Components of *Torpedo* Electric Organ and Muscle That Cause Aggregation of Acetylcholine Receptors on Cultured Muscle Cells

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ABSTRACT The synaptic portion of a muscle fiber's basal lamina sheath has molecules tightly bound to it that cause aggregation of acetylcholine receptors (AChRs) on regenerating myofibers. Since basal lamina and other extracellular matrix constituents are insoluble in isotonic saline and detergent solutions, insoluble detergent-extracted fractions of tissues receiving cholinergic input may provide an enriched source of the AChR-aggregating molecules for detailed characterization. Here we demonstrate that such an insoluble fraction from *Torpedo* electric organ, a tissue with a high concentration of cholinergic synapses, causes AChRs on cultured chick muscle cells to aggregate. We have partially characterized the insoluble fraction, examined the response of muscle cells to it, and devised ways of extracting the active components with a view toward purifying them and learning whether they are similar to those in the basal lamina at the neuromuscular junction.

The insoluble fraction from the electric organ was rich in extracellular matrix constituents; it contained structures resembling basal lamina sheaths and had a high density of collagen fibrils. It caused a 3- to 20-fold increase in the number of AChR clusters on cultured myotubes without significantly affecting the number or size of the myotubes. The increase was first seen 2–4 h after the fraction was added to cultures and it was maximal by 24 h. The AChR-aggregating effect was dose dependent and was due, at least in part, to lateral migration of AChRs present in the muscle cell plasma membrane at the time the fraction was applied. Activity was destroyed by heat and by trypsin. The active component(s) was extracted from the insoluble fraction with high ionic strength or pH 5.5 buffers. The extracts increased the number of AChR clusters on cultured myotubes without affecting the number or degradation rate of surface AChRs. Antiserum against the solubilized material blocked its effect on AChR distribution and bound to the active component.

Insoluble fractions of *Torpedo* muscle and liver did not cause AChR aggregation on cultured myotubes. However a low level of activity was detected in pH 5.5 extracts from the muscle fraction. The active component(s) in the muscle extract was immunoprecipitated by the antiserum against the material extracted from the electric organ insoluble fraction. This antiserum also bound to extracellular matrix in frog muscles, including the myofiber basal lamina sheath. Thus the insoluble fraction of *Torpedo* electric organ is rich in AChR-aggregating molecules that are also found in muscle and has components antigenically similar to those in myofiber basal lamina.

Each muscle fiber in skeletal muscles is ensheathed by basal lamina, a component of extracellular matrix that lies adjacent to the myofiber plasma membrane. The portion of the basal lamina in the synaptic cleft at the neuromuscular junction is highly specialized. For example, it contains acetylcholinesterase and other antigens that are far less concentrated in extra-

synaptic basal lamina (2, 18, 35, 50). Moreover, the synaptic basal lamina plays a role in regeneration of the neuromuscular junction. Damaged muscle fibers regenerate within the basal lamina sheaths of the original muscle fibers and regenerating axons grow precisely to original synaptic sites on the sheaths. Molecules tightly adherent to the synaptic basal lamina cause the formation of active zones in regenerating motor nerve terminals and the aggregation of acetylcholine receptors (AChRs)[1] in the subsynaptic plasma membrane of regenerating myofibers (8, 51).

The experiments presented here are part of a study aimed at identifying the basal lamina molecules that cause the aggregation of AChRs at synaptic sites in regenerating muscle. Our approach is to prepare fractions from tissues that contain cholinergic synapses, screen for those fractions that are both enriched for extracellular matrix constituents and cause aggregation of AChRs on cultured myotubes, purify the active components, and determine by immunological techniques whether or not the active components are similar to those in the synaptic basal lamina at the neuromuscular junction.

Several years ago Meezan et al. (34) demonstrated that basal lamina was the major constituent of insoluble detergent-extracted fractions from kidney and blood vessels. As described in the experiments presented here (see also reference 47), we applied to cultured myotubes a skeletal muscle fraction prepared in a similar way, but we did not observe AChR-aggregating activity. We therefore turned to an insoluble fraction from the electric organ of *Torpedo californica*. Electrocytes of torpedine rays are similar to muscle fibers in that they are ensheathed by basal lamina and AChRs are aggregated opposite nerve terminals (53). Moreover, the electric organ has a much greater concentration of synapses than muscle, which has made it a useful preparation for isolating and characterizing other molecular components of cholinergic synapses, including the acetylcholine receptor and acetylcholinesterase (30, 44). We found that the insoluble fraction from the electric organ was rich in extracellular matrix components and contained a proteinaceous molecule (or molecules) that aggregated AChRs on cultured chick myotubes. We have partially purified and characterized this molecule. These studies have been reported in brief elsewhere (22, 37, 47, 58), and our observation that an insoluble fraction from electric organ contains AChR-aggregating molecules has now been confirmed by others (13). Here we present a detailed account of the preparation and characterization of the insoluble fraction of the electric organ, the extraction of the AChR-aggregating molecule(s) from it, our method of assaying its activity, and its effect on cultured chick myotubes. We also demonstrate that *Torpedo* muscle contains similar molecules.

MATERIALS AND METHODS

Preparation of Insoluble Fractions: Large *Torpedo californica* (40–70 cm across, supplied by Pacific Bio-Marine Laboratories Inc., Venice, CA, and J. R. Scientific, Woodland, CA) were pithed and the electric organs, liver, and muscles of the back and tail were quickly removed. The tissues were cut into pieces ~3 cm diam and frozen immediately in liquid nitrogen. They were stored up to several months at −60°C. (On several occasions, we have used electric organs frozen by the supplier. In no case did these preparations provide as much AChR-aggregating activity as those we froze ourselves.)

The insoluble fraction of the electric organ was prepared by a sequence of homogenizations at 4°C in buffers designed to eliminate most of the soluble and membrane-associated components; this procedure was based on the method of Meezan et al. (34). Initially, 2,000 g of frozen pieces of electric organ

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[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor.

were smashed with a hammer. The tissue was then homogenized in a Waring blender (2 min) in 3,200 ml buffered saline containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-Cl, pH 7.5, and 0.1 mg/ml phenylmethylsulfonyl fluoride (dissolved in 8 ml 100% ethanol and added just before homogenization). This "crude homogenate," which was used for some of the experiments described below, was centrifuged (17,700 g; 30 min) and the supernatant was discarded. The pellet and a loosely associated fibrous layer that sometimes appeared above the liquid were homogenized in the blender (2 min) in 1,200–1,800 ml of buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-Cl, pH 7.5, and 0.1 mg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged (17,700 g; 30 min) and the supernatant was discarded. The pellet was homogenized in the blender (2 min) with 400–1,000 ml of 10 mM Tris-Cl, pH 7.5, and then centrifuged (30,000 g; 30 min). The pellet was resuspended in 10 mM Tris-Cl, pH 7.5 buffer containing 3% (vol/vol) Triton X-100 with either a Teflon-glass homogenizer or a Waring blender (2 min) to a final volume of 500–1,000 ml, then stirred for 30 min. The suspension was centrifuged (30,100 g; 30 min), and the pellet was reextracted with the buffered Triton solution, then centrifuged (30,100 g; 30 min). The pellet was twice homogenized and centrifuged (30,100 g; 30 min) in 500 ml of saline buffer containing 150 mM NaCl and 10 mM Tris-Cl, pH 7.5. Before use the material was resuspended in the same saline buffer to a final volume of 200 ml by homogenization in a Teflon-glass homogenizer followed by extrusion through a 16-gauge needle (once) and a 20-gauge needle (twice). The suspension, referred to as the "insoluble fraction," was stored either frozen (−20°C) or refrigerated (4°C) with 0.02% NaN₃.

Insoluble fractions of *Torpedo* liver and muscle were prepared by the same procedure. The insoluble fractions from these tissues contained 10 times more protein per wet weight of tissue than corresponding fractions of electric organ.

Electron Microscopy of the Insoluble Fraction of the Electric Organ: The insoluble fraction was suspended in 0.8% glutaraldehyde in 0.09 M phosphate buffer (pH 7.1) for 1 h. It was then treated with 1% osmium textroxide in phosphate buffer (1 h), dehydrated in ethanol, and embedded in a mixture of Epon and Araldite. Before each solution change the insoluble fraction was pelleted by centrifugation (10,000 g) in a microfuge for 2–3 s. Thin sections were stained with uranyl acetate and lead citrate (56).

Extraction of AChR-aggregating Activity from Insoluble Fractions: Insoluble fractions of electric organ, muscle, and liver were centrifuged (30,100 g; 30 min) and were resuspended in buffer containing 50 mM NaCl, 0.01% NaN₃, and 50 mM sodium citrate, pH 5.5, to a final volume of 200–350 ml using a Teflon-glass homogenizer or Waring blender (2 min) followed by extrusion three times through a 20-gauge needle. This mixture was stirred overnight at 4°C, then centrifuged (48,400 g; 30 min). The clear supernatant is referred to as the "pH 5.5 citrate extract." We routinely concentrated the extract 10- to 15-fold by vacuum dialysis against two changes (2 liters each) of buffer containing 500 mM NaCl, 5% (vol/vol) glycerol, 0.01% NaN₃, 50 mM sodium citrate, pH 5.5. The dialyzed solution was centrifuged (48,400 g; 30 min) to remove a small amount of precipitate which formed. Citrate extraction solubilized a similar percentage of protein from insoluble fractions of electric organ, muscle, and liver.

For some experiments the insoluble fractions were extracted with buffer containing 2 M MgCl₂ and 10 mM Tris-Cl, pH 7.5, instead of the citrate buffer, following the same protocol ("MgCl₂ extract").

Chick Myotube Cultures: Myotube cultures were prepared from hind limb muscles of 11- to 12-d White Leghorn chick embryos by the method of Fischbach (19) with the following minor modifications: the cells were exposed to 0.02% trypsin for 15 min and the medium in which they were plated contained 2% chick embryo extract. About 3 × 10⁵ cells were plated on 35-mm plastic tissue culture dishes coated with collagen (either calf skin collagen from Calbiochem-Behring Corp., La Jolla, CA or rat-tail tendon collagen [5]). Culture medium consisted of Eagle's minimal essential medium (Gibco Laboratories, Grand Island, New York) supplemented with 10% (vol/vol) horse serum (Gibco), 2% (vol/vol) chick embryo extract (prepared from 11-d chick embryos with an equal volume of Puck's saline G [43]), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml Fungizone (Gibco). Medium was replaced every 2 d. After the initial 2 d in culture, cells were exposed to 10⁻⁵ M cytosine arabinoside for 2 d to eliminate rapidly dividing cells. Myotubes were used to assay AChR-aggregating activity after a total of 4–8 d in culture. Unless otherwise specified, the volume of culture medium was 1.5 ml.

Quantitation of AChR Aggregation: In order to assay the effect of various fractions on AChR distribution, we incubated myotube cultures with 6 × 10⁻⁸ M rhodamine-α-bungarotoxin (45) in culture medium for 1 h at 37°C. The cultures were then washed two times (1 ml each) with Puck's saline G at room temperature and fixed in chilled 95% ethanol for 10 min at −20°C. Glass coverslips were affixed to the surface of the cultures with a drop of glycerol. AChR clusters were counted at × 250 magnification with a Leitz microscope equipped with fluorescence optics. For each culture, counts from 10–12 evenly

spaced fields were averaged, composing ~0.4% of the surface area of the bottom of the culture dish. We counted as a cluster any distinct fluorescent patch whose longest axis was >2 μm. In some platings, numerous relatively small clusters (<½ μm diam) freckled the myotubes in both treated and untreated cultures; they are not included in our results.

AChR Number and Degradation Rate: AChRs on cultured myotubes were labeled with 2 × 10^{-8} M ^{125}I-α-bungarotoxin (16 μCi/μg; New England Nuclear, Boston, MA) in culture medium for 1 h at 37°C. Unbound toxin was removed by washing three times in Puck's saline G. To quantitate AChR number, we scraped cultures into 0.5 ml of 1 N NaOH and determined the radioactivity with a gamma counter. To examine the rate of AChR degradation, we returned labeled cultures to culture medium after washing and put them back in the incubator; at different times the cells were washed once and the amount of radioactivity remaining associated with the cells was determined.

Size and Number of Myotubes per Culture: Camera lucida drawings were made of the outlines of all myotubes in 12 microscope fields from each culture. The cultures were viewed with bright-field optics at the same magnification used to count AChR clusters (× 250). The area of the myotubes was measured with a HIPad Digitizer (Houston Instruments, Austin, TX) coupled with an Apple II computer (Apple Computer, Inc., Cupertino, CA). Measurements from the 12 fields were averaged. The number of myotubes per field was determined from the same drawings.

Protein and DNA Content of Cultures: Cultures were scraped into 0.5 ml of 1 N NaOH and the protein concentration was determined by the method of Bradford (6) with BSA as a standard. Other cultures were scraped into 0.2 ml of 0.5 M perchloric acid and the method of Burton (9) was used to determine DNA concentration.

Biochemical Analysis of Tissue Fractions: Protein concentration was assayed by the method of Bradford (6) using bovine serum albumin as a standard. Hydroxyproline was determined by the method of Huszar et al. (25) with either hydroxyproline or calf skin collagen (Calbiochem-Behring Corp.) as a standard. Uronic acid was measured as described by Bitter and Muir (4) with heparin from porcine intestinal mucosa (Sigma Chemical Co., St. Louis, MO., Grade I) as a standard; we took 52% of the weight of heparin to be uronic acid (29).

To determine the number of AChRs in the crude homogenate or insoluble fraction, we incubated aliquots in 1.0 ml of 10^{-8} M ^{125}I-α-bungarotoxin in Puck's saline G with 1 mg/ml BSA (P-BSA) for 2 h at room temperature. The mixture was centrifuged (10,000 g; 5 min) and then washed three times by resuspension in 1 ml P-BSA followed by centrifugation. The supernatant was removed and the radioactivity remaining in the pellet was determined with a gamma counter. Nonspecific binding was determined by incubation in the presence of a 100-fold excess of native α-bungarotoxin. The number of AChRs in *Torpedo* electric organ determined in this way was comparable to published values (44).

Heat, Trypsin, and DNase Treatments of the Electric Organ Insoluble Fraction and Extract: The insoluble fraction (520 μg; 31 U) or pH 5.5 citrate extract (4 μg; 36 U) of the electric organ was exposed to one of the following treatments and then assayed in triplicate: (a) The material was put in a glass test tube in a boiling water bath for 5 min. (b) Material was exposed to 1 U (5 μg) trypsin (Worthington Biochemical Corp., Freehold, NJ) for 30 min at 37°C, followed by 70 μg soybean trypsin inhibitor (Worthington Biochemical Corp.) for an additional 20 min at 37°C. Trypsin premixed with trypsin inhibitor had no effect on AChR-aggregating activity. (c) Material was exposed to 1.8 U (1 μg) DNase I (Sigma Chemical Co.) for 90 min at 37°C. To test the effectiveness of the enzyme treatment, insoluble material was pelleted (10,000 g; 5 min) and analyzed for DNA; >70% of the DNA had been removed from this fraction.

Immunization: Two rabbits were immunized with several 1–3-mg injections of the MgCl$_2$ extract of the electric organ insoluble fraction that had been emulsified with an equal volume of adjuvant (Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for subsequent injections). The injections were made at intervals of 1–3 mo; rabbits were bled 11–28 d after each injection. Blocking antibodies were first detected in serum of both rabbits after two injections. The experiments reported here utilize antiserum obtained after the fourth injection from one rabbit; it was heat inactivated (56°C; 30 min) before use.

Immunohistochemistry: The thin (two to three muscle fibers thick) cutaneous pectoris muscles from adult frogs were pinned out in Sylgard-coated Petri dishes and incubated 1 h in rabbit antiserum raised against the MgCl$_2$ extract. The antiserum was diluted 1:100 with frog Ringer's solution. Muscles were then rinsed with several changes of Ringer's solution (1 h), incubated in horseradish peroxidase-coupled goat anti-rabbit IgG (1 h), rinsed with Ringer's solution (1 h), fixed in 1% glutaraldehyde in 0.09 M phosphate buffer, pH 6.9

(0.5 h), treated with 0.05% 3,3-diaminobenzidine and 0.02% H$_2$O$_2$ in cacodylate buffer, briefly rinsed with cacodylate buffer, fixed further and stained with 1% osmium tetroxide in phosphate buffer (3 h), dehydrated in ethanol, and embedded in a mixture of Epon and Araldite.

Immunoprecipitation of AChR-aggregating Activity: Sepharose beads activated with cyanogen bromide were coupled with goat antirabbit γ-globulin antibodies (Antibodies Inc., Davis, CA). The resultant bead suspension contained 15 mg protein/ml. The antibody-coupled beads were incubated for 30 min in 150 mM NaCl, 10 mM sodium phosphate, pH 7.5, containing 1 mg/ml BSA and 10% (vol/vol) goat serum (PBS-BSA-GS), then mixed (2:1 vol/vol) with rabbit antiserum or normal rabbit serum for 2–3 h at room temperature. The beads were washed ten times (1 ml each) with PBS-BSA-GS to remove unbound antibodies and mixed with AChR-aggregating extract in PBS-BSA-GS for 2 h at room temperature. The beads were removed by centrifugation (10,000 g; 1 min) and the supernatant was applied to myotube cultures to determine the AChR-aggregating activity left in solution.

RESULTS

Aggregation of AChRs by the Insoluble Fraction of the Electric Organ

When the insoluble fraction was added to chick muscle cultures, it settled on both the myotubes and the exposed collagen substrate as widely scattered particles and particle aggregates which ranged in length from a fraction of a micrometer to 30 μm (Fig. 1). To determine the effect of the insoluble material on the distribution of AChRs in the myofiber plasma membrane, we treated cultures with rhodamine-conjugated α-bungarotoxin and examined them by fluorescence microscopy. The insoluble material caused a 3–20-fold increase in the mean number of AChR clusters per microscope field (Fig. 2). The increase was detected as early as 2 h after application of the insoluble fraction and reached a plateau by 24 h (Fig. 3a). Compared with controls, there was no difference in the number of myotubes per microscope field or the proportion of the field covered by myotubes (Table I). Thus the increase in AChR clusters per field in cultures treated with the insoluble fraction must have been due to an increase in the number of clusters per unit area of myotube surface.

The AChR clusters in treated and control cultures were similar in shape and size (Fig. 2); however, the clusters on myotubes exposed to the insoluble fraction generally fluoresced more intensely than those in control cultures, indicating a higher density of AChRs. Together these findings mean that the increase in clusters on treated myotubes did not result simply from fragmentation of the clusters present prior to treatment but rather from the accumulation of receptors into aggregates.

In seven experiments, the AChRs on myotubes were labeled with rhodamine-α-bungarotoxin before the addition of the insoluble fraction. Under these conditions, the fraction still caused a 2–11-fold increase over controls in the number of fluorescent AChR clusters. Thus, the induced clusters are formed, at least in part, by the lateral migration of AChRs in the myofiber membrane.

Dose-Response Relationship

The response of myofibers to the insoluble material was dose dependent. Fig. 4a shows that as the amount of the insoluble fraction added to the cultures was increased, the number of AChR clusters on the myotube surface also increased and then reached a plateau. The magnitude of the response to saturating amounts of extract was similar from dish to dish for cultures plated at the same time, but varied widely from plating to plating. However, there was little
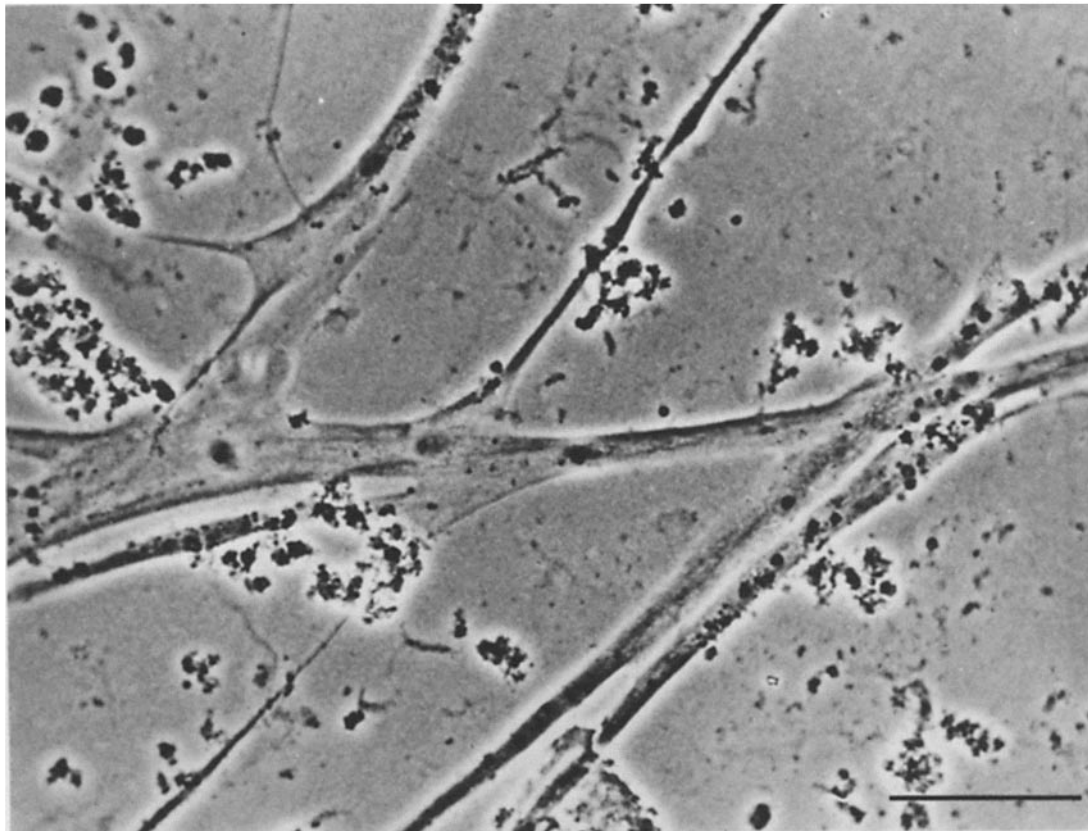
FIGURE 1 The insoluble fraction from the *Torpedo* electric organ, when added to chick muscle cultures, settles on the myofibers as widely scattered particles. Phase-contrast micrograph. Bar, 50 μm.

TABLE I

Lack of Effect of Electric Organ Fractions on Myotube Number, Size, and DNA and Protein Content

	Myotubes per microscope field	Microscope field covered by myotubes	DNA	Protein
		%	μg/culture	
a. Control	7.2 ± 0.4	31.9 ± 1.8	ND	ND
Insoluble fraction	7.1 ± 0.6*	26.2 ± 1.3‡	ND	ND
b. Control	10.0 ± 0.2	22.7 ± 0.4	2.0 ± 0.2	160 ± 4
pH 5.5 citrate extract	10.2 ± 0.2*	23.5 ± 0.8*	2.2 ± 0.1*	158 ± 7*

To determine effects on myotube number and size, cultures were exposed overnight to (a) the insoluble fraction (20 μg; 2.5 U) or (b) the pH 5.5 citrate extract (3 μg; 11 U) from *Torpedo* electric organ. The insoluble and soluble fractions caused increases in the number of AChR aggregates of 10.5- and 7.3-fold, respectively. In another experiment cultures were exposed to 4 U of pH 5.5 citrate extract, and DNA and protein content were determined. Each entry is the mean ± SEM derived from three cultures; a and b are from different myotube platings. ND, not determined.
* $P > 0.4$, two-sided Student's t test.
‡ $P > 0.066$, two-sided Student's t test.

variability from plating to plating in the dose dependence of the response. By defining one unit of activity as the amount of material needed to cause a half-maximal response, the amount of AChR-aggregating activity could be measured reproducibly from experiment to experiment.

Characteristics of the Insoluble Fraction of the Electric Organ

Properties of the crude homogenate and the insoluble fraction are given in Table II. Compared with the initial homogenate, the insoluble fraction was enriched fivefold in AChR-aggregating activity. It was enriched threefold for hydroxyproline, an amino acid found almost exclusively in collagen (15,

41). If hydroxyproline composes $^1/_{10}$ (wt/wt) of the amino acid residues in *Torpedo* collagen, as in the collagen of mammals (10, 16, 23), then 20% of the protein in the insoluble fraction was collagen. Electron microscopy of the fraction (Fig. 5) revealed that it contained numerous collagen fibrils and material resembling basal lamina (11, 53) which is known to contain Type IV collagen (46, 55). (In our initial studies [22, 37, 58] we prepared the insoluble fraction by a slightly different method than the one described here, which probably accounts for the dearth of collagen fibrils reported for those preparations.) The fraction had few AChRs (0.2% of those in the crude homogenate) as expected since detergent treatment had been used to solubilize membrane components. To assess the distribution of proteoglycans, which are constituents both
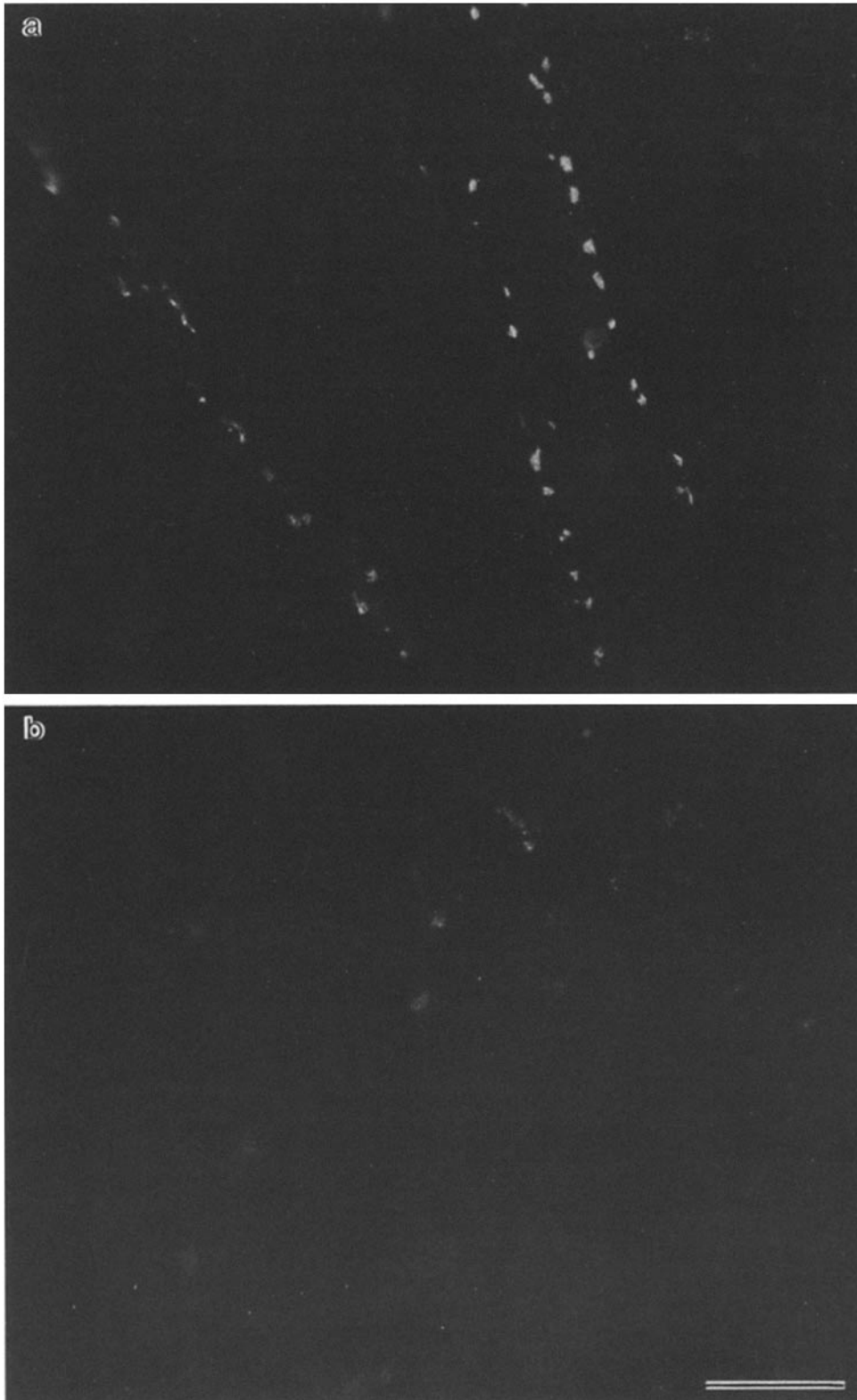
FIGURE 2 The insoluble fraction from the electric organ causes the aggregation of AChRs on cultured chick myotubes. Fluorescence micrographs of muscle cultures in which AChRs were labeled with rhodamine-conjugated α-bungarotoxin. (a) The insoluble fraction was added 16 h before labeling the AChRs. The two myotubes in the field exhibit numerous intensely fluorescent AChR patches. (b) Control culture, processed in parallel with that of a but without the insoluble fraction. The two myotubes have few patches, which fluoresce less intensely than those in a. Bar, 50 μm.

of cell membranes and extracellular matrix (28, 38), we measured uronic acid. Only 0.5% of the uronic acid in the crude homogenate was present in the insoluble fraction, indicating that most proteoglycans had been removed. The fraction contained DNA (82 μg/mg protein), as found in similar fractions from other sources (34); incubation of the fraction with DNase had no effect on receptor-aggregating activity (Table III).

The ability of the insoluble fraction to aggregate AChRs into clusters was destroyed by heating it to 100°C for 5 min or by treating it with trypsin (Table III). Thus, the active components are heat labile and probably proteinaceous.

Insoluble Fractions of Liver and Muscle

We made insoluble fractions from Torpedo liver and muscle, tissues with a far lower density of synapses than the
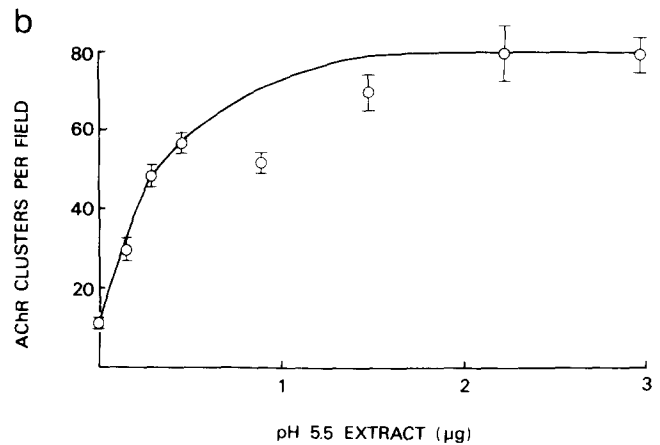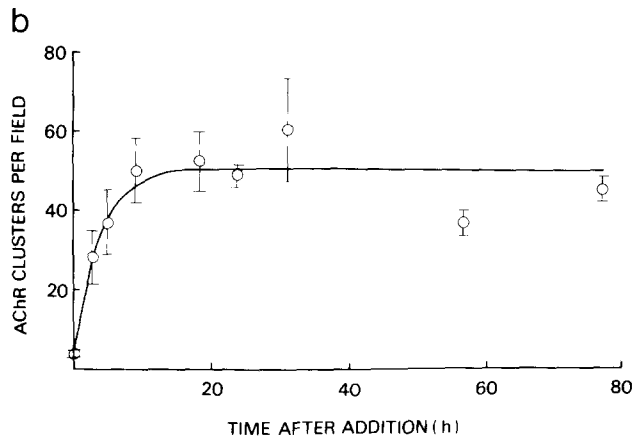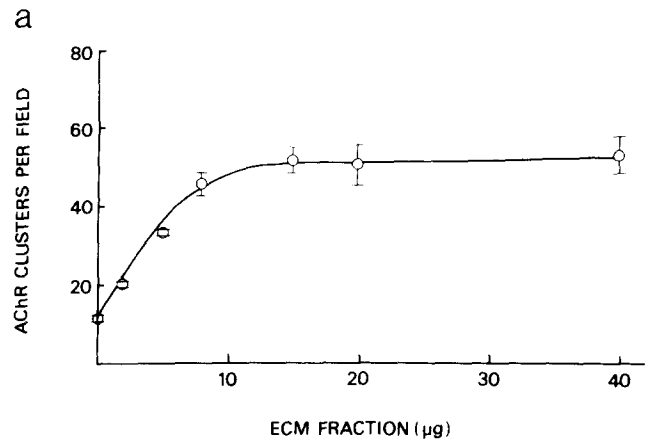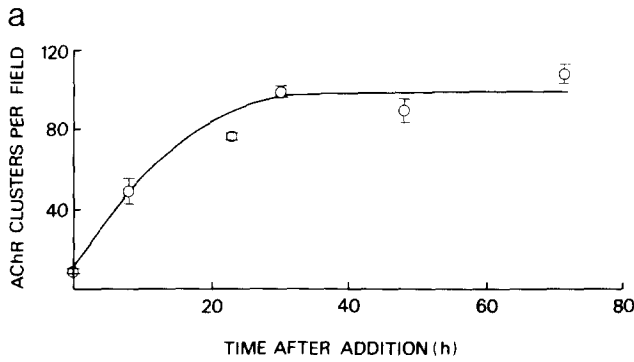
FIGURE 3   Time course of the increase in number of AChR aggregates per microscope field on myotubes treated with (a) the insoluble fraction or (b) the pH 5.5 citrate extract of Torpedo electric organ. Cultures were treated for the times indicated; incubations were staggered so that they were all terminated together. In each case a saturating amount of AChR-aggregating activity was used (see Fig. 4 and text): 70 μg (5 U) of insoluble fraction, 2 μg (5 U) of pH 5.5 citrate extract. Each point represents mean ± SEM from three cultures. In other experiments, cultures were assayed after shorter periods of time than those shown here; each type of fraction was found to cause an increase in the number of receptor aggregates as early as 2 h.

FIGURE 4   The response of myotubes to fractions of electric organ is dose dependent. Myotube cultures were treated for 15 h with different amounts of (a) the insoluble (ECM) fraction or (b) the pH 5.5 citrate extract, and the number of AChR clusters was then determined. Values represent micrograms of protein per culture dish; a and b are from different myotube platings. Each point is the mean ± SEM from three cultures. One unit of activity, defined as the amount of material needed to evoke a half-maximal response, is 4.0 μg for the insoluble fraction and 0.27 μg for the pH 5.5 citrate extract.

electric organ, following the protocol used for the electric organ. As shown in Table IV, the insoluble material did not cause AChR aggregation on cultured myofibers even when the amount of protein added was 100 times greater than that required to see a response with the electric organ insoluble fraction. Thus, myotubes respond selectively to the insoluble fraction from the synapse-rich electric organ.

Extraction of AChR-aggregating Activity from the Insoluble Fraction of the Electric Organ

The molecules that aggregate AChRs are firmly bound to components of the insoluble fraction of the electric organ. For example, they remained associated with the insoluble material during the repeated saline and detergent extractions in the fractionation procedure. Moreover, when stored for several months at 4°C, most of the activity still remained associated with the insoluble material. However, as shown in Table V, active molecules could be extracted from the insol-

TABLE II
Characterization of the Electric Organ Crude Homogenate and Insoluble Fraction

Component	Crude homogenate	Insoluble fraction	Enrichment
Protein	30.4 g	1.7 g	(1)
AChRs	1.9 μmol	0.0031 μmol	0.03
Hydroxyproline	124 mg	19 mg	2.8
Uronic acid	1040 mg	5.9 mg	0.1
AChR-aggregating activity	1.0×10^6 U*	2.9×10^5 U	5.2

AChR-aggregating activity was determined from dose-response curves as described in the text. The crude homogenate was prepared from 2.1 kg (wet weight) of Torpedo electric organ. Enrichment of components in the insoluble fraction is normalized to protein content.
* Prelabeled cultures were used to determine activity.

uble fraction with acidic buffer or 2 M salt, treatments shown to solubilize extracellular matrix components such as collagen (55), acetylcholinesterase (30), and proteoglycans (24). The
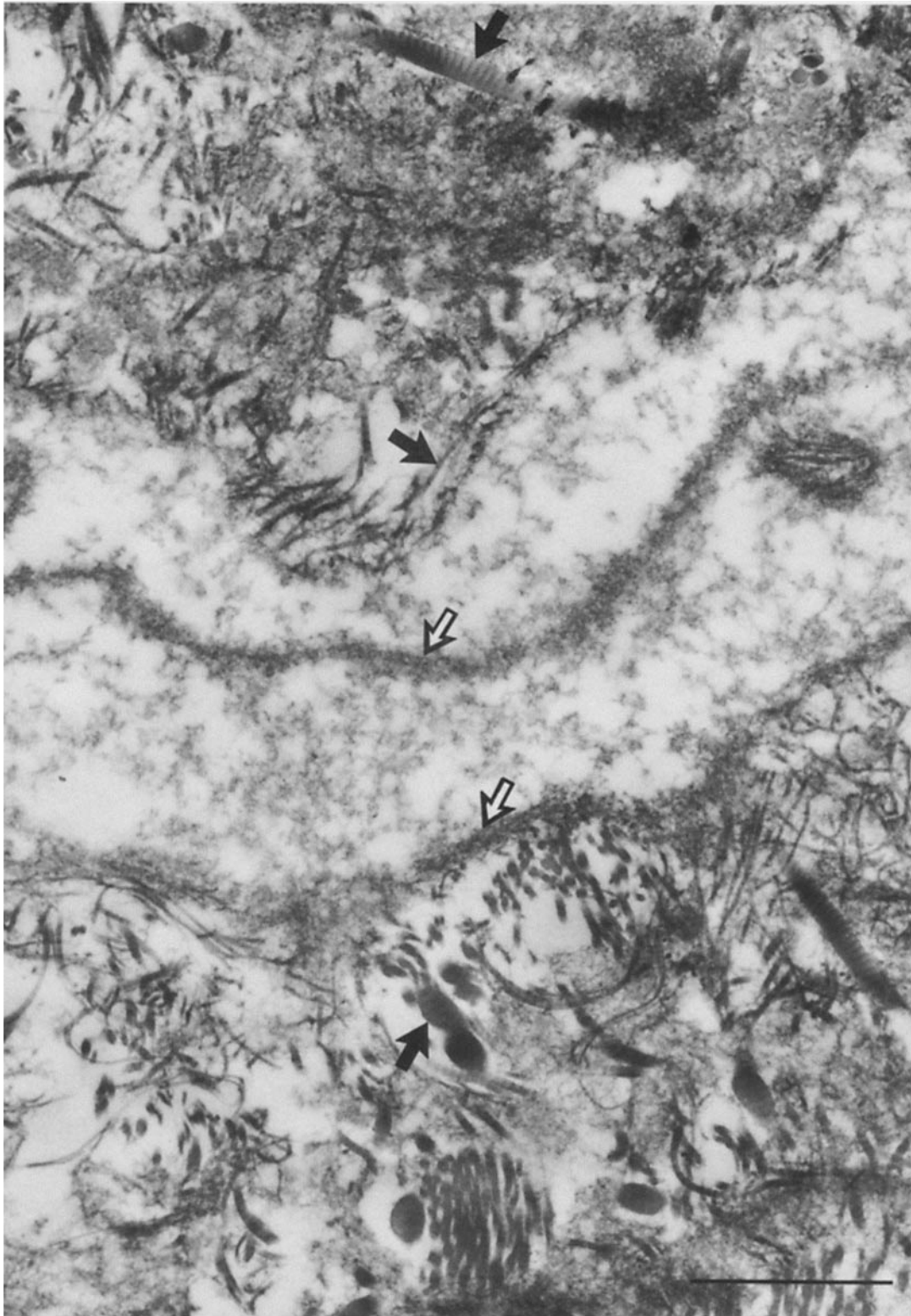
FIGURE 5 Electron micrograph of the insoluble fraction of the electric organ. Closed arrows, collagen fibrils; open arrows, basal lamina-like material. Bar, 1 μm.

activity in the extracts, as in the insoluble fraction, was destroyed by boiling and by treatment with trypsin (Table III).

The response of cultured myotubes to the solubilized molecules was similar to that for the insoluble fraction. The increase in receptor clusters evoked by solubilized material arose at least in part by lateral migration of AChRs. The solubilized material did not affect myotube number or size (Table I). The time course of AChR aggregation after exposure to extracts was similar to that after addition of the insoluble fraction; in both cases AChR aggregation was detectable as early as 2 h (Fig. 3). The dose-response curve for solubilized

Table III

Sensitivity of Electric Organ AChR-aggregating Activity to Heat, Trypsin, and DNase

	Activity remaining	
Treatment	Insoluble fraction	pH 5.5 citrate extract
	%	
Boiling	0.9 ± 0.3	0.4 ± 1.1
Trypsin	0.4 ± 0.1	0.3 ± 0.8
DNase	112.6 ± 5.8	ND

Each entry represents the mean ± SEM from three cultures, expressed as a percentage of the activity (12 U) initially present in the fractions. ND, not determined.

Table IV

Insoluble Fractions from Muscle and Liver Did Not Cause AChR Aggregation

Source of insoluble fraction	AChR clusters per field
Control (no fraction added)	2.8 ± 0.8
Electric organ	
70 μg	64.0 ± 3.7
7 μg	18.4 ± 1.2
Muscle	
500 μg	—*
200 μg	3.8 ± 0.8
Liver	
1,000 μg	1.9 ± 0.7
400 μg	2.5 ± 0.2

Insoluble fractions were prepared from Torpedo muscle and liver by the procedure used for the electric organ. (The amounts shown represent protein added per dish.) Each entry represents the mean ± SEM from three cultures.
* Myotubes were disrupted by this concentration of the insoluble material from muscle.

material was similar in shape to that of the insoluble fraction (Fig. 4), and when both fractions were tested on cultures from the same plating, the maximum increase in the number of AChR clusters was similar (data not shown). Comparison of half-maximal values revealed that the pH 5.5 citrate extract had a 15-fold greater specific activity than the insoluble fraction (Fig. 4).

We examined further the similarity between the active components of the insoluble fraction and extracts by raising an antiserum against the 2 M $MgCl_2$ extract and determining its effect on the activity of the pH 5.5 citrate extract and the insoluble fraction. The antiserum, at a dilution of 1:300, completely blocked AChR aggregation caused by one unit of the $MgCl_2$ extract. At the same dilution it also blocked AChR aggregation by the insoluble fraction and the pH 5.5 citrate extract (Table VI). To learn whether the antiserum contained antibodies directed against the active molecules in the extracts and not simply against myotube components involved in AChR aggregation, we bound immunoglobulins from the antiserum to goat anti-rabbit γ-globulin that had been covalently coupled to Sepharose beads. As shown in Table VII, incubating the electric organ pH 5.5 citrate extract with the antiserum-coated beads completely removed the AChR-aggregating activity. Moreover, the following results indicate that the antiserum did not contain antibodies that directly interfered with the myofiber's ability to aggregate AChRs (see Table VIII): (a) AChR aggregation was not affected when

cultures were incubated with antiserum, briefly rinsed, and then exposed to the pH 5.5 citrate extract; (b) the ability of the antiserum to block AChR aggregation was not significantly reduced by absorbing the serum with muscle cultures; and (c) inhibition of AChR aggregation by the antiserum was reversed by adding excess electric organ extract. Altogether, our findings lead to the conclusions that the antiserum against the $MgCl_2$ extract contained antibodies against all AChR-aggregating molecules in both the $MgCl_2$ and pH 5.5 citrate extracts and that the active molecules in the extracts were antigenically similar to all active components in the insoluble fraction.

Specificity of Action

Consistent with the observation that the insoluble fraction and the extracts active in aggregating AChRs did not change the number or size of myotubes, we found that the pH 5.5 citrate extract had no effect on the DNA or protein content of the cultures (Table I). We examined further the influence of the extract on myotubes to learn if it affected specific aspects of AChR metabolism. As shown in Figure 6 a, during the period when receptor aggregation was occurring there was no change in the number of surface AChRs on cultured myotubes, as measured by binding of ^{125}I-α-bungarotoxin. Over the next 2 d there was only a slight increase in the

Table V

Extraction of AChR-aggregating Activity from the Insoluble Fraction

Extraction condition	Activity extracted	Protein extracted
	%	
150 mM NaCl, pH 7.5	9.9 ± 4.0	0.9 ± 0.3
2 M NaCl, pH 7.5	44.7 ± 4.9	4.4 ± 0.3
2 M $MgCl_2$, pH 7.5	21.3 ± 4.3	3.6 ± 2.1
150 mM NaCl, pH 5.5	30.5 ± 5.6	1.5 ± 0.5
2 M NaCl, pH 5.5	49.7 ± 3.8	5.0 ± 0.8

The insoluble fraction from the electric organ (600 μg protein) was incubated in 0.2 ml of the indicated solution (50 mM Tris-Cl was used to buffer at pH 7.5 and 50 mM Na-citrate at pH 5.5) for 16 h at 4°C with constant mixing. The material was centrifuged (10,000 g; 5 min), the supernatant was dialyzed against 150 mM NaCl, 5% glycerol, 10 mM Tris-Cl, pH 7.5, and the number of units of AChR-aggregating activity in each extract was determined from dose-response curves. Recovery of activity and protein is calculated as the percentage of that initially present in the insoluble fraction and is expressed as the mean ± SEM for three or four separate experiments, each involving triplicate cultures.

Table VI

Effect of Antiserum on AChR Aggregation

Torpedo electric organ fraction	Antiserum	AChR-aggregating activity remaining
	μl	%
a. Insoluble	0	100
	1	43 ± 10
	5	0 ± 3
b. pH 5.5 citrate extract	0	100
	1	60 ± 14
	5	8 ± 8

The indicated amount of rabbit antiserum against the $MgCl_2$ extract of electric organ was mixed (1 h; 37°C) with 1 U of AChR-aggregating activity as (a) the insoluble fraction (4 μg) or (b) the pH 5.5 citrate extract (1.2 μg), and the mixture was then added to a culture (final volume 1.5 ml). Each entry is the mean ± SEM for three cultures. Normal rabbit serum (100 μl per culture) had no effect on AChR-aggregating activity of either fraction.

TABLE VII

Immunoprecipitation of AChR-aggregating Activity

Condition	Activity remaining in supernatant
	%
Electric organ extract (control)	100
Electric organ extract + antiserum-beads	−1.1 ± 2.3
Electric organ extract + normal rabbit serum-beads	103 ± 11.3
Muscle extract (control)	100
Muscle extract + antiserum-beads	0.0 ± 6.2

Normal rabbit serum or antiserum against the $MgCl_2$ extract of electric organ (0.1 ml) was mixed with a suspension of Sepharose beads coupled with goat antibodies against rabbit γ-globulins (0.1 ml). After washing, the beads were mixed with pH 5.5 citrate extracts of electric organ (6.5 U; 3.4 µg) or muscle (2 U; 600 µg) in a total volume of 0.7 ml. The beads were centrifuged and 0.2 ml of the supernatant was assayed on each of three cultures. To determine if the inhibition of activity was due to antibodies released from beads and transferred to the myotube cultures, supernatant from antiserum-beads was mixed with electric organ extract and assayed: 86.1 ± 3.4% of AChR-aggregating activity remained, indicating that little, if any, antibody was released from the beads under these conditions. Values given are means ± SEM for three cultures.

TABLE VIII

Inhibition of AChR Aggregation by Antiserum Is Not Due to a Direct Effect on Myotubes

Assay conditions	AChR-aggregating activity
	%
Extract	100
Extract + antiserum	2 ± 1
Extract on antiserum pretreated cultures	96 ± 13
Extract + absorbed antiserum	1 ± 2
10X extract + antiserum	31 ± 7

pH 5.5 citrate extract (5 U) and/or rabbit antiserum against the $MgCl_2$ extract of electric organ (15 µl) were applied to myotube cultures in a final volume of 1.0 ml. Pretreated cultures were exposed to 15 µl of antiserum in 1.0-ml culture medium for 1 h and then washed twice in culture medium before applying extract. Absorbed antiserum had been incubated with three myotube cultures for 1–2 h each in 1.0-ml culture medium prior to mixing with the extract. Each entry is the mean ± SEM of triplicate cultures.

number of receptors on the myotubes. Similarly, in cultures treated with extracts, the rate of AChR degradation was not significantly different from control (Fig. 6b).

AChR-aggregating Molecules Can Be Extracted from Muscle

Although the insoluble fraction from muscle did not affect receptor distribution, our ability to detect activity in this fraction was limited by the amount of material that could be added to the cultures. Having found that the AChR-aggregating activity of the electric organ insoluble fraction could be extracted with pH 5.5 citrate-buffered saline and concentrated, we treated the insoluble fraction from *Torpedo* muscle in the same way and analyzed the concentrated extract for AChR-aggregating activity. The muscle extract caused detectable AChR-aggregation at 20 µg per culture, a protein concentration 100–1,000-fold higher than that necessary to see a response using electric organ extracts. Similar extracts of the insoluble fraction from liver were without effect, even at a protein concentration of 1,700 µg per culture, an 80-fold higher concentration than that of the muscle extract. The maximal effect was the same for extracts from both electric

organ and muscle. The AChR-aggregating activity solubilized from muscle could be blocked and precipitated by the antiserum prepared against the $MgCl_2$ extract of electric organ

LENGTH OF EXPOSURE TO EXTRACT (h)
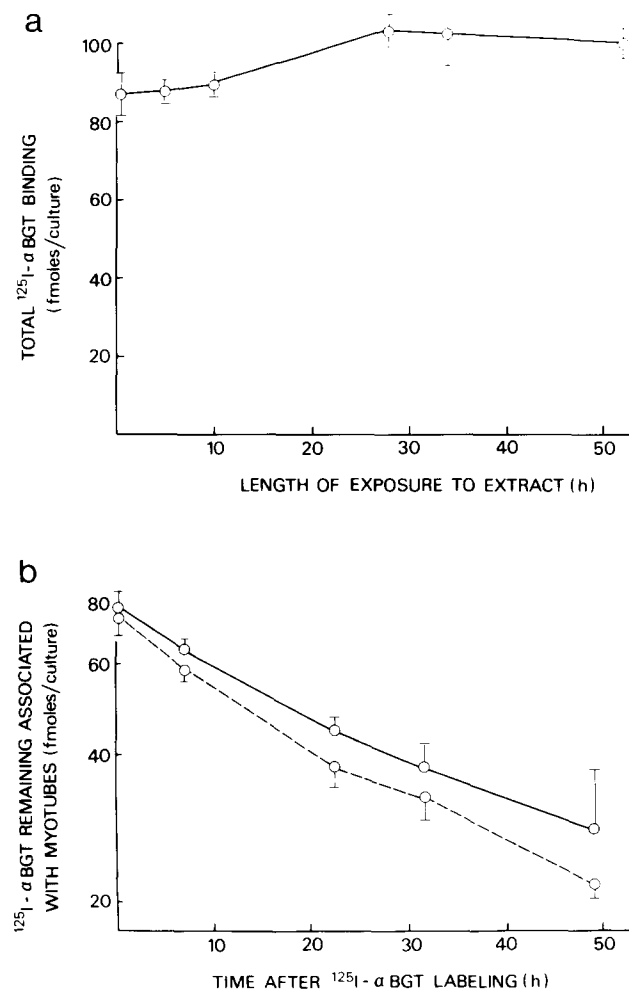
TIME AFTER ^{125}I-α BGT LABELING (h)

FIGURE 6 Effect of pH 5.5 citrate extract of the electric organ on AChR number and degradation rate. (a) After the indicated times of incubation with pH 5.5 citrate extract (0.9 µg; 2 U), myotube cultures were labeled with ^{125}I-α-bungarotoxin (BGT). They were then washed and the amount of toxin that remained bound to the myofibers was determined as described in Materials and Methods. The first point represents exposure to extract for 10 min. Each point equals the mean ± SEM (n = 3). During the first 10 h of exposure, when AChR aggregation was occurring, there was no significant change in ^{125}I-α-bungarotoxin binding (P>0.4, two-sided Student's t test); at later times there was a slight increase (P<0.05, one-sided Student's t test). (b) Myotube cultures were labeled with ^{125}I-α-bungarotoxin then washed to remove unbound toxin. At the indicated times cultures were washed and the amount of toxin associated with the cells was determined (the first point represents 0.25 h). The solid line is based on data from cultures that were exposed to pH 5.5 citrate extract (0.9 µg; 2 U) for 24 h prior to ^{125}I-α-bungarotoxin labeling, and throughout the experimental period. The dotted line is data from control cultures. At each time analyzed there is no significant difference between the mean number of AChRs remaining on control and pH 5.5 citrate extract-treated cultures (P>0.25, two-sided Student's t test). If it is assumed that the loss of AChRs follows a simple exponential (14) and the data are fit by the method of least squares, then there is no significant difference between the slopes of the regression lines (P>0.25, two-sided Student's t test) for control and extract-treated cultures. In each case the half-time of degradation is ~30 h. Each point represents the mean ± SEM from three cultures.
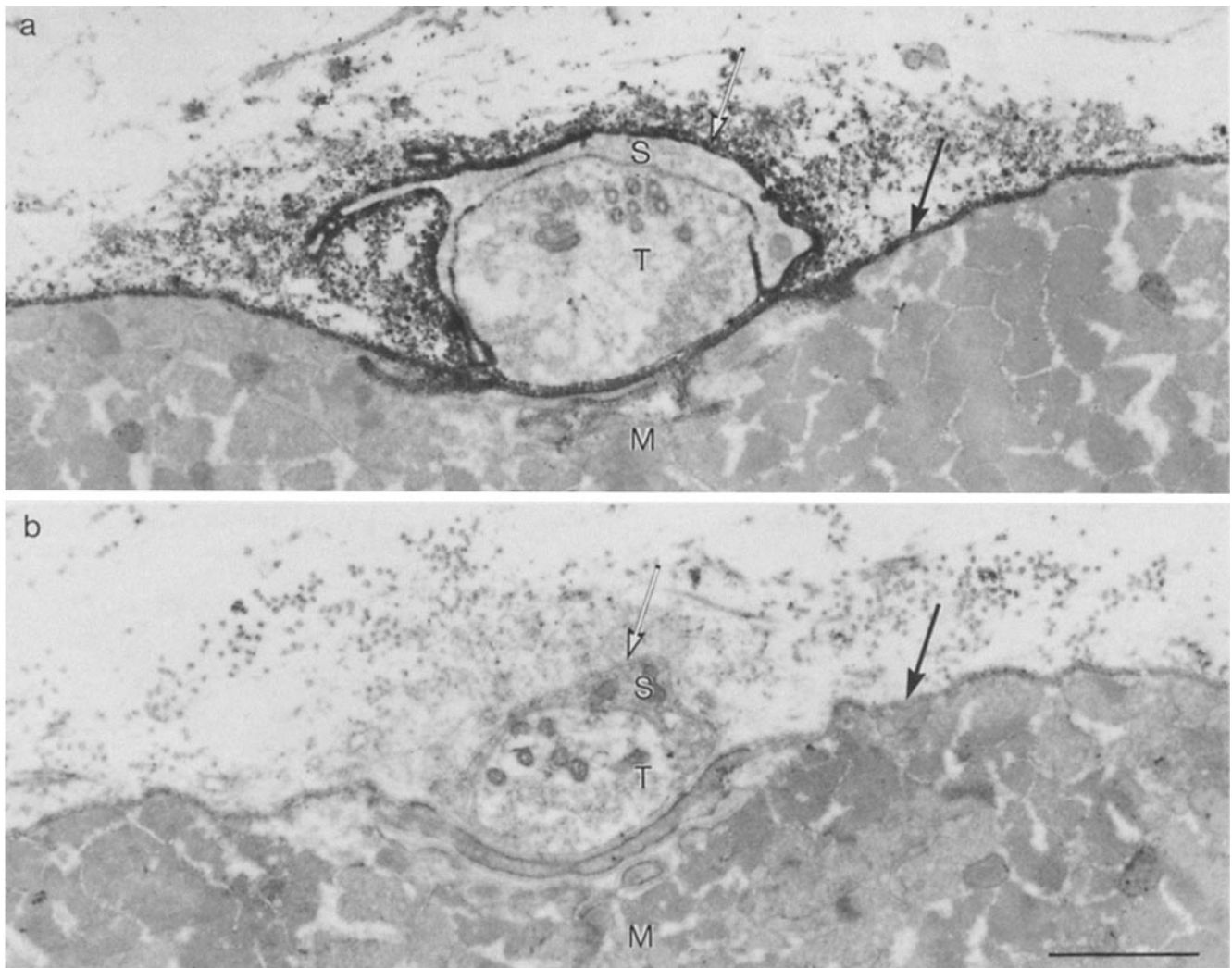
FIGURE 7 Antiserum against the MgCl₂ extract from the *Torpedo* electric organ binds to basal lamina sheaths in frog muscle. (a and b) Electron micrographs of cross sections through neuromuscular junctions. *T,* axon terminal; *M,* muscle fiber; *S,* Schwann cell; filled arrow, muscle fiber basal lamina; open arrow, Schwann cell basal lamina. The muscle in a was incubated with the antiserum and the sites of bound antibodies were stained by indirect immunoperoxidase histochemistry. The muscle in b was treated in parallel with a but with preimmune serum. Antibodies in the antiserum bound to the myofiber basal lamina, Schwann cell basal lamina, and to other extracellular matrix material near the junction. Bar, 1 μm.

insoluble fraction (Table VII). Thus, *Torpedo* muscle contains AChR-aggregating molecules functionally and antigenically similar to those present in the electric organ.

Antiserum against an Electric Organ Extract Binds to Muscle Basal Lamina

One of our aims is to determine, using immunological techniques, whether the AChR-aggregating molecules extracted from *Torpedo* electric organ are similar to the active components of the myofiber basal lamina at neuromuscular junctions. To learn whether our extracts of the insoluble fraction of the electric organ contained any components similar to those in the extracellular matrix of neuromuscular junctions, we treated frog muscles with the antiserum against the MgCl₂ extract. Frog muscles were used because the simple and orderly pattern of innervation makes the junctions easy to find in histological preparations. Bound antibodies were made visible by incubating the preparations with horseradish peroxidase-coupled goat anti-rabbit γ-globulin and staining

for horseradish peroxidase. Fig. 7*a* shows a cross section of a neuromuscular junction from such a preparation. The extracellular matrix, including the myofiber basal lamina in both synaptic and extrasynaptic regions, is far more heavily stained than in the accompanying section from a muscle treated with preimmune rabbit serum (Fig. 7*b*). The antigenic components are tightly adherent to the basal lamina; experiments we have presented elsewhere (22, 37, 58) demonstrate that the antiserum binds to frog myofiber basal lamina weeks after all cellular components of the neuromuscular junction—myofiber, nerve terminal, and Schwann cell—have been removed from the muscle.

DISCUSSION

In vivo experiments show that even after muscles have been frozen or crushed and phagocytes have removed the myofibers including their plasma membranes, the synaptic basal lamina is still capable of aggregating AChRs on regenerating myofibers (36). Thus, one might expect that the AChR-aggregating

molecules would remain attached to synaptic basal lamina during the sequence of homogenization and centrifugation in isotonic saline and detergent solutions that we used to prepare insoluble fractions from *Torpedo* electric organ and muscle. Our principal finding is that, in fact, an insoluble fraction of the synapse-rich electric organ causes AChRs to aggregate on cultured myofibers. Moreover, we show that antigenically and functionally similar molecules can be extracted from an insoluble fraction of muscle. The active molecules from both muscle and electric organ are extracted by methods that solubilize components of extracellular matrix. Together these findings focus attention on these extracted proteins as candidates for the AChR-aggregating molecules at neuromuscular synapses and provide encouragement for purifying and characterizing them further.

There is no doubt that our insoluble fraction from electric organ contained extracellular matrix material. Electron microscopy revealed a high density of collagen fibrils in the fraction, and an assay for hydroxyproline, which is found almost exclusively in collagen (15, 41), indicated that collagen composed ~20% of the protein in the fraction, a threefold enrichment over the crude homogenate. We have not yet examined the fraction using assays for specific molecules known to be in high concentration in basal lamina, such as Type IV collagen and laminin (20, 46, 49, 59). However, the insoluble fraction contained structures resembling basal lamina and the antiserum we prepared against material extracted from the insoluble fraction bound to the basal lamina of frog muscles, making it very likely that the insoluble fraction contained basal lamina constituents.

Only 20–50% of the activity in the insoluble fraction of the electric organ was recovered in our extracts of it. Thus it is conceivable that the insoluble fraction contained several sorts of molecules capable of aggregating AChRs in vitro, and those specifically associated with synaptic basal lamina were not removed by our extraction procedures. We show, however, that the active components in the extracted material were similar to those in the insoluble fraction with respect to the time course of receptor aggregation, the magnitude of the response, the shape of the dose-response curve, and sensitivity to trypsin and heat. These findings coupled with our observation that antiserum raised against the extracted material blocks all activity in the insoluble fraction provide strong evidence that all active components of the insoluble fraction were represented in the extracts.

Extracts that cause AChR aggregation on cultured myotubes have been obtained by others from a variety of neuron-rich tissues, including fetal rat brain (3, 27, 42, 48), embryonic pig brain (39), embryonic chick brain and spinal cord (7, 26), cultured nerve cells (3, 12, 52), chick sciatic nerve (33), and *Torpedo* electric lobe and organ (13). It remains to be learned whether or not any or all of the active molecules in these extracts cause AChR aggregation at synapses in vivo, and if they share structural similarities as well as functional ones. (The importance of such problems is emphasized by the fact that clearly nonphysiological agents, such as latex beads [40], can cause AChR aggregation on myotubes.) Initial characterization indicates that there are some similarities and differences between these molecules and those we have extracted from the electric organ, which we summarize as follows.

(*a*) Selectivity of action. The active molecules extracted from rat brain by Podleski, Salpeter, and their colleages (42, 48) and from conditioned media of neuronal cell lines initially by Christian et al. (12), like those we have extracted from *Torpedo* electric organ, have a selective effect on myotubes; they cause the aggregation of AChRs without influencing the number or (for the rat brain extract) the degradation rate of surface AChRs. On the other hand, Fischbach and co-workers (7, 13, 26) have prepared soluble fractions of embryonic chick brain and spinal cord and of *Torpedo* electric lobe and organ that not only cause AChR aggregation but also increase the number of surface AChRs. An active molecule purified from the sciatic nerve by Markelonis et al. (31), which was initially called sciatin but is now known to be identical with the serum protein transferrin (32), likewise not only causes the aggregation of AChRs but also increases the number of surface AChRs and the protein content of muscle cultures (33).

(*b*) Molecular weight. The active molecules extracted from rat brain (42) and neuronal cell lines (3) have apparent molecular weights >50,000. Gel filtration studies on the molecules we have extracted from the *Torpedo* electric organ, as reported elsewhere (37), revealed that they also have an apparent molecular weight >50,000; ~80% of the activity is found in the range of 50,000–100,000 mol wt. Transferrin from chick sciatic nerve is ~80,000 mol wt (31). On the other hand, the active factor(s) in the soluble fractions of *Torpedo* electric organ and lobe and chick brain studied by Fischbach's group appears to have a molecular weight of <5,000 (7, 13).

(*c*) Specific activity. We have purified the active molecules we extracted from the *Torpedo* electric organ more than 1,000-fold using gel filtration and ion-exchange chromatography (22, 37). Our most purified fraction causes AChR aggregation at a protein concentration of 10^{-10} M. Chick transferrin has thus far been shown to aggregate AChRs on chick myotubes only at a 100-fold greater concentration (33). Although the electric organ molecules have an apparent molecular weight near that of transferrin, the marked differences in specific activity and selectivity of action, as described above, show that the active component in the electric organ extract is not transferrin.

(*d*) Source. Except for transferrin, which has been shown by immunohistochemistry to be associated with neurons and Schwann cells (32) and laminin (see below), the in vivo location of AChR-aggregating molecules studied by us and others is not known. However, the AChR-aggregating molecule we are studying (as well as laminin) is the only one derived from fractions that are insoluble in isotonic saline and detergent solutions, which would be expected if this molecule were associated with basal lamina in vivo.

Since our initial reports appeared on the role of basal lamina in aggregating AChRs at regenerating neuromuscular junctions and the isolation of an active insoluble fraction from the electric organ (8, 47), Vogel et al. (57) have applied purified components of extracellular matrix to cultured myotubes to learn if they can cause AChR aggregation. Fibronectin, collagen types IV and V, and a heparan sulfate proteoglycan had no effect. On the other hand, laminin, found both in synaptic and extrasynaptic regions of myofiber basal lamina (49), as well as in the basal lamina of tissues other than nerve and muscle (20), caused a small increase in the number of AChR aggregates. The concentration of purified laminin required to obtain a response from rat myotubes was 3 nM. We estimate that our partially purified extract of the electric organ causes AChR aggregation on chick myotubes at a concentration of 0.1 nM (22, 37). Moreover, the apparent molecular weight of the electric organ AChR aggregating

molecules (50,000–100,000) is much smaller than that of laminin (950,000 [54]) or its subunits (220,000 and 440,000 [17]).

It is not yet known how basal lamina molecules cause AChR aggregation on regenerating myofibers. However, it is known that the density of AChRs aggregated by synaptic basal lamina in regenerating muscles can be the same as at normal neuromuscular junctions (36). This raises the possibility that AChR-aggregating molecules in synaptic basal lamina are related to those that mediate the aggregation of AChRs caused by nerve terminals at developing neuromuscular junctions. If this were the case, it would be reasonable to expect that AChR aggregation on cultured myofibers would occur by the same mechanism when induced by synaptic basal lamina as when induced by nerve terminals. We demonstrate here that the active molecules we have extracted from the electric organ, like those from other sources (12, 48, 52), cause AChR aggregation at least in part by lateral migration of AChRs in the plasma membrane of cultured muscle, just as nerve terminals do (1, 21).

Blocking and immunoabsorption assays with our antiserum against an extract of the insoluble fraction from electric organ clearly show that the active molecules are highly antigenic. Attempts to make specific immunological reagents against the active molecules are now in progress (37). Such reagents will greatly aid in determining whether or not the molecules we have extracted from *Torpedo* muscle and electric organ are structurally similar to those in synaptic basal lamina, and, if so, in learning how the basal lamina molecules are regulated and how they cause AChR aggregation.

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