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Subunit Composition of Bovine Muscle Acetylcholine Receptor†

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ABSTRACT: Acetylcholine receptors from fetal calf muscle were purified to homogeneity (specific activity up to 7500 nmol/g of protein), in reasonable yields (20–50%), and near-milligram quantity. Purification was by affinity chromatography on *Naja naja siamensis* toxin coupled to agarose by using methods similar to those for receptors from fish electric organs, but with modifications to account for the low concentration of receptor in muscle and the high probability of proteolysis. Immunochemical methods are described for approximating the extent of proteolysis in receptor preparations. Bovine acetylcholine receptor is composed of four glycoprotein subunits designated α ($M_r \simeq 41\,000$), β ($M_r \simeq 50\,000$), γ ($M_r \simeq 53\,000$), and δ ($M_r \simeq 56\,000$) which correspond immunochemically to the four glycoprotein subunits of fish electric organ acetylcholine receptors of the same designations. Electron micrographs of purified bovine receptor show that it has the same size and shape as receptors from fish electric organs. Immunization of rats with receptor from bovine and human muscle is very effective at inducing experimental autoimmune myasthenia gravis. Acetylcholine receptors purified from rat muscle are composed of subunits which correspond immunochemically to the α, β, γ, and δ subunits of receptor from *Torpedo californica*. The evidence presented strongly suggests that acetylcholine receptors from fish electric organ tissue and mammalian muscle share a fundamentally similar shape, antigenic structure, and $\alpha_2\beta\gamma\delta$ subunit structure.

Striated muscle fibers are usually innervated at a single site; thus, although the acetylcholine receptor is a major component of the postsynaptic membrane, the 10^7 (Fambrough, 1979) receptors contained in this patch of membrane a few micrometers long are a very small component of a muscle fiber which might be nearly 100 μm in diameter and several centimeters long. The electric organs found in some fish are thought to have evolved from striated muscle by increasing the number of synapses on the muscle derivative to 10^4, or so, and by eliminating the contractile proteins and sarcoplasmic reticulum that compose the bulk of a muscle cell (Mellinger et al., 1978). The proposed phylogenic link between receptors from electric organs and skeletal muscle and their observed pharmacological and physiological similarity suggest that receptors from these sources should be structurally similar (Changeux, 1981; Karlin, 1980). Receptors from fish electric organs are much better characterized than are those from mammalian muscle because receptors from electric organs are available in much larger amounts and are less subject to proteolytic degradation. In order to put the problems encountered during the purification of mammalian striated muscle acetylcholine receptors into perspective, it is useful to first briefly consider what is known about receptor from fish electric organs.

The various species of the marine electric rays contain receptor at a concentration of 1–2 nmol/g of electric organ tissue (Karlin, 1980; Changeux, 1981). Acetylcholine receptors from the electric organs of *Torpedo californica* have been affinity purified in amounts up to 50 mg/batch and, consequently, have been relatively well characterized (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1978; Lindstrom et al., 1981). *Torpedo* receptors are composed of four kinds of partly homologous glycoprotein subunits of apparent molecular weights α = 38 000, β = 49 000, γ = 57 000, and δ = 64 000 organized in "monomers" of apparent molecular weight 250 000 consisting of two α subunits and one each of β, γ, and δ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). The receptor monomers contain two acetylcholine binding sites (Damle & Karlin, 1978; Neubig & Cohen, 1979) and the cation-specific channel whose opening they regulate (Anholt et al., 1980; Changeux et al., 1979; Wu & Raftery, 1979; Lindstrom et al., 1980a). The acetylcholine binding sites are formed at least partially by amino acids from the α subunits (Karlin, 1980). However, which subunits form the cation channel is not known. The *Torpedo* acetylcholine receptor is 110 Å long, about 55 Å of which is on the external surface of the membrane and about 15 Å on the cytoplasmic side, and there appears to be a channel running across the membrane through the center of the molecule (Kistler et al., 1982). Initially, in some laboratories, proteolysis of receptor during purification cleaved β, γ, and δ subunits into fragments so that only α subunits were recognized by electrophoresis on acrylamide gels in sodium dodecyl sulfate (Sobel et al., 1977). Despite proteolytic nicking, so that only fragments the size of α and smaller remain, the subunit fragments remain associated and capable of agonist-triggered channel opening (Lindstrom et al., 1980c).

The freshwater teleost *Electrophorus electricus* contains receptor at a concentration of 0.1 nmol/g of electric organ tissue. As a consequence of its lower concentration, it is usually only purified a few milligrams at a time. Initially, only two subunits corresponding to α ($M_r \simeq 41\,000$) and β ($M_r \simeq$

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50 000) were recognized (Lindstrom & Patrick, 1974); later, a third corresponding to γ ($M_r \simeq 55000$) was identified (Karlin et al., 1976), and recently, adequate control of proteolysis permitted recognition of a fourth subunit corresponding to δ ($M_r \simeq 62000$) (Lindstrom et al., 1980b). N-Terminal amino acid sequence analysis reveals that, like receptor from *Torpedo*, these four subunits are partially homologous and present in the mole ratio $\alpha_2\beta\gamma\delta$ (Conti-Tronconi et al., 1982). Proteolysis of *Electrophorus* receptor with trypsin causes it to appear as only a single band on gels, but little material is removed from the receptor as judged by its sedimentation on sucrose gradients (Lindstrom et al., 1976b). Another lesson to be learned from studies of receptor from *Electrophorus* is that relying on apparent molecular weights of bands on gels for identifying subunits can be deceiving. For example, at one point, it was reported that receptor from *Electrophorus* consisted of subunits of apparent molecular weights 110 000, 60 000, 53 000, and 48 000 and lacked an α subunit (Patrick et al., 1975). The identities of the corresponding subunits in receptors from *Torpedo* and *Electrophorus* were determined by affinity labeling of the α subunits (Karlin, 1980), by cross-reaction of each of the *Electrophorus* receptor subunits with antiserum raised against *Torpedo* receptor subunits of similar molecular weights (Lindstrom et al., 1980b), and by N-terminal amino acid sequence analysis (Conti-Tronconi et al., 1982). Receptors from *Electrophorus* have essentially the same size and shape as receptor monomers from *Torpedo*, as indicated by their 9S sedimentation on sucrose gradients (Conti-Tronconi et al., 1981) and their appearance when negatively stained of being 90-Å diameter doughnuts (Cartaud et al., 1973). Like receptors from *Torpedo*, receptors from *Electrophorus* have a main immunogenic region located on their α subunits (Tzartos & Lindstrom, 1980; Tzartos et al., 1981), and each receptor can bind two monoclonal antibodies directed at this region (Conti-Tronconi et al., 1981). All this evidence supports the idea that the acetylcholine receptors from the electric organ tissue of *Electrophorus* have the same basic $\alpha_2\beta\gamma\delta$ subunit structure as the acetylcholine receptors from *Torpedo* electric organ tissue.

Mammalian muscle acetylcholine receptors are present in normally innervated muscle at a concentration of about 0.0003 nmol/g of muscle (Lindstrom & Lambert, 1978), and even after denervation of the muscle, the concentration only increases 1 order of magnitude to about 0.006 nmol/g (Berg &, Hall, 1975), still several hundred-fold less than the receptor concentration of *Torpedo* electric organ. The limited amount of receptor from muscle is not the worst problem. Controlling proteolysis of *Torpedo* receptor was difficult for some laboratories, and controlling proteolysis of *Electrophorus* receptor, which is present at 10-fold lower concentrations, was even more difficult. Therefore, it is not surprising that controlling proteolysis of receptor from mammalian muscle, which is present at 5000-fold lower concentration than *Torpedo* electric organ receptor, is an extremely difficult problem. Repeating the experience with electric organ receptors, some laboratories have observed only a single subunit in muscle receptor (Merlie et al., 1977; Shorr et al., 1978, 1981). Other laboratories have observed between two and five bands on electrophoresis on acrylamide gels in sodium dodecyl sulfate (Boulter & Patrick, 1977; Nathanson & Hall, 1979; Merlie & Sebbane, 1981; Kemp et al., 1980; Stevenson et al., 1981). A major problem is to determine which of these bands are receptor subunits, which are contaminants, which are proteolytic artifacts, and which correspond to subunits observed in receptor from electric organ tissue. We have previously demonstrated that receptors from bovine muscle and human muscle have the same size on sucrose gradient centrifugation (Lindstrom et al., 1982), have antigenic determinants corresponding to all four *Torpedo* receptor subunits (Lindstrom et al., 1978b, 1979b), have α subunits which have a reactive disulfide bond near the acetylcholine binding site (Lindstrom et al., 1979b), and have a main immunogenic region (Tzartos et al., 1982) and that two monoclonal antibodies to the main immunogenic region can bind per receptor monomer (Lindstrom et al., 1982). All these results are consistent with the idea that receptors from mammalian muscle have an $\alpha_2\beta\gamma\delta$ subunit structure similar to that of receptors from fish electric organs but do not prove that receptor from muscle actually has four corresponding types of subunits. In addition to the resolution of this problem, there is the major problem that most preparations of receptor purified from muscle have consisted of only a few micrograms or even submicrogram amounts of radioactively labeled protein.

Here we report a method for purifying near-milligram amounts of receptor from fetal calf muscle. We show that this receptor has the electron microscopic appearance of receptor from fish electric organs and is composed of four subunits which correspond immunochemically to those of receptors from electric organs. We also show that receptor from rat muscle is composed of four subunits homologous to those of electric organ receptor. We demonstrate that proteolysis of β, γ, and δ subunits during purification is a problem and report an immunochemical technique for measuring the extent of this proteolysis. Our results strongly support the concept that acetylcholine receptors from mammalian muscle have fundamentally the same size, shape, and $\alpha_2\beta\gamma\delta$ subunit composition as receptors from fish electric organ tissue.

Materials and Methods

Purification. Receptors from *Torpedo californica* and *Electrophorus electricus* were purified by affinity chromatography on *Naja naja siamensis* toxin conjugated to agarose as previously described (Lindstrom et al., 1981).

Fetal calves 10–20 in. long were used as the source of bovine muscle acetylcholine receptor because of the high receptor content. Fetal muscle was obtained within an hour of death, placed in plastic bags, and frozen on dry ice. Denervated rat muscle was prepared as described by Nathanson & Hall (1979) and stored frozen. Human muscle was obtained from amputated legs.

Receptor was purified from muscle as follows. Frozen tissue was shattered with a hammer, mixed with 6 volumes of 4 °C buffer [10 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM iodoacetamide, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride, and 1 mL/L aprotinin (Sigma)], and homogenized for 1 min at high speed in a Waring blender. After centrifugation for 40 min at 10 000 rpm (17000*g*) in a Beckman JA10 rotor, the pellet was resuspended in 6 volumes of 10 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, and 10 mM NaN_3. After a second centrifugation, the pellet was homogenized again for 10 s with 1.25 volumes of 10 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, and 10 mM NaN_3, and then Triton X-100 was added to 2%. After moderate agitation for 2 h at 4 °C, the extract was centrifuged as before. The supernate was then recentrifuged for 30 min at 45 000 rpm (138000*g*) in a Beckman 50.2 rotor to clarify it. The lipid layer on the surface was aspirated and discarded, and the clear detergent extract containing solubilized receptors was retained. The extract was recirculated through a small column of toxin–agarose (1–2 mL of agarose and 0.5 mg of

Naja naja siamensis toxin/mL, capacity 4 nmol/mL) at 700 mL/h for 15 h at 4 °C. The column was then washed with 2000 volumes of 0.5% Triton X-100, 100 mM NaCl, 10 mM NaN_3, and 10 mM sodium phosphate, pH 7.5. The Triton buffer was subsequently exchanged for cholate buffer (10 mM sodium phosphate buffer, pH 7.5, 10 mM NaN_3, and 0.2% cholate). Cholic acid was obtained from Sigma, recrystallized from ethanol, and prepared as a 10% stock solution titrated to pH 7.0 with NaOH. Cholate is a more convenient but weaker detergent than Triton X-100, so it was not used for extraction or washing. Receptor was eluted by recirculating the high-affinity antagonist benzoquinonium (a gift from Sterling Winthrop Pharmaceuticals) at 1 mM in cholate buffer through the affinity column onto a small (200–500 μL) DEAE-cellulose column for 15 h at 4 °C at 700 mL/h. Small disposable columns (Bio-Rad) joined by Tygon tubing were used. The DEAE-cellulose column was washed free of benzoquinonium with a few milliliters of cholate buffer before the receptor was eluted with 1–2 mL of 0.5 M NaCl in cholate buffer applied to the column over about 5 min.

Protein concentration was measured by the Lowry et al. (1951) method. Receptor concentration was measured in moles of ^{125}I-labeled α-bungarotoxin binding sites per liter by radioimmunoassay using a rat antiserum to bovine muscle acetylcholine receptors as previously described (Lindstrom et al., 1981).

Electrophoresis. Electrophoresis on 10% acrylamide slab gels in sodium dodecyl sulfate using the Laemmli discontinuous buffer system was conducted as previously described (Lindstrom et al., 1979a,b).

Electrophoretic transfer of proteins from gels to activated paper was performed at 0.8 A and 7.5 V for 3 h at 23 °C on an electroblot apparatus (E-C Apparatus Corp., St. Petersburg, FL) essentially as described by Symington et al. (1981). The buffer was 25 mM sodium phosphate, pH 6.5. Diazophenyl thioether (DPT) paper was prepared according to the method of Seed (1982). After the transfer, excess reactive groups on the paper were quenched by incubation in 100 mL of 10% ethanolamine, 0.25% gelatin, and 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 9.0, for 2 h at 23 °C.

Antibody binding to receptor subunits on DPT paper was done over 18 h at 23 °C in 10 mL of 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, and 0.05% Triton X-100 to which was added 10 μL of one or more antisera to *Torpedo* receptor subunits [each $\simeq 5 \times 10^{-6}$ M (Lindstrom et al., 1979b)]. The paper was washed with 2 × 100 mL of the same buffer for 1 h at 23 °C and then incubated for 2 h in 10 mL of this buffer containing 5×10^5 cpm/mL ^{125}I-labeled goat anti-rat IgG (specific activity 1×10^{18} cpm/mol). After the paper was washed with 2 × 100 mL of the same buffer supplemented with 0.1% sodium dodecyl sulfate and 0.5% Triton X-100, it was covered with saran wrap and autoradiographed for 18 h at −70 °C by using preflashed X-Omat AR film from Kodak and an intensifying screen. Antibodies were removed from the paper before reuse by washing the paper in 100 mL of 50 mM sodium phosphate buffer, pH 7.5, 2% sodium dodecyl sulfate, and 0.1 M β-mercaptoethanol for 1 h at 60 °C.

Immunoprecipitation of ^{125}I-Labeled Subunits. Receptor from muscle was labeled with ^{125}I to specific activities of approximately 6×10^{18} cpm/mol by using lactoperoxidase conjugated to agarose according to the method previously described for receptors from fish electric organs (Lindstrom et al., 1979b). ^{125}I-Labeled receptor was dissociated into subunits with 1% sodium dodecyl sulfate and then diluted to 5×10^6 cpm/mL in 10 mM sodium phosphate buffer, pH 7.5, 0.1% sodium dodecyl sulfate, 0.5% Triton X-100, 100 mM NaCl, and 10 mM NaN_3. Aliquots of 1 mL were then incubated with 5 μL of antisera to *Torpedo* receptor subunits, goat anti-rat antibody coupled to agarose was added, and the bound ^{125}I-labeled subunits were eluted with sodium dodecyl sulfate and identified by electrophoresis followed by autoradiography as previously described for ^{125}I-labeled electric organ receptor subunits (Lindstrom et al., 1979a,b).

Electron Microscopy. Receptor preparations were dialyzed against 200 mM sodium bicarbonate buffer, pH 9.5, containing 0.1% Triton X-100. Bacitracin was added at 10 μg/mL to improve spreading. Carbon-coated copper grids were treated with polylysine, and then a drop of receptor solution was added. Excess solution was blotted with filter paper, and then the grids were washed with 0.1% ammonium acetate, stained with 1% uranyl acetate, and dried. The specimens were examined with a JEOL 100B or 100 CX electron microscope at 80 kV by using a 100-μm objective aperture. Electron micrographs were taken at 100000× or 130000× direct magnification and subsequently enlarged with a point source illumination enlarger.

Table I: Purification of Acetylcholine Receptor from 2615 g of Fetal Calf Muscle

	volume (mL)	protein (mg)	receptor nmol	receptor %	sp act. (nmol/mg of protein)
detergent extract	1720	7568	8.75	100	1.2×10^{-3}
extract after passage over affinity column	1720	7568	2.78	32	
eluate of DEAE column	4	0.572	4.0	46	7.0

Results

Purification of Receptor. Receptor was purified from bovine muscle by affinity chromatography on toxin–agarose by essentially the same procedure used with fish electric organs. There are several factors which seem to be critical. The use of 10–20-in. fetuses provides a concentration of receptor [$(4.1 \pm 2.4) \times 10^{-12}$ mol/g of tissue] comparable with that of denervated rat muscle, but far larger amounts of muscle are conveniently obtained than from denervated rats, and at no animal cost. The high concentration of receptor is probably due to the presence of extrajunctional receptors in fetal muscle (Reiness & Hall, 1981) and to the small size of fetal muscle fibers. A complex cocktail of protease inhibitors in the initial homogenization buffer reduces but does not, unfortunately, eliminate proteolysis. Washing of the particulate fractions and high-speed centrifugation of the detergent extract give a clear extract that minimizes trapping of extraneous material on the affinity column. Use of a small affinity column reduces excess receptor binding capacity, thereby making elution more efficient. Extensive recirculation through the columns also helps to ensure good receptor binding and elution.

A typical preparation from fetal calf tissue is summarized in Table I. A one-step affinity purification on toxin–agarose gave about 6000-fold purification from the initial detergent extract at a 67% yield of the receptor which bound to the column. The specific activity of the purified receptor was 7 nmol of toxin binding sites/mg of protein, which compares favorably with the value of 8 nmol/mg expected of receptor from electric organ tissue. The average specific activity for 30 bovine receptor preparations was 5 nmol/mg. Many kilograms of fetal calf muscle can be readily obtained, but the amount of receptor which can be purified at one time is limited. Centrifuge capacity for the initial homogenate limits prepa-

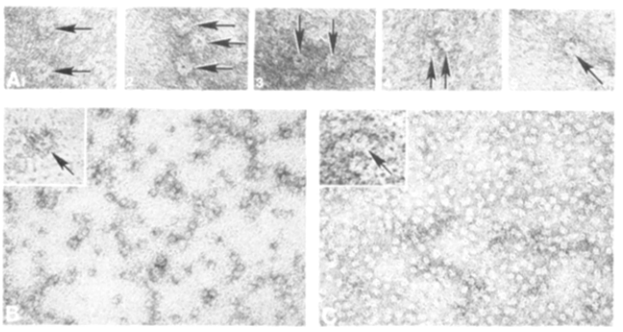

FIGURE 1: Electron micrographs of acetylcholine receptors: (A) bovine muscle, (B) *Electrophorus* electric organ, and (C) *Torpedo* electric organ, all negatively stained with uranyl acetate. Doughnut-shaped particles of about 9-nm diameter are visible in all three preparations. (A1–5) 157500× magnification; (B and C) 67500× magnification; (B and C insets) 157500× magnification.

ration to 1–2 kg of muscle which yields 0.5–1 mg of purified receptor.

Frozen muscle can be stored for long periods at −70 °C without significant loss of activity. For example, three normal rat carcasses freshly extracted contained $(0.54 \pm 0.04) \times 10^{-12}$ mol of receptor/g of tissue, whereas three others extracted after freezing contained $(0.53 \bullet 0.05) \times 10^{-12}$ mol/g. Note that the receptor content of this normally innervated muscle tissue is an order of magnitude below that of fetal calf muscle. Crude extracts of purified receptor can also be stored without loss of activity by adding 10% glycerol and freezing inside an insulated container to reduce the rate of cooling to about 1 °C/min.

Size and Shape of Bovine Receptor. Negatively stained bovine acetylcholine receptor has the appearance of a doughnut approximately 90 Å in diameter with a central pit (Figure 1). Its appearance is essentially identical with that of receptors purified from the electric organs of *Torpedo* or *Electrophorus* viewed under the same conditions, except that receptors from *Torpedo* are frequently observed as dimers (Figure 1). This appearance of negatively stained receptor from electric organ tissue has been described by many laboratories [e.g., see Cartaud et al. (1973), Potter & Smith (1977), and Kistler et al. (1982)]. We have previously demonstrated that even very extensive proteolytic nicking of electric organ receptor does not substantially alter the appearance of the receptors, dissociate any of the subunits, or prevent their function (Lindstrom et al., 1980c). Thus, our results suggest that receptor from bovine muscle has essentially the same size and shape as receptor from electric organ tissue, but this method is insensitive to any proteolytic cleavage of the primary structure of the receptor subunits.

Subunit Structure of Receptors from Muscle. In the best of preparations, purified bovine acetylcholine receptors show four distinct subunits of apparent molecular weights 41 000, 50 000, 52 000, and 56 000, all of which contain carbohydrate (Figure 2). These apparent molecular weights are similar to, but distinct from, those of receptor from *Torpedo*. Other preparations either at the beginning or on storage show loss of the higher molecular weight bands but retention of the α band (Figure 2). Intentional proteolysis of electric organ receptor causes preferential loss of the higher molecular weight bands without actually dissociating significant amounts of the resulting peptides (Lindstrom et al., 1980c). Also, there is evidence that spontaneous proteolysis of δ during purification can produce fragments the size of β (Wennogle et al., 1981) and that spontaneous proteolysis of γ can produce fragments the size of α (Gullick & Lindstrom, 1982).

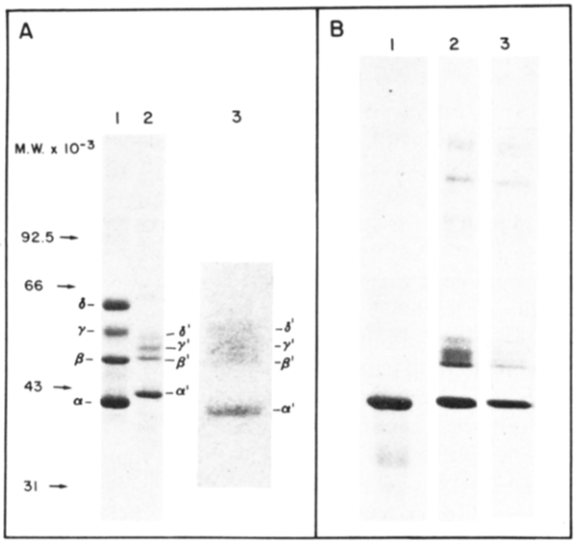

FIGURE 2: Electrophoresis on acrylamide gels containing sodium dodecyl sulfate of bovine acetylcholine receptor compared with *Torpedo* acetylcholine receptor. Panel A: (1) *Torpedo* receptor and (2) bovine receptor, both from a gel stained with Coomassie blue for protein (the molecular weight scale applies only to this gel); (3) bovine receptor from another gel stained with periodic acid–Schiff base for carbohydrate. Panel B: (1–3) Bovine receptor suffering various degrees of proteolytic damage due to warming during purification (1), normal vicissitudes of purification (2), or prolonged storage at −20 °C (3). Tracts 1–3 in panel B are from different gels aligned for comparison.

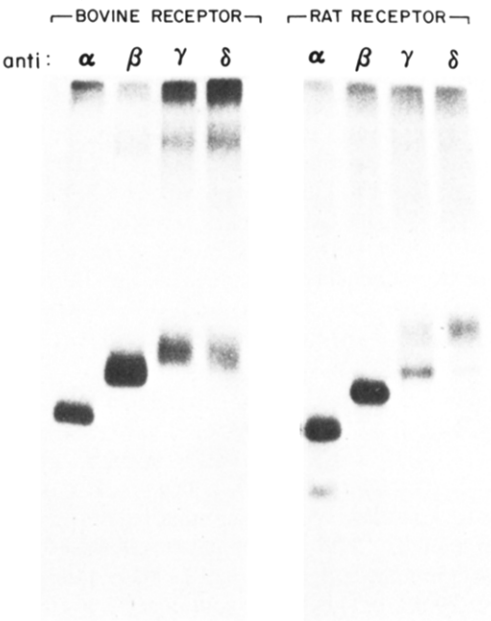

FIGURE 3: Reaction of ^{125}I-labeled receptor subunits from bovine or rat muscle with antisera to receptor subunits from *Torpedo*. ^{125}I-Labeled receptors were dissociated into subunits with sodium dodecyl sulfate and immunoprecipitated with antisera to each of the subunits of receptor from *Torpedo*, and the immunoprecipitated ^{125}I-labeled subunits were then identified by electrophoresis and autoradiography as described under Materials and Methods.

It was important to determine whether the bands seen on gels corresponded to the subunits of *Torpedo* receptor to whose molecular weights they were similar. In order to do this, we labeled bovine receptor with ^{125}I and dissociated it into its subunits with sodium dodecyl sulfate, and antisera to each of the *Torpedo* receptor subunits were added to determine which bands they would precipitate (Figure 3). Antiserum to α specifically bound to the bovine receptor subunit of apparent molecular weight slightly greater than *Torpedo* α, showing

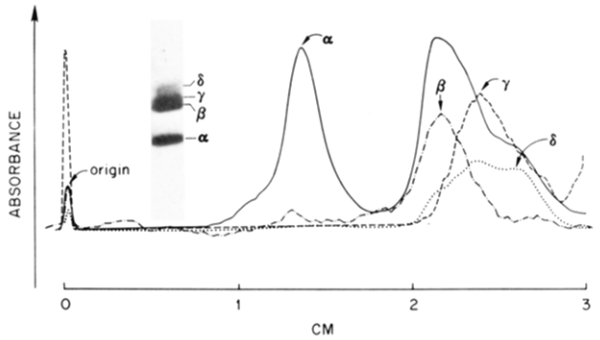

FIGURE 4: Reaction of bovine receptor subunits bound to DPT paper with antisera to receptor subunits from *Torpedo*. The inset shows the pattern of bands obtained when 100 ng of bovine receptor was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and then transferred to DPT paper. The transfer was visualized by incubating in a mixture of antisera raised against individual subunits of *Torpedo* receptor followed by a second layer of ^{125}I-labeled goat anti-rat antisera and then autoradiography for 18 h. The same paper was probed with each anti-*Torpedo* receptor subunit serum individually, and the autoradiographs were scanned. The traces obtained were aligned to a common reference point marked origin. (—) Mixture of all four antisubunit sera (as in the inset); (·····) antiserum to *Torpedo* β subunit; (- - -) antiserum to *Torpedo* γ subunit; (···) antiserum to *Torpedo* δ subunit.

that it corresponded structurally to α. In further confirmation, we had previously shown that this subunit was specifically affinity labeled with [^{3}H]MBTA [[4-(*N*-maleimido)benzyl]-trimethylammonium] (Lindstrom et al., 1979b). Antiserum to *Torpedo* β subunits specifically bound the bovine receptor subunit with a molecular weight similar to β, showing that this subunit was homologous to β. The γ and δ subunits of bovine receptor could not be clearly resolved by this technique. This is probably due in part to the cross-reaction between γ and δ which we have previously described (Lindstrom et al., 1979b; Tzartos & Lindstrom, 1980) and which is due to their extensive sequence homology (Raftery et al., 1980). Also, it may be that proteolytic nicking of δ reduced its apparent molecular weight somewhat, making it difficult to resolve from γ. In order to determine that bovine receptor did in fact contain distinct components corresponding to γ and δ, we used another technique to study the binding of antibodies to *Torpedo* receptor subunits to the subunits of bovine receptor. The subunits of bovine receptor were separated electrophoretically, and then the proteins on the gel were electrophoretically transferred to activated paper to which they were covalently bound. Then antisubunit sera were allowed to bind, labeled with ^{125}I-labeled anti-antibody, and visualized by autoradiography (Figure 4).

The complete pattern of immunoreactive material was visualized by using a mixture of sera raised against the individual subunits of *Torpedo* acetylcholine receptor (Figure 4, inset). The paper was then erased and probed sequentially with each antisubunit serum individually. The autoradiographs obtained were then scanned to give a graphic representation of the band pattern (Figure 4). The total pattern obtained with all four antisubunit sera gave a discrete band corresponding to the α subunit and a skewed slower moving band that was not well resolved into separate components. Inspection of the autoradiograph (Figure 4, inset) shows this to consist of two closely spaced bands followed by a weaker staining area. Probing with anti-*Torpedo* β-subunit serum visualized a single symmetrical peak which contributed to the leading edge of the skewed peak. Anti-*Torpedo* γ-subunit serum gave a peak with a trailing shoulder, the faster moving element being the predominant component. As shown above,

Table II: Binding of Intact Receptors from Muscle by Antisera to the Subunits of *Torpedo* Receptor[a]

	% of ^{125}I-labeled α-bungarotoxin-labeled receptors bound				
	bovine receptor preparation				
antiserum to	1	2	3	4	rat receptor
α	74	94	85	98	94
β	75	74	86	100	85
γ	35	43	54	73	35
δ	38	49	57	84	42

[a] In the assay mix, antisera were at 1×10^{-9} M, and receptor was at 2×10^{-10} M.

this serum cross-reacts between the γ and δ subunits of *Torpedo* AcChR. The same paper probed with antiserum to *Torpedo* δ, however, gave a double humped trace, but in this case, the peaks are equivalent in size, indicating in this case a predominant reaction with the δ subunit. The results of these experiments are complicated by three factors: (1) poor resolution of bovine receptor subunits due to the similar molecular weights of their β, γ, and δ subunits; (2) immunological cross-reaction of γ and δ subunits due to their previously described extensive structural homology; and (3) selective proteolytic degradation of γ and δ subunits (to be discussed below and in Table II) which reduces the amount of antibody bound and broadens the peaks. Nevertheless, these results suggest that bovine AcChR contains four subunits corresponding immunochemically to the four subunits of *Torpedo* AcChR.

We also studied immunoprecipitation of the ^{125}I-labeled subunits of receptor from rat muscle in order to determine whether the ^{125}I-labeled subunits previously described by Froehner et al. (1977b) and Nathanson & Hall (1979) in fact corresponded to the subunits of *Torpedo* receptor to whose apparent molecular weights they were similar (Figure 3). Subunits corresponding to α, β, γ, and δ were clearly identified. Antiserum to *Torpedo* γ reacted strongly with rat γ and weakly with rat δ, while antiserum to *Torpedo* δ reacted strongly with rat δ and weakly with rat γ. This shows that these subunits in rat receptor are extensively homologous, as they are in *Torpedo* and *Electrophorus* (Lindstrom et al., 1980b; Tzartos & Lindstrom, 1980). Froehner et al. (1977a) and Nathanson & Hall (1979) reported a second α subunit labeled with [^{3}H]MBTA that electrophoresed near β but had a peptide map similar to that of the lower molecular weight α. We found no immunochemical evidence of such a second α component near β (Figure 3). This is consistent with our observation of only a single [^{3}H]MBTA-labeled component in bovine receptor (Lindstrom et al., 1979a,b). These results strongly support the idea that acetylcholine receptors from mammalian muscle are composed of four kinds of subunits homologous to those composing receptor from electric organs. These results do not support the report of Shorr et al. (1978, 1981) that mammalian muscle consists only of α subunits but suggest that their report is the result of proteolytic artifacts of the type shown in Figure 2B.

Immunochemical Detection of Proteolysis. The fraction of ^{125}I-labeled α-bungarotoxin-labeled bovine acetylcholine receptors which could be precipitated by antisera to each of the subunits of *Torpedo* receptor decreased in preparations which appeared more proteolytically degraded by electrophoresis (Table II). This effect was greatest with antisera to γ and δ and least with antisera to α, presumably reflecting their relative sensitivity to proteolysis. In the worst of preparations, no γ or δ was visible on electrophoresis, and more

Table III: Binding of Monoclonal Antibodies to ^{125}I-Labeled α-Bungarotoxin-Labeled *Torpedo* Receptor before and after Proteolysis[a]

monoclonal antibody	specificity[b]	% of receptors bound by monoclonal antibody after indicated treatment of receptor: control	plus trypsin	plus bovine muscle homogenate supernatant for 24 h	plus bovine muscle homogenate supernatant for 144 h
6	α, main immunogenic region	103	95	98	100
3	α, site different from 6 or 8	97	5.7	77	5.5
8	α, site different from 3 or 6	95	7.3	84	2.8
11	β	90	5.9	84	0
7	γ and δ	89	5.6	78	8.1

[a] Receptor in alkaline-extracted membrane fragments was resuspended in buffer, trypsin (1 mol of trypsin/100 mol of receptor), or the supernatant of the initial homogenate of a bovine muscle receptor preparation for 24 or 144 h. Trypsinization was terminated after 24 h with a 100-fold excess of phenylmethanesulfonyl fluoride. Receptor was then solubilized in 2% Triton X-100, diluted to 1×10^{-9} M, and labeled with 2×10^{-9} M α-bungarotoxin. Monoclonal antibody at 2.5×10^{-8} M was added overnight to triplicate aliquots, and after precipitation by goat anti-rat IgG, the washed pellets were assayed for ^{125}I. Polyclonal antiserum to *Torpedo* receptor at 2.5×10^{-7} M was used as the 100% value. [b] Antibody specificities were reported in Tzartos & Lindstrom (1980) and Gullick et al. (1981).

than 60% of the receptors did not bind antisera to γ or δ. For the first time, this method permits a quantitative estimate of the extent to which muscle acetylcholine receptors are degraded by protease before purification. Incidentally, the observation that antiserum to any one of the receptor subunits can bind most or all of the toxin-labeled receptors indicates that all four kinds of muscle receptor subunits are intimately associated in complexes of uniform subunit composition, as are those of electric organ receptor (Lindstrom et al., 1980c).

Proteolysis of *Torpedo* receptor with papain did not have such a great effect on reaction with antisubunit sera as did proteolysis of bovine receptor (Lindstrom et al., 1980c), presumably because the antisera to *Torpedo* receptor subunits recognize many more antigenic determinants on *Torpedo* receptor than they do on bovine receptor. By testing the ability of single monoclonal antibodies to *Torpedo* receptor to bind ^{125}I-labeled α-bungarotoxin-labeled receptor, it was possible to demonstrate loss of some antigenic determinants due to proteolysis (Table III). Antibodies to the main immunogenic region (Tzartos & Lindstrom, 1980), like number 6, bound to all receptors despite proteolysis with trypsin or exposure to the supernatant of a crude homogenate of bovine muscle. Thus, the main immunogenic region is not sensitive to proteolysis, and one would expect that even extensively proteolyzed receptor would be a good immunogen. This is, in fact, observed (Bartfield & Fuchs, 1979). Antibodies to other parts of α (Gullick et al., 1981), like numbers 3 and 8, are directed at sites which are cleaved and/or dissociated from the receptor by proteolysis either by trypsin or by endogenous proteases in homogenates of bovine muscle. Other determinants on β, γ, and δ recognized by monoclonal antibodies 11 and 7 (Tzartos & Lindstrom, 1980) are similarly sensitive to proteolysis both by trypsin and by the endogenous proteases in bovine muscle. Note that this experiment demonstrates that there are soluble proteases in bovine homogenates which are not inhibited by the mixture of inhibitors used and which are capable of proteolyzing receptor in membranes. It also provides an assay for conveniently determining what are good protease inhibitors in this system.

Myasthenogenicity of Receptor from Mammalian Muscle. It is known that receptor from mammalian muscle is myasthenogenic (Lindstrom et al., 1976b; Granato et al., 1976). We found that receptor purified from bovine or human muscle by the protocol described was at least equal to and probably better than receptor from *Torpedo* or *Electrophorus* electric organs in its ability to induce experimental autoimmune myasthenia gravis in rats (Table IV). Rats immunized with receptor from bovine or human muscle produced a greater fraction of these antibodies (5–10%) which cross-reacted with receptor from rat muscle than did rats immunized with receptor from *Torpedo* or *Electrophorus* electric organs (1–2%). This confirms previous observations (Lindstrom et al., 1978a) which showed that receptors from mammalian species are, not surprisingly, more closely structurally related to one another than they are to receptors from fish electric organs. We have observed (data not shown) that bovine acetylcholine receptors cross-react extensively with antibodies to human acetylcholine receptor from myasthenia gravis patients, some up to 50%, whereas these antibodies cross-react negligibly with receptors from fish electric organs (Lindstrom et al., 1978a).

Discussion

There is now evidence that acetylcholine receptors from mammalian muscle are fundamentally structurally similar to acetylcholine receptors from fish electric organs. They have a similar specific activity, size, and shape (Table I, Figure 1; Lindstrom et al., 1982). They are composed of four kinds of subunits with similar but distinct molecular weights (Figures 2–4, Table II). There appear to be two α subunits per receptor (Lindstrom et al., 1982) which can be affinity labeled by cholinergic analogues (Lindstrom et al., 1979a,b; Wolosin et al., 1980). The α subunits contain a main immunogenic region (Tzartos et al., 1981, 1982) whose antigenicity is not affected

Table IV: Myasthenogenicity of Acetylcholine Receptors from Several Species in Lou Rats[a]

immunogen	dose (μg) day 0	dose (μg) day 30	sacrifice (day)	weakness (rats/total)	muscle receptor content (% control)	antibody-bound receptor (% total)	concn of serum antibody to rat receptor (nM)	concn of serum antibody to antigen (nM)
	0	0	35	0/3	100	0	0	0
Torpedo receptor	12	12	44	3/3	40	86	86	6800
Electrophorus receptor	50	30	37	3/3	28	97	127	6300
bovine receptor	15	5	35	6/6	30	94	230	3640
human receptor	2	1	35	3/3	38	75	46	500

[a] Rats greater than 8 weeks old were injected intradermally at several sites on the back with 0.2 mL of receptor emulsified in complete Freund's adjuvant.

by proteolysis (Tables II and III). Antisera to γ and δ subunits are highly cross-reactive, suggesting that γ and δ have greater structural homology than α and β (Figures 3 and 4). All these results are consistent with the idea that acetylcholine receptors from mammalian muscle have the same $\alpha_2\beta\gamma\delta$ subunit structure as receptors from fish electric organ tissue.

Proteolysis is a severe problem during purification of receptor from mammalian muscle. There are soluble proteases in muscle capable of proteolyzing membrane-bound receptor which are not easily inhibited (Table III). There is selective proteolysis of the higher molecular weight subunits so that the purified material may appear to consist only of α subunits on electrophoresis in gels containing sodium dodecyl sulfate (Figure 2) when in fact the proteolytic fragments of the other subunits and the size, shape, and function of the receptor remains basically unaltered (Figure 1; Lindstrom et al., 1980c). The greater proteolytic susceptibility of β, γ, and δ causes severely proteolyzed receptor to appear to consist only of α subunits. This no doubt accounts for the persistent reports by Shorr et al. (1978, 1981) that receptor from cat muscle is composed only of α subunits. This is very similar to early experiences in some laboratories with electric organ receptors. Other workers have reported muscle receptor preparations with two (Kemp et al., 1980; Stevenson et al., 1981), four (Boulter & Patrick, 1977), five (Nathanson & Hall, 1979), and six or seven bands (Froehner et al., 1977b), but the data did not discriminate which bands corresponded to receptor subunits. Previously, we have described four sets of antigenic determinants corresponding to α, β, γ, and δ in bovine and human receptors (Lindstrom et al., 1978b, 1979b). Here we show that these correspond to four distinct subunits in the purified receptors (Figures 3 and 4). It has been reported that receptor from rat muscle contained two kinds of α subunits with identical peptide maps but differing in molecular weight (Nathanson & Hall, 1979; Froehner et al., 1977a). We detect only one size of α subunit in receptors from bovine or rat muscle (Figures 3 and 4; Lindstrom et al., 1979b). We cannot confirm the report (Stevenson et al., 1981) that human receptor is not myasthenogenic (Table IV). Our results strongly suggest that the subunit structure of receptor from mammalian muscle does not differ fundamentally from that of receptors from fish electric organs and that such differences as have been reported are artifactual, primarily due to proteolysis.

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Neurotoxins from *Bungarus fasciatus* Venom: A Simple Fractionation and Separation of α- and β-Type Neurotoxins and Their Partial Characterization†

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ABSTRACT: The crude venom of *Bungarus fasciatus* has been fractionated by column chromatography, and the fractionation characteristics of three different resins have been compared. A minimum of 21 fractions can be identified under optimum conditions on Bio-Gel CM-30. Of the major fractions tested for neurotoxic activity, three showed postsynaptic (α) and four showed presynaptic (β) neurotoxic activity. The major protein component (an α-neurotoxin) has an isoleucyl N terminus and a calculated molecular weight of 14 200 based on amino acid composition. This main component contains 127 amino acid residues including 16 cysteine residues. A second less abundant α-neurotoxin of similar molecular weight has a methionyl N terminus. The isoelectric points of these toxins are 9.1 and 8.8, respectively. A third fraction also has postsynaptic (α) activity. Four other, very basic proteins have presynaptic (β) activity. Their apparent molecular weights are approximately 10 800 (two fractions), 13 100, and 19 100 as determined by sodium dodecyl sulfate gel electrophoresis. All α-toxin fractions showed a high tendency to aggregate in aqueous media; however, the presence of L-cysteine in molar excess prevents dimer formation. In the absence of L-cysteine, freeze/thaw cycling of aqueous solutions of α-toxins invariably leads to the formation of dimers which can be dissociated only under reducing conditions (β-mercaptoethanol). Conversely, only one out of four β-toxins examined tended to form dimers.

Low molecular weight neurotoxins have proven very useful in the study of biochemical aspects of neural impulse transmission (Changeux et al., 1970; Raftery et al., 1975; Heidmann & Changeux, 1978). In particular α-bungarotoxin (α-Bgt),[1] a protein which can be isolated from the crude venom of the elapid snake *Bungarus multicinctus*, was shown by Lee (1970, 1972) to block neural transmission by its action at the postsynaptic membrane of the neuromuscular junction where it binds selectively to the acetylcholine receptor (AChR).

α-Bgt was purified by Clark et al. (1972) and analyzed with respect to several characteristics including amino acid composition, isoelectric point, N-terminal amino acid, and electrophysiological properties. Isoleucine was identified as the N-terminal residue. More recently Hanley et al. (1977) reported the purification to homogeneity of nine neurotoxic proteins obtained from the venom of *Bungarus multicinctus*. These workers characterized the molecular weights, amino acid composition, and N-terminal residues of each protein. The purified fractions include α-Bgt, plus two other α-neurotoxins, and β-bungarotoxin, plus five other β-neurotoxins. Although α- and β-bungarotoxins have been studied extensively, much less is known about the activity or specificity of the other postsynaptic (α) and presynaptic (β) neurotoxins from *B. multicinctus*.

The availability of toxins other than α-Bgt with complementary pharmacological or biochemical activities would enhance the usefulness of toxin binding studies and has encouraged us to examine other venoms as sources of such toxins. Venom from *Bungarus fasciatus* was reported by Moore & Loy (1972) to contain an α-neurotoxin, but the toxin was not extensively characterized. *B. fasciatus* venom has, however,

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[1] Abbreviations: α-Bgt, α-bungarotoxin; AChR, acetylcholine receptor; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEMED, *N,N,N′,N′*-tetramethylethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; HSA, human serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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