Identification of Agrin, A Synaptic Organizing Protein from *Torpedo* Electric Organ

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Abstract. Extracts of the electric organ of *Torpedo californica* contain a proteinaceous factor that causes the formation of patches on cultured myotubes at which acetylcholine receptors (AChR), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE) are concentrated. Results of previous experiments indicate that this factor is similar to the molecules in the synaptic basal lamina that direct the aggregation of AChR and AChE at regenerating neuromuscular junctions in vivo. We have purified the active components in the extracts 9,000-fold. mAbs against four different epitopes on the AChR/AChE/BuChE–aggregating molecules each immunoprecipitated four polypeptides from electric organ extracts, with molecular masses of 150, 135, 95, and 70 kD. Gel filtration chromatography of electric organ extracts revealed two peaks of AChR/AChE/BuChE–aggregating activity; one comigrated with the 150-kD polypeptide, the other with the 95-kD polypeptide. The 135- and 70-kD polypeptides did not cause AChR/AChE/BuChE aggregation. Based on these molecular characteristics and on the pattern of staining seen in sections of muscle labeled with the mAbs, we conclude that the electric organ–aggregating factor is distinct from previously identified molecules, and we have named it "agrin."

FINDINGS from studies conducted in this laboratory have led to the hypothesis that a proteinaceous factor extracted from the synapse-rich electric organ of *Torpedo californica* (15, 16, 29, 34, 45) resembles the extracellular synaptic organizing molecules in the basal lamina at the neuromuscular junction that direct the formation of acetylcholine receptor (AChR)[1] and acetylcholinesterase (AChE) aggregates on regenerating muscle fibers (4, 25). For example, the factor is found in basal lamina–containing fractions of the electric organ; it induces the formation of patches on cultured muscle fibers that contain a high concentration of AChRs and AChE; low levels of a similar factor are found in extracts of muscle; and mAbs against the factor recognize molecules concentrated in the synaptic cleft of neuromuscular junctions in vivo. Patches induced by the electric organ factor also include butyrylcholinesterase (BuChE), a third component of the postsynaptic apparatus at the neuromuscular junction (43). It may well be that the factor is capable of triggering the formation of a complete postsynaptic apparatus (43, 45).

We have now made additional mAbs against the electric organ factor. As described in the accompanying report, we have shown that these antibodies bind to components of the synaptic basal lamina at the neuromuscular junction, providing further evidence that the electric organ factor resembles the AChR/AChE–aggregating molecules in the synaptic basal lamina. The aims of the studies described here were to use these mAbs to identify the electric organ factor. On the basis of its biochemical properties and the distribution of anti–agrin staining in muscles, we conclude that the electric organ AChR/AChE/BuChE–aggregating factor differs from previously identified AChR-aggregating molecules and identified components of the synaptic cleft at the neuromuscular junction. Therefore, we name the AChR/AChE/BuChE–aggregating factor in our extracts of electric organ "agrin" (from the Greek "ageirein," to assemble). Brief accounts of some of these results have been reported previously (22, 29, 40).

Materials and Methods

Acetylcholine Receptor-, Acetylcholinesterase-, and Butyrylcholinesterase-aggregating Activity

The assay for AChR-aggregating activity has been described in detail (16). In brief, 4–7-d-old cultures of chick myotubes were exposed to test solutions for 12–18 h, incubated for 1 h at 37°C with 10^{-8} M rhodamine-α-bungarotoxin to label AChRs, rinsed with Puck's saline, fixed for 10 min at −20°C in 95% ethanol, mounted in glycerol, and examined by fluorescence microscopy. The level of AChR-aggregating activity in a sample was determined by counting the mean number of AChR aggregates per field (or per myotube segment). AChR-aggregating activity is expressed in units; 1 U of AChR-

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1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; AChR, acetylcholine receptor; BuChE, butyrylcholinesterase.

aggregating activity is the amount of material needed to achieve a half-maximal increase in the number of aggregates.

AChE- and BuChE-aggregating activities were assayed as previously described (43). In brief, cultures were treated with extracts and labeled with rhodamine-α-bungarotoxin as described above, fixed with 1% paraformaldehyde, and labeled with a mAb against chick AChE (35) followed by fluorescein-conjugated second antibody, or stained histochemically for sites of BuChE activity. Cultures were examined by phase and fluorescence microscopy and the levels of AChE- and BuChE-aggregating activity were determined by the same method as for AChR-aggregating activity.

Protein Assay

Protein concentration was determined by the method of Bradford (11), using BSA as the standard.

Extraction and Purification

An insoluble fraction enriched in extracellular matrix components was prepared by extracting homogenates of electric organ with isotonic saline and detergent at pH 7.5 (16). The insoluble fraction was resuspended and homogenized in bicarbonate buffer (0.2 M sodium bicarbonate, 5% glycerol, 0.02% sodium azide, pH 9.0 [300 ml/kg of electric organ]), stirred overnight at 4°C, rehomogenized, and centrifuged (30,000 g; 20 min). In some experiments, the pellet was reextracted in the same manner and the supernatants were combined.

The supernatant was applied to a 9-ml column of Cibacron Blue 3GA-agarose (Affi-gel Blue Gel, 100-200 mesh [Bio-Rad Laboratories, Richmond, CA]) equilibrated in bicarbonate buffer. The column was washed with 20 ml of bicarbonate buffer and eluted with a 40-ml linear gradient of 0-3 M NaCl in bicarbonate buffer, followed by 20 ml of 3 M NaCl. In some experiments, the supernatant was loaded onto a 25-ml column of Cibacron Blue 3GA-agarose, washed with 10 column volumes of bicarbonate buffer, and eluted with 30 ml of 1.5 M NaCl in bicarbonate buffer. Fractions with the highest specific activity were pooled.

An aliquot of the pooled fractions was applied to either a 540-ml column (110 × 2.5 cm) of Bio-Gel A 1.5m agarose (Bio-Rad Laboratories) or a 94-ml (120 × 1 cm) column of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated and eluted with bicarbonate buffer containing 0.5 M NaCl. Columns were calibrated with Blue Dextran (>1000 kD = void volume [Pharmacia Fine Chemicals]), β-galactosidase (540 kD [Sigma Chemical Co., St. Louis, MO]), glucose 6-phosphate dehydrogenase (104 kD [Sigma Chemical Co.]), BSA (66 kD [Sigma Chemical Co.]), ovalbumin (43 kD [Sigma Chemical Co.], and cytochrome c (12.4 kD [Sigma Chemical Co.]). Fractions from the Bio-Gel A 1.5m agarose column with highest specific activity were pooled and concentrated 5-10-fold by vacuum dialysis against a phosphate buffer (10 mM sodium phosphate, 5% glycerol, 0.02% sodium azide, pH 8.0).

The concentrated gel filtration pool was applied to a 1-ml column of DEAE cellulose (Whatman Inc., Clifton, NJ) equilibrated in phosphate buffer. After washing with 5 column volumes of phosphate buffer, the bound material was eluted with a 10-ml linear gradient of 0-250 mM NaCl in phosphate buffer.

Radiolabeling of Proteins

Proteins eluted from the ion exchange column were covalently labeled with radioactive iodine by a solid-phase method (23). Briefly, 1-50 μg of protein (corresponding to 200 U of activity) were reacted with 1 mCi of ^{125}I-sodium iodide (Amersham Corp., Arlington Heights, IL) in the presence of 2 μg of immobilized IODO-GEN (Pierce Chemical Co., Rockford, IL). After 20-30 min free iodide was separated from the protein by gel filtration on a small column of Sephadex G-25 (PD-10 column [Pharmacia Fine Chemicals]).

Immunoprecipitation

Aliquots of ^{125}I-labeled ion exchange pool (∼10^7 cpm) were incubated with 0.05 ml of either a test hybridoma supernatant, a control hybridoma supernatant, or normal mouse serum in PBS-GS (150 mM NaCl, 5% glycerol, 0.5% NP-40 [Sigma Chemical Co.], 10% goat serum, in 50 mM sodium phosphate buffer, pH 7.0). After 2 h at room temperature, 10 μl of Sepharose beads (Sepharose CL-4B-200 [Sigma Chemical Co.]) to which goat anti-mouse IgG (Antibodies, Inc., Davis, CA) had been covalently coupled (16) were added. After an additional 1-h incubation at room temperature the

beads were washed three times in 1 ml PBS-GS, then boiled for 5 min in 50 μl of SDS sample buffer. Material eluted by this treatment was analyzed by SDS-PAGE (18) and autoradiography. Parallel experiments demonstrated that under these conditions mAbs against the AChR/AChE/BuChE-aggregating factor removed virtually all aggregating activity from solution.

Immunoblotting

Samples were subjected to electrophoresis on an SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose paper after the method of Towbin et al. (41). The paper was washed four times for 15 min each time in PBSA-T (150 mM sodium chloride, 0.02% sodium azide, 0.05% Tween-20, in 50 mM sodium phosphate buffer, pH 7.5), blocked for 2-3 h in PBSA-T containing 0.1% goat serum and 3% BSA, and then incubated overnight in undiluted hybridoma supernatant, or a 1:1,000 dilution of ascites or normal mouse IgG in hybridoma growth medium containing 0.05% Tween-20. The paper was washed four times for 15 min each time in PBSA-T containing goat serum, incubated for 4 h with alkaline phosphatase-coupled goat anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:1,000 in PBSA-T containing 0.1% normal goat serum, rinsed four times for 15 min each time in PBSA-T, and reacted for alkaline phosphatase activity. This procedure allowed detection of <200 U of AChR-aggregating activity.

Immunoaffinity Purification

Normal mouse IgG or mAbs from hybridoma supernatants or ascites fluid were covalently coupled to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) by the method of Schneider et al. (39). Aliquots of the Cibacron pool (3,000 U) were incubated overnight at 4°C with gentle agitation with 10-50 μl of mAb or normal mouse IgG beads. The beads were washed sequentially in 100 vol each of 10 mM triethanolamine buffer, pH 8.2, containing 150 mM NaCl, 0.5% NP-40, and 5% glycerol (wash buffer); triethanolamine buffer containing 1 M NaCl, 0.5% NP-40, and 5% glycerol; wash buffer; triethanolamine buffer containing 150 mM NaCl, 5% glycerol, and 0.5% sodium deoxycholate; and wash buffer. After washing, the beads were eluted with 2 × 0.5-ml of 50 mM triethylamine, pH 11.5, containing 150 mM NaCl, 5% glycerol, and 0.5% NP-40. The eluates were pooled and proteins were precipitated with 20% TCA, washed three times with 0.6 ml acetone at 4°C, separated by SDS-PAGE, and visualized by silver staining (27). Under these conditions, >75% of the activity present in the extract bound to the antibody beads.

Results

As a first step toward purifying the AChR/AChE/BuChE-aggregating factor agrin from homogenates of the electric organ of *Torpedo californica*, we isolated material that is insoluble in isotonic saline and detergent at neutral pH.

Table I. Purification of AChR-aggregating Activity from Torpedo Electric Organ

Fraction	Protein	AChR-aggregating activity	Purification	Yield
	mg	*U*	*fold*	*%*
Electric organ homogenate	14,000	845,000	1	100
Insoluble fraction	2,700	760,000	5	90
pH 9 bicarbonate extract	545	695,000	22	82
Cibacron pool	9.0	380,000	715	45
Gel filtration pool	1.2	230,000	3,300	28
Ion exchange pool	0.070	36,000	8,800	4

AChR-aggregating activity was purified from 1.33 kg (wet weight) of *Torpedo californica* electric organ by procedures described in Materials and Methods. Values listed above were typical; other preparations generally were within a factor of three with respect to purification and yield.
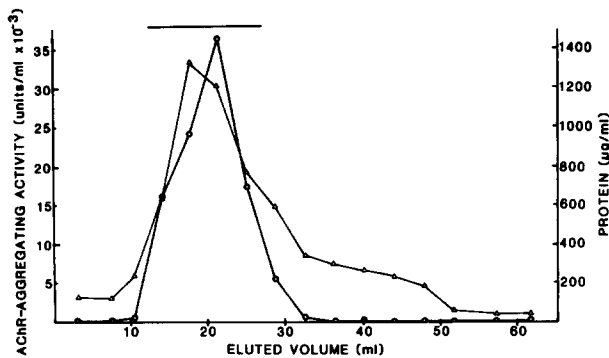
Figure 1. Affinity chromatography on Cibacron Blue 3GA-agarose. Bicarbonate extract (545 mg protein; 695,000 U) was applied (80 ml/h) to a 9-ml column of Cibacron Blue 3GA-Agarose; ∼65% of the protein and 4% of the activity were recovered in the flow-through volume. The column was washed with bicarbonate buffer (20 ml). Shown above is the elution of the column (40 ml/h) with a 40-ml linear gradient of 0–3 M NaCl followed by 20 ml of 3 M NaCl; fractions were assayed for AChR-aggregating activity (*circles*) and protein concentration (*triangles*). Material eluting at 0.3–1.1 M NaCl (indicated by horizontal bar) was combined as the Cibacron pool; it accounted for 54% of the applied activity, 2% of the applied protein.

Preparation and characterization of the insoluble fraction has been described in detail elsewhere (16).

Alkaline Extraction and Affinity Chromatography

Agrin was solubilized by homogenizing the insoluble fraction in bicarbonate buffered saline, pH 9.0 (Table I). The bicarbonate extract was applied to a column of Cibacron Blue 3GA-Agarose. Most (≥95%) of the AChR-aggregating activity bound to the column while ∼65% of the protein in the extract passed through. The column was eluted with a 0–3-M gradient of NaCl; agrin eluted together with the major peak of protein at ∼0.5 M NaCl (Fig. 1). Aliquots having the highest specific activity were combined to form the Cibacron pool.
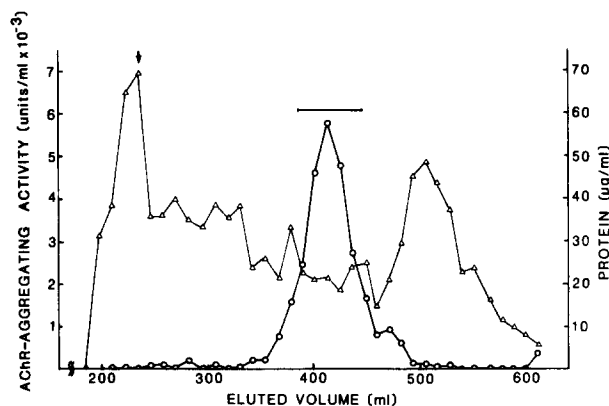
Figure 2. Gel filtration on Bio-Gel agarose 1.5m. Cibacron pool (16.5 ml; 9.0 mg protein; 377,000 U) was fractionated on a 540-ml (110 × 2.5-cm) column of Bio-Gel A 1.5m eluted at 50 ml/h. Fractions (12 ml) were assayed in triplicate for activity (*circles*) and in duplicate for protein concentration (*triangles*). The horizontal bar indicates the fractions combined to give the "gel filtration pool"; it contained 62% of the applied activity, 14% of the protein. Arrow indicates the position of the void volume.
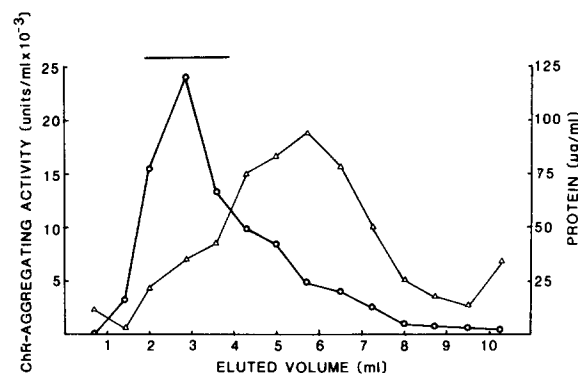
Gel Filtration Chromatography

The Cibacron pool was fractionated by gel filtration chromatography on a Bio-Rad 1.5m agarose column. Much of the protein eluted in the void volume (Fig. 2), as is characteristic of the large molecules expected in an extracellular matrix extract. The major portion (∼62%) of the AChR-aggregating activity applied to the column eluted as a single peak in the included volume at a position characteristic of a globular protein with a molecular mass of 98 ± 17 kD (mean ± SEM; $n = 3$). In some preparations an additional, smaller peak of activity was observed at a position corresponding to higher molecular mass proteins (see reference 29). Fractions comprising the major peak of activity were combined ("gel filtration pool") and concentrated by vacuum dialysis. Due to precipitation from solution and/or adsorption of protein to the dialysis tubing, 50–75% of the protein and AChR-aggregating activity were lost at this step.

Ion Exchange Chromatography

The dialyzed gel filtration pool was fractionated by ion exchange chromatography on DEAE-cellulose at pH 8.0. More than 80% of the activity initially bound to the resin while >70% of the protein passed through (Fig. 3). The column was eluted with a linear gradient of 0–250 mM NaCl. A broad peak of AChR-aggregating activity (comprising ∼40% of the applied activity) and protein (∼6% of that applied) eluted between 55 and 90 mM NaCl (Fig. 3). Fractions having the highest specific activity were combined ("ion exchange pool").

The purification protocol outlined above is summarized in Table I. Purifications of several thousand-fold were routinely obtained; the overall yield was low. Analysis of the ion exchange pool by SDS-PAGE (Fig. 4) revealed that it was far from homogeneous. In fact, in spite of the high specific activity of the ion exchange pool (0.5 U/ng protein), agrin is

Figure 3. Ion exchange chromatography on DEAE-cellulose. Dialyzed gel filtration pool (1.2 mg protein; 89,000 U) was applied (5–10 ml/h) to a 1-ml DEAE cellulose column. About 78% of the protein and 3% of the activity passed through the column. The column was washed (5 ml) in phosphate buffer. Shown above is the elution of the column with a 10-ml linear gradient of 0–250 mM NaCl. Fractions were collected and assayed in duplicate for AChR-aggregating activity (*circles*) and protein concentration (*triangles*). Fractions eluting between 55 and 90 mM NaCl (indicated by the *horizontal bar*) were combined to give the ion exchange pool. It contained 40% of the applied activity, 6% of the protein.
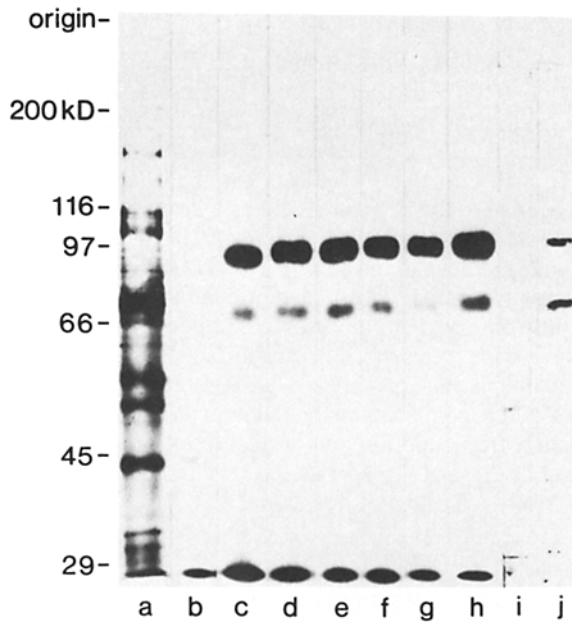
Figure 4. mAbs against AChR-aggregating activity immunoprecipitate polypeptides of 95 and 70 kD. All lanes represent proteins subjected to electrophoresis through a 7.5% polyacrylamide-SDS gel. (a) Silver stain of 0.4 μg (80 U) of a radiolabeled preparation of the ion exchange pool. (b–h) Autoradiogram of polypeptides immunoprecipitated from the radiolabeled ion exchange pool by (b) a control mAb and (c–h) mAbs that immunoprecipitate AChR-aggregating activity. Based on their properties as immunohistochemical reagents, each mAb (3B5, 4B1, 5B1, 11D2, 2F6, and 17A6) recognizes a different epitope (see reference 34). (i and j) Immunoblot of aliquots of the gel filtration pool (3.7 μg, 200 U) probed with (i) a control mAb and (j) mAb 11D2.

a relatively minor component of this pool, as discussed below.

Identification of Agrin Polypeptides

To characterize further the components in our electric organ extracts that mediate AChR, AChE, and BuChE aggregation, we raised a library of mAbs directed against antigens in the ion exchange pool (15, 34). Thirteen antibodies were selected for their ability to immunoprecipitate AChR-aggregating activity from electric organ extracts. Based on sensitivity to fixation and cross-reactivity in frog and/or chicken, these antibodies recognize at least seven different epitopes (34).

We next sought to identify the polypeptides in our extracts to which the mAbs bound. In initial experiments we radiolabeled proteins in the ion exchange pool and immunoprecipitated them with mAbs that immunoprecipitate AChR-aggregating activity. mAbs recognizing six different epitopes each specifically precipitated polypeptides with apparent molecular masses of 95 and 70 kD (Fig. 4). Although the relative amounts of the two polypeptides varied somewhat from preparation to preparation, in each case most of the label was associated with the 95-kD polypeptide. In other experiments, an aliquot of the ion exchange pool was subjected to electrophoresis on an SDS–polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted. Only one of the 13 mAbs bound to the immunoblots; it again stained poly-

peptides of 95 and 70 kD (Fig. 4). These results indicate that either or both of the polypeptides (95 and 70 kD) is agrin.

As mentioned above, AChR-aggregating activity was not always confined to a single peak on gel filtration column chromatography (e.g., reference 29). However, in our previous immunoprecipitation experiments mAbs removed all of the AChR/AChE/BuChE-aggregating activity, even from relatively crude extracts such as the Cibacron pool (15, 43, 45). Therefore, we were concerned that AChR/AChE/BuChE-aggregating molecules related to those in the ion exchange pool might be present in cruder fractions but excluded by the conventional chromatographic purification protocol. To investigate this possibility we immunoaffinity purified proteins from the Cibacron pool and analyzed them by SDS-PAGE and silver staining. Fig. 5 shows that five mAbs recognizing at least four different epitopes each immunoprecipitated AChR-, AChE-, and BuChE-aggregating activities from the Cibacron pool. Silver stains of the gels confirmed that the mAbs bound polypeptides of 70 and 95 kD, but in addition demonstrated that each mAb specifically immunoprecipitated polypeptides of 135 and 150 kD as well (Fig. 6). Thus, polypeptides of 150, 135, 95, and 70 kD are specifically immunoprecipitated from crude extracts of electric organ by mAbs that immunoprecipitate AChR/AChE/BuChE-aggregating activity.
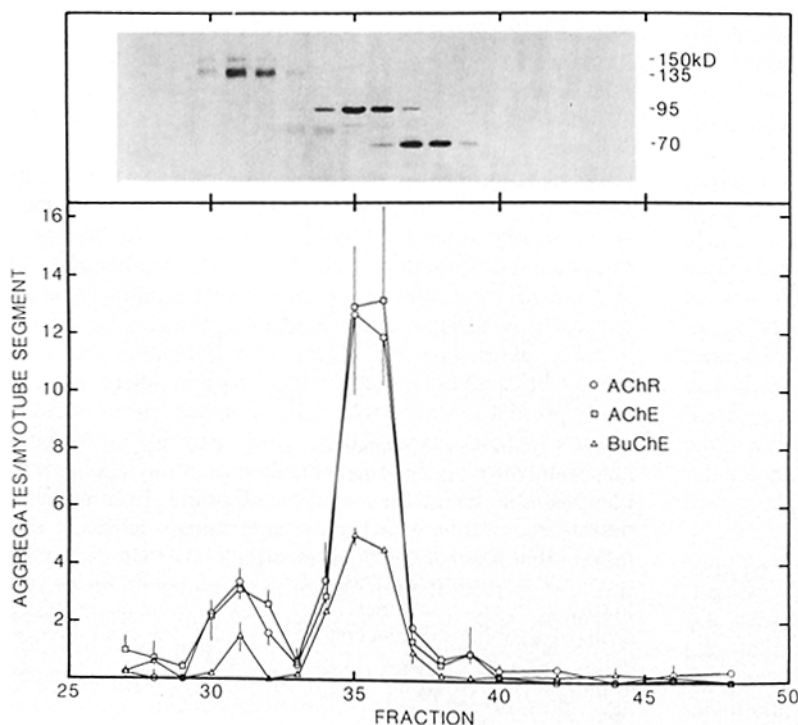
Recent immunohistochemical studies have shown that mAbs that immunoprecipitate agrin from extracts of *Torpedo* electric organ not only bind in high concentration at the neuromuscular junction, but also recognize molecules at other sites in muscle (15, 34). This raises the possibility that one or more of the polypeptides immunoprecipitated by the mAbs might not cause AChR/AChE/BuChE aggregation. Alternatively, different aggregating activities might reside in different polypeptides. To investigate these possibilities, an aliquot of the Cibacron pool was fractionated on a Sephacryl S-200 column and samples of the fractions were analyzed by immunoblotting and assayed for AChR-, AChE-, and BuChE-aggregating activities to correlate activity with the distribution
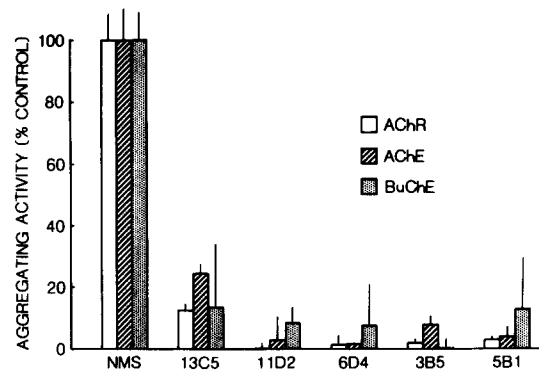
Figure 5. Immunoprecipitation of AChR/AChE/BuChE-aggregating activities. An aliquot of the Cibacron pool (62 U) was combined with 5 μl of Sepharose beads carrying mAbs or normal mouse serum in 50 μl of culture medium. After incubation for 2 h at 37°C with constant gentle agitation, the beads were removed by centrifugation and the supernatants tested for residual AChR-, AChE-, and BuChE-aggregating activities. Each of five different mAbs, recognizing at least four different epitopes, bound >75% of all three activities. Data, expressed as mean ± SEM (n = 3), is normalized to the level of activity in extracts exposed to normal mouse serum-coupled beads.
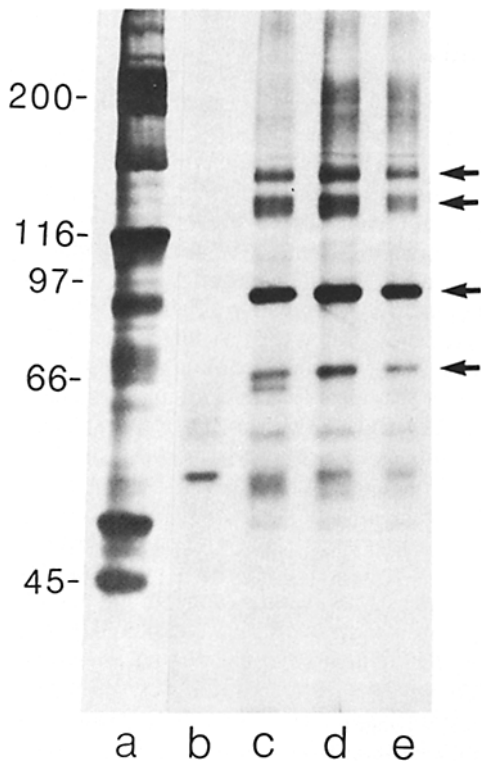
Figure 6. Antibodies against agrin bind to polypeptides of 150, 135, 95, and 70 kD. Photograph of a silver-stained SDS–polyacrylamide gel. (a) 30 U Cibacron pool; (b–e) peptides equivalent to 3,000 U, eluted from (b) normal mouse serum or (c–e) anti–agrin mAb-coupled beads (mAbs 6D4, 11D2, and 5B1). Numbers at the left indicate positions of molecular mass standards (kD); arrows indicate positions of the 150-, 135-, 95-, and 70-kD polypeptides consistently immunoprecipitated by all mAbs. Similar results were obtained with two other mAbs (3B5, 13C5) recognizing at least 1 additional epitope (data not shown).

of the 150-, 135-, 95-, and 70-kD polypeptides. As can be seen in Fig. 7, the peaks of AChR-, AChE-, and BuChE-aggregating activity comigrate. Comparison of the activity profile with the distribution of polypeptides bound by the anti–agrin mAb indicates that the 150- and 95-kD polypeptides are responsible for the aggregating activities, while the 135- and 70-kD polypeptides are inactive. In addition, under the buffer conditions in which the column was run, each of the four polypeptides appears to migrate independently of the others as a monomeric protein. Thus, we conclude that our extracts of electric organ contain two forms of agrin (150 and 95 kD) and two inactive proteins that are antigenically related to agrin.

Discussion

Molecules in the basal lamina at the neuromuscular junction direct the accumulation of AChRs and AChE at synaptic sites on regenerating muscle fibers (4, 25). In an effort to identify these molecules we have purified and characterized two proteins from an extracellular matrix–enriched fraction of *Torpedo* electric organ that each cause AChR, AChE, and BuChE aggregation in vitro. Results of previous studies (15, 16, 29, 45) and those reported in the accompanying paper (34) provide evidence that these proteins, which we call agrin, resemble the AChR/AChE-aggregating molecules in the synaptic basal lamina at the neuromuscular junction.

We chose the electric organ of *Torpedo* as a source from which to purify synaptic organizing molecules because the electric organ is densely innervated and has been useful for isolating and characterizing other components of cholinergic synapses, such as AChRs and AChE. The first step in our

Figure 7. AChR/AChE/BuChE–aggregating activities comigrate with polypeptides of 150 and 95 kD. (*Bottom*) An aliquot of the Cibacron pool (0.75 ml, 9,000 U) was fractionated on a 120 × 1-cm column of Sephacryl S-200 (void volume = 28 ml). 1-ml fractions were collected and assayed for AChR-, AChE-, and BuChE-aggregating activities. The data is expressed as mean ± SEM ($n = 3$). (*Top*) A photograph of an immunoblot in which an 0.5-ml aliquot of each column fraction was treated with TCA, and the precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mAb 11D2. The two peaks of AChR/AChE/BuChE–aggregating activity comigrate with polypeptides of 150 and 95 kD; the 135- and 70-kD polypeptides do not appear to possess any aggregating activity. The correlation between activity and the 95-kD polypeptide is easily seen. We reached the conclusion that the 150-kD polypeptide is active and that the 135-kD polypeptide is probably inactive by comparing, for example, fractions 30 and 33. Each has a similar amount of the 135-kD polypeptide, yet fraction 30 has readily detectable activity and fraction 33 has no activity. On the other hand, the 150-kD polypeptide, like the activity, is present in fraction 30 and not detectable in fraction 33. Likewise, fractions 30 and 32 have comparable levels of activity and comparable levels of the 150-kD polypeptide, but very different levels of the 135-kD polypeptide.

purification protocol, a modification of a method used by others to extract basal lamina from kidney and blood vessels (26), was selected to provide a fraction enriched in components of the extracellular matrix (16, 36). In initial reports we solubilized agrin from the extracellular matrix–enriched fraction by treatment with high salt buffers (16); such buffers are known to solubilize another extracellular matrix–associated molecule, acetylcholinesterase, from extracts of electric organ and muscle (21). We have now found, as indicated in our protocol, that agrin is more selectively and efficiently solubilized in 0.2 M bicarbonate buffer at pH 9. Although we recover >90% of the AChR/AChE/BuChE–aggregating activity detected in the insoluble fraction in the pH 9 extract, we have recently found (unpublished observations) that repeated extraction of the insoluble fraction can yield up to 10-fold more agrin activity. Apparently most of the agrin in the insoluble fraction is bound to particulate material in such a way that its activity is not expressed in our in vitro assay and it is difficult to solubilize.

We find that each of five different anti–agrin mAbs immunoprecipitates polypeptides of 150-, 135-, 95-, and 70-kD from electric organ extracts; both the 150- and 95-kD polypeptides have all three aggregating activities, while the 135- and 70-kD polypeptides are not active. A simple explanation for the existence of four agrin-related polypeptides is that the smaller are proteolytic fragments of the largest. We cannot rule out that some proteolysis occurs during our purification protocol, although care is taken to prepare extracts in the cold and in the presence of protease inhibitors. On the other hand, agrin may be a member of a family of antigenically, and perhaps functionally, related molecules that have a broad distribution in electric organ and muscle. Indeed, immunohistochemical studies have shown that anti–agrin antibodies stain not only the synaptic basal lamina at the neuromuscular junction but also some of the basal lamina–coated surfaces of other structures in muscle and electric organ (15, 22, 34).

Three polypeptides that cause up to a twofold increase in the number of AChR aggregates and a 1.4–6-fold increase in the rate of AChR insertion into the plasma membrane of cultured myotubes have been identified in neural tissues by others. Any or all of these molecules may play a role in the formation of AChR aggregates at the neuromuscular junction. They are a 42-kD polypeptide (ARIA) extracted from chick brain (42); an 84-kD polypeptide, called sciatin, which is extracted from sciatic nerves and is virtually identical to transferrin (e.g., reference 30); and calcitonin gene–related peptide, designated CGRP, which is 23 kD and is present in motor neurons (e.g., reference 28). The molecular mass of each clearly differs from that of the two forms of agrin. Moreover, agrin routinely causes a much more pronounced (up to 20-fold) increase in the number of receptor patches on myotubes and has little, if any, effect on the rate of receptor insertion (16). Thus, agrin is distinct from any of these molecules. Agrin also appears to be distinct from two molecules known to be concentrated in the synaptic cleft at the neuromuscular junction and at the electric organ synapse: AChE and a heparan sulfate proteoglycan. For example, the molecular mass of the extracellular, A_{12} form of *Torpedo* AChE is $\sim 10^6$ D (catalytic subunit: monomer, 68 kD; dimer, 131 kD; tail subunit: 55 kD [19, 24]); the molecular mass of a heparan sulfate proteoglycan from the neuromuscular junction is ~ 500 kD (3, 9) and that of an apparently similar mole-

cule at synapses in the electric organ is $\sim 10^6$ D (12). The pattern of staining seen with anti–agrin antibodies clearly distinguishes agrin from common basal lamina components such as laminin, type IV collagen, and entactin (34). On the other hand, agrin may be similar to as yet unidentified AChR-aggregating factors from rat brain (33), cultured embryonic neurons (38), neuronal cell lines (8), and rat diaphragm (7) which have apparent molecular masses of >50 kD.

We are just beginning to examine how agrin causes the formation of AChR/AChE/BuChE aggregates (44). We have shown that agrin-induced aggregation of AChRs occurs at least in part by lateral migration (16), as does nerve-induced AChR aggregation at developing neuromuscular junctions (2). One way agrin could cause aggregation is by directly cross-linking AChR, AChE, and BuChE molecules together, as is seen with antibody- and biotinylated α-bungarotoxin–avidin–induced AChR aggregation (5, 17). Such a scheme would require ~ 1 agrin molecule for each AChR, AChE, and BuChE molecule in an aggregate. We estimated the amount of agrin in one unit of aggregating activity in two ways. First, we compared the intensity of staining of the 95-kD polypeptide with that of BSA standards on silver-stained SDS–polyacrylamide gels of immunoaffinity-purified agrin (see Fig. 6). This gave $1.2(\pm 0.2) \times 10^{-11}$ g/U 95-kD agrin (mean $\pm$ SEM; $n = 8$). A similar estimate was obtained by calculating the fraction of the total radioactivity specifically immunoprecipitated by anti–agrin mAbs from a ^{125}I-labeled preparation of ion exchange pool (Fig. 4). Thus, in our muscle cultures agrin acts at $\sim 10^{-13}$ M. Extrapolating from the agrin-induced increase in the number of AChR aggregates per microscope field in our standard assay (16), we calculate that 1 U of agrin can induce as many as 1.5×10^5 patches, which is equivalent to 400 agrin molecules per patch. We can estimate the number of AChRs per patch to be $\sim 64,000$, based on an average size of 16 μm^2 (unpublished observations) and a concentration of 4,000 AChRs/μm^2 in a patch. (We know of no direct measurement of the concentration of AChRs in agrin-induced patches on chick myotubes: 2,000/μm^2 is the concentration seen in spontaneously occurring patches on rat myotubes; rat brain extract induces patches with 10,000 AChRs/μm^2 [37].) Thus, if every molecule of agrin added to a culture dish were bound in an aggregate, there would be at most 1 agrin for every 160 AChRs in each aggregate. Even if we have overestimated the specific activity of agrin and the number of AChRs in a patch each by a factor of four, there would still be at least a 10-fold excess of AChRs over agrin in a patch, not to mention an undetermined number of AChE and BuChE molecules. Therefore, it seems unlikely on numerical grounds that agrin induces aggregates by binding AChRs, AChE, and BuChE together, but rather that agrin acts either catalytically or by triggering some mechanism already present in the myotube. For example, agrin could be an enzyme that causes AChRs, AChE, and BuChE to aggregate spontaneously by modifying them or some other surface component to which they subsequently bind. In frog nerve-muscle cocultures, growing nerve terminals release a proteinase that degrades a heparan sulfate proteoglycan on the myotube surface; the removal of the heparan sulfate proteoglycan is proposed to be an early step in neurite-induced AChR aggregation (1). Alternatively, agrin could bind to a receptor on the myotube surface that, in turn, initiates a cascade of events leading to AChR/AChE/BuChE aggregation.

Such a receptor-mediated mechanism might also be activated by nonphysiological signals, which would account for the observation that positively charged latex beads induce the formation of specializations on cultured myotubes that resemble the postsynaptic apparatus (31).

In view of the constancy of the postsynaptic apparatus from embryo to adult and the reestablishment of a nearly identical apparatus during regeneration it is reasonable to suggest that the same molecules that direct the formation of the postsynaptic apparatus during embryogenesis also maintain it in the adult and direct its formation again during regeneration. Several lines of evidence indicate that the nerve terminal induces the formation of the postsynaptic apparatus during embryogenesis (13). Thus, it seems likely that the molecules that induce and maintain postsynaptic differentiation are synthesized by motor neurons and released from their axon terminals. We suggest that agrin is such a molecule and present the following hypothesis for how it might act. As motor axons approach and grow over the surface of developing myofibers they release agrin. Receptors for agrin are scattered over the surface of the myotubes. When agrin binds to its receptor it causes AChRs, AChE, BuChE, and other components of the postsynaptic apparatus, including components of the synaptic basal lamina, to aggregate on the myotube surface in the vicinity of the activated receptor. (Indeed, a heparan sulfate proteoglycan [9], one component of basal lamina, accumulates at agrin-induced AChR/AChE/BuChE patches on cultured myotubes [unpublished observations]). Agrin becomes associated with this nascent synaptic basal lamina and thus is bound at the synaptic site. Such localization might have two consequences: (a) it might prolong the interaction of agrin with its receptor, and (b) it might prevent agrin from diffusing away from the synaptic site to induce unneeded synaptic specializations elsewhere on the myofiber. Release of agrin from motor nerve terminals at the adult neuromuscular junction and its incorporation into the synaptic basal lamina would help maintain the postsynaptic apparatus by insuring that newly synthesized components become concentrated at the synaptic site, while release of agrin by regenerating axons would account for their ability to induce postsynaptic apparatus at ectopic sites on denervated myofibers (e.g., reference 20). As we have previously suggested, agrin stably bound to the synaptic basal lamina at the adult neuromuscular junction would induce the aggregation of AChRs and AChE that occurs when myofibers regenerate in the absence of nerve terminals (4, 25). Consistent with our scheme is the finding that motor neurons contain agrin-like molecules: anti–agrin antibodies stain the cell bodies of embryonic and adult motor neurons and extracts of regions of the central nervous system rich in motor neurons contain molecules that are both antigenically similar to agrin and cause AChR aggregation on myofibers (40).

This scheme does not eliminate the need for other factors that regulate the amount and turnover of molecules of the postsynaptic apparatus: for example, electromechanical activity (see reference 13) and molecules such as the 42-kD polypeptide, ARIA, and the calcitonin gene–related peptide, designated CGRP. Nor does it obviate a role for components of the cytoskeleton, which may be linked to AChRs and thus could be involved in the formation and/or stabilization of AChR aggregates (6, 10, 14, 32). Our hypothesis is that agrin triggers the formation of the postsynaptic apparatus and specifies where on the myofiber it is to be assembled. No doubt this simple scheme will require modification as knowledge about synapse formation in general and agrin in particular increases. Identifying agrin and developing antibodies to it, as reported here and in the accompanying paper (34), are important steps toward making direct tests of this hypothesis.

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