresponding subunits of junctional and extrajunctional receptors. To accomplish this, the method described by Cleveland et al. (1977) was adapted for use as a two-dimensional technique. The modified procedure has the advantage of being easy, fast, and highly reproducible, and does not require isolation of the poorly resolved muscle subunits from the $NaDodSO_4$–polyacrylamide gel used for their separation. Its major disadvantage is that the partial digestion products that are compared are quite large. Thus, small differences in homologous proteins or regions of homology in dissimilar proteins may not be detected.

The method was first applied to the *Torpedo* receptor both because it was available in large amounts and because of uncertainty about the origin (Patrick et al., 1975) and number (Karlin et al., 1976; Raftery et al., 1976; Sobel et al., 1977) of its subunits. After treatment with each of several proteases, each of the four *Torpedo* subunits gave a distinctive pattern of peptides, suggesting that there is relatively little homology between them. Thus, the four *Torpedo* subunits appear to be structurally unrelated, a conclusion also reached by Claudio & Raftery (1977) and Lindstrom et al. (1978) from experiments with antibodies to the isolated subunits and by Rafto & Froehner (1979) on the basis of peptide mapping.

In contrast, peptide mapping of the extrajunctional muscle receptor indicated that two of the subunits, those that bind MBTA, are highly similar. This similarity could arise because of homologous, although distinctive, amino acid sequences. Alternatively, the two subunits could differ in the extent of glycosylation or one could be proteolytically derived from the other during purification. The latter two possibilities are suggested by the observation that, as noted above, the α_2 band sometimes appeared to be heterogeneous. The peptide patterns derived from β and γ subunits were different from the pattern of the α subunits and from each other. Thus, it is unlikely that they are related by proteolysis. In addition, when peptide patterns for α_1 and β subunits of junctional and extrajunctional receptors were compared, no differences were found. Thus, the main subunits of the junctional and extrajunctional receptor appear to have similar polypeptide sequences.

The Structural Difference between Junctional and Extrajunctional Receptor. One of the sources of interest in studying the two forms of the muscle receptor is the suggestion that they may be related by a posttranslational modification (Brockes & Hall, 1975; Changeux & Danchin, 1976). Because we found no difference in subunit molecular weights between the two receptor types, we attempted to extend the comparison by two-dimensional gel analysis (O'Farrell, 1975; Ames & Nikaido, 1976). Such experiments could unequivocally establish whether the two receptors are different in covalent structure and whether one or more subunits are responsible for the difference. We were unsuccessful, however, in obtaining satisfactory gels, either with muscle or with *Torpedo* receptors, due partly to incomplete dissociation of the receptor subunits in the isoelectric focusing dimension (unpublished experiments).

Our results do suggest that there are not extensive differences in glycosylation between the two receptors. If such differences were present, differences in the apparent subunit molecular weights or in the positions of the cleavage products produced by proteolysis might have been detected, because of effects of sugar residues on mobility of polypeptides in $NaDodSO_4$ polyacrylamide gels. Such effects would also be present with the lower molecular weight cleavage products produced by proteolysis. The inability to detect such differences in mobility suggests, but does not prove, that the two receptors do not differ extensively in glycosylation. The acetylcholine receptor from the electric organs of both *Electrophorus* (Teichberg et al., 1977) and *Torpedo* (Gordon et al., 1977) can be phosphorylated, and it has been suggested (Teichberg & Changeux, 1976, 1977) that junctional and extrajunctional muscle receptors may differ in the extent of phosphorylation. Such differences in phosphorylation, if present, would not necessarily have been detected by the methods used here.

Comparison of the subunit structures of junctional and extrajunctional receptors thus strengthens the conclusion derived from study of the properties of the partially purified receptors (Brockes & Hall, 1975) that they are highly similar proteins. The basis of the differences in isoelectric points, immunological properties, and susceptibility to radioiodination that have been found for the native proteins remains to be determined. The availability of purified mammalian junctional and extrajunctional receptors will permit further investigation of these differences and of their importance in the development of the neuromuscular junction.

Acknowledgments

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Steady-State Kinetics of Mouse DNA Polymerase β†

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ABSTRACT: DNA polymerase β from mouse myeloma has been purified to near homogeneity, and its properties have been examined. The enzyme did not catalyze a detectable level of dNTP turnover, pyrophosphate exchange, pyrophosphorolysis, 3′-exonuclease degradation, or 5′-exonuclease degradation. Steady-state kinetic studies point to an ordered bibi mechanism for the polymerization reaction. Metal activation, which is required for polymerization, did not alter the K_m for either the dNTP or the template–primer.

In subunit composition, size, and catalytic repertoire, DNA polymerase β is the simplest naturally occurring DNA polymerase known. This enzyme is a single polypeptide of approximately 40 000 daltons, and it does not degrade nucleic acids or nucleoside 5′-triphosphates [see below; Chang & Bollum (1973)]. Earlier kinetic studies have indicated that homopolymer replication by calf thymus β-polymerase involves just one binding site for dNTP and one binding site for the primer (Chang, 1973b); in addition, the enzyme can be inhibited by pyrophosphate, one of the products of the polymerization reaction (Chang & Bollum, 1973). Because of these properties and the availability of methods for preparing near homogeneous β-polymerase (Chang, 1973a; Wang et al., 1974, 1977; Stalker et al., 1976), this enzyme is a good candidate for use in steady-state kinetic studies of the DNA polymerase mechanism.

Here we describe the preparation of near homogeneous mouse β-polymerase and the characterization of its catalytic activity. Aided by the conceptual framework described by McClure & Jovin (1975), we undertook a steady-state kinetic evaluation of the mechanism of this enzyme. Substrate kinetics measured with three different template–primer/dNTP systems indicated a sequential mechanism for polymerization. These results together with product inhibition studies using pyrophosphate suggest an ordered bibi mechanism.

Materials

Chemicals and Enzymes. DNA polymerase I from *Escherichia coli* and terminal deoxynucleotidyltransferase from calf thymus were kindly provided by Drs. L. Loeb and R. L. Ratliff, respectively. Polynucleotide kinase from T4 phage infected *E. coli* was obtained from P-L Biochemicals. Unlabeled deoxynucleoside 5′-triphosphates and 5′-monophosphates were from Calbiochem and P-L Biochemicals, respectively. Labeled nucleotides were from ICN Pharma-

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