Aggregating Factor from *Torpedo* Electric Organ Induces Patches Containing Acetylcholine Receptors, Acetylcholinesterase, and Butyrylcholinesterase on Cultured Myotubes

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Abstract. A factor in extracts of the electric organ of *Torpedo californica* causes the formation of clusters of acetylcholine receptors (AChRs) and aggregates of acetylcholinesterase (AChE) on myotubes in culture. In vivo, AChRs and AChE accumulate at the same locations on myofibers, as components of the postsynaptic apparatus at neuromuscular junctions. The aim of this study was to compare the distribution of AChRs, AChE, and butyrylcholinesterase (BuChE), a third component of the postsynaptic apparatus, on control and extract-treated myotubes. Electric organ extracts induced the formation of patches that contained high concentrations of all three molecules. The extract-induced aggregation of AChRs, AChE, and BuChE occurred in defined medium, and these components accumulated in patches simultaneously. Three lines of evidence indicate that a single factor in the extracts induced the aggregation of all three components: the dose dependence for the formation of patches of AChRs was the same as that for patches of AChE and BuChE; the AChE- and BuChE-aggregating activities co-purified with the AChR-aggregating activity; and all three aggregating activities were immunoprecipitated at the same titer by a monoclonal antibody against the AChR-aggregating factor. We have shown previously that this monoclonal antibody binds to molecules concentrated in the synaptic cleft at neuromuscular junctions. Taken together, these results suggest that during development and regeneration of myofibers in vivo, the accumulation at synaptic sites of at least three components of the postsynaptic apparatus, AChRs, AChE, and BuChE, are all triggered by the same molecule, a molecule similar if not identical to the electric organ aggregating factor.

THE postsynaptic apparatus at the vertebrate skeletal neuromuscular junction is composed of several cytoplasmic, plasma membrane, and extracellular matrix specializations. These include accumulations of acetylcholinesterase (AChE)[1] and several unidentified antigens in the synaptic portion of the myofiber's sheath of basal lamina (11, 47, 63), junctional folds and clusters of acetylcholine receptors (AChRs) in the myofiber plasma membrane (6, 19, 48, 52), and electron-dense material and associated filaments in the cytoplasm beneath the postsynaptic membrane (15, 26). Butyrylcholinesterase (BuChE) is also concentrated at the neuromuscular junction (14, 29, 34), although it has yet to be determined whether BuChE is associated with the myofiber's plasma membrane or its sheath of basal lamina.

During regeneration of muscles in the adult, molecules associated with the synaptic portion of the myofiber's sheath of basal lamina direct the formation of at least three components of the postsynaptic apparatus: aggregates of AChRs, accumulations of AChE, and infoldings in the myofiber's plasma membrane resembling junctional folds (2, 7, 46).

These findings have focused attention on the roles molecules associated with the synaptic basal lamina play in the regeneration of the neuromuscular junction.

As a step toward identifying the number and nature of the signals that regulate synaptic differentiation, we isolated a basal lamina–containing fraction from the electric organ of *Torpedo californica* and assayed its effects on chick myotubes in culture. The basal lamina-containing fraction induced aggregates of both AChRs and AChE to form on the myotube surface (23, 50, 59, 69). Several lines of evidence indicate that the same factor in the electric organ extract directs both AChR and AChE accumulation: the AChR- and AChE-aggregating activities co-purify, the dose dependence for the induction of AChR and AChE aggregates is the same at each stage of the purification, and two different monoclonal antibodies immunoprecipitate and inhibit both activities (69). Moreover, at least one of these monoclonal antibodies, 6D4, binds in high concentration in the synaptic cleft at the neuromuscular junction, indicating that the active component in the extract is related antigenically to molecules concentrated in or adjacent to the synaptic basal lamina (18).

The present studies were undertaken to compare the effects of the electric organ extract on the distribution of AChRs,

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[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; AChRs, acetylcholine receptors; BuChE, butyrylcholinesterase; BW284c51, 1,5-bis-(4-allyl-dimethylammoniumphenyl) penton-3-one dibromide; ChE, cholinesterase; iso-OMPA, tetraisopropylpyrophosphoramide; MSF, methane sulfonyl fluoride.

AChE, and BuChE. The results reported here show that the electric organ extract causes the formation of patches that contain both AChRs, AChE, and BuChE, and that these three components accumulate simultaneously. In addition, the BuChE-aggregating activity co-purifies with the AChR/AChE aggregating factor and all three aggregating activities are immunoprecipitated by 6D4 at the same titer. Thus, a single factor in the basal lamina-containing extract of electric organ induces the formation of specializations on cultured myotubes that are similar to the postsynaptic apparatus at the neuromuscular junction in that they contain AChRs, AChE, and BuChE.

Materials and Methods

Chick Myotube Cultures

Myotube cultures were prepared from hind limb or pectoral muscles of 11- to 12-d White Leghorn chick embryos by the method of Fischbach (21) with minor modifications (23). In brief, minced muscles were dissociated for 15 min in 0.02% trypsin at 37°C, the cell suspension was preplated to reduce the number of nonmuscle cells, and $\sim 1 \times 10^5$ cells were plated onto 35-mm plastic tissue culture dishes coated with calf skin collagen (Calbiochem-Behring Corp., La Jolla, CA). Culture medium (1.5 ml) consisted of Eagle's minimum essential medium (Gibco, Grand Island, NY) 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 [54]), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml Fungizone (Gibco). Medium was replaced every 2 d; 10^{-5} M cytosine arabinoside was added from day 2 to 4 to reduce the number of rapidly dividing cells. Cultures were used 4 to 6 d after plating.

To ensure that the formation of cholinesterase ChE aggregates induced by electric organ extracts involved myotube ChE and was not due to ChE present in the culture medium or the extract itself, 2 mM methane sulfonyl fluoride (MSF, an irreversible inhibitor of ChE [39]) was routinely added to the extracts and culture medium. The extracts were incubated with MSF for 1 h at room temperature immediately before being added to the myotube cultures. The rapid spontaneous hydrolysis of unreacted MSF and the large dilution of MSF-treated extracts on addition to the cultures prevented any significant inactivation of myotube ChE. Before extracts were added to cultures, the culture medium was replaced with medium, lacking chick embryo extract, that had been treated with MSF to inactivate ChE present in horse serum. Medium was usually stored at least 24 h after MSF treatment before being used to allow all unreacted MSF to be hydrolyzed. In some experiments (see Results) horse serum and chick embryo extract were omitted for the last 24 h, and the culture medium was supplemented with 3 mg/ml bovine serum albumin (BSA). Cultures incubated for 24 h in the absence of horse serum and embryo extract stained much more intensely for ChE, both histochemically and immunohistochemically.

Quantitation of AChR, AChE, and BuChE Aggregation

To determine the effect of electric organ extracts on AChR and ChE distribution, myotube cultures were incubated for 1 h at 37°C with 6×10^{-8} M rhodamine-α-bungarotoxin (55) in either normal culture medium that had been treated with MSF or in Eagle's minimum essential medium supplemented with 3 mg/ml BSA. The cultures were washed twice with Puck's saline G at room temperature, fixed for 10 min in ice cold 1% paraformaldehyde dissolved in 90 mM sodium phosphate, pH 7.2, containing 160 mM sucrose, rinsed twice with Puck's saline, and stained for ChE for 1.5 h at room temperature (33). The staining solution contained, in final concentration, sucrose, 205 mM; sodium maleate, 32.5 mM; sodium citrate, 5 mM; potassium ferricyanide, 0.5 mM; cupric sulfate, 3 mM; acetylthiocholine iodide, 1.7 mM. After staining, cultures were rinsed with Puck's saline, water, 50% ethanol, 95% ethanol, and glass coverslips were mounted with a drop of glycerol.

In experiments where the contribution of AChE and BuChE was determined pharmacologically, cultures were labeled with rhodamine-α-bungarotoxin, fixed, and incubated for 15 min with either 10^{-4} M tetraisopropylpyrophosphoramide, a specific inhibitor of BuChE, or 10^{-5} M 1,5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide, an inhibitor selective for AChE (iso-OMPA or BW284c51, respectively; Sigma Chemical Co., St. Louis,

MO). They were then stained as above either with acetylthiocholine as substrate in the presence of 10^{-4} M iso-OMPA to demonstrate sites of AChE activity or with 1 mM butyrylthiocholine iodide as substrate in the presence of 10^{-5} M BW284c51 to stain sites of BuChE activity.

To compare more accurately the distribution of AChRs and AChE, AChE was localized by immunohistochemical techniques. Cultures were incubated in $1-2 \times 10^{-8}$ M rhodamine-α-bungarotoxin in Eagle's minimum essential medium supplemented with 3 mg/ml BSA for 1 h at 37°C, rinsed twice with Puck's saline at room temperature, and fixed for 30 min in 1% formaldehyde as described above. Cultures were then rinsed twice with Puck's saline and incubated for 1 h in 6.7 μg/ml 1A2 anti-AChE dissolved in Puck's saline containing 10% horse serum. 1A2 anti-AChE is a monoclonal antibody specifically against chick AChE (58). To assess nonspecific staining, cultures were labeled with an equal concentration of the IgG secreted by the parent myeloma SP3 (58). The cultures were then rinsed for 15 min in two changes of Puck's saline, incubated for 1 h in fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) diluted 1:200 in Pucks saline containing 10% horse serum, rinsed for 15 min in two changes of Puck's saline, rinsed in 50% ethanol, 95% ethanol, and mounted under a glass coverslip in a drop of Citifluor mountant medium AF1 (City University, London).

To compare the distribution of AChRs, AChE, and BuChE on individual myotubes, cultures were labeled with $1-2 \times 10^{-8}$ M rhodamine-α-bungarotoxin, rinsed, fixed for 15 min at room temperature, and incubated with 1A2 anti-AChE and fluorescein-conjugated goat anti-mouse IgG as above, then rinsed with Puck's saline, incubated for 15 min in Puck's saline containing 10^{-5} M BW284c51, and stained histochemically for 1.5 h with butyrylthiocholine as substrate in the presence of 10^{-5} M BW284c51. After being stained, the cultures were rinsed in Puck's saline, 50% ethanol, 95% ethanol, and mounted with mountant medium AF1.

Cultures were examined at magnifications of 250-312 with either a Zeiss or a Nikon microscope equipped with phase and fluorescence optics. Myotubes were chosen at random, and the patches of ChE reaction product along an unbranched 400-μm segment of the myotube were counted under phase optics; then the AChR and/or AChE clusters were counted under fluorescence optics, and the number of co-patches was recorded. Only patches that appeared to be on the bottom of the myotubes were counted; most patches in control and extract-treated cultures occurred on this surface of the myotubes. For each culture, counts from 10-20 myotubes, scattered over the surface of the dish, were averaged. Any distinct fluorescent or ChE-stained patch whose shortest axis was >2 μm was counted as an aggregate. To quantitate AChR/ChE-aggregating activity, dose-response curves were constructed (see Fig. 6); 1 U activity was taken as the amount of material required to give a half-maximal response. Previous experiments have shown that the electric organ extracts used in this study do not affect the size or number of myotubes per culture, their protein or DNA content, or the number of AChRs on their surface (23). Similarly, we found extract treatment caused little change in the total ChE content of the myotubes (extract-treated/control = 1.04 ± 0.04, mean ± SEM; $n = 5$) or in the amount of ChE they released into the medium (extract-treated/control = 1.04 ± 0.07, mean ± SEM; $n = 4$), as determined by assaying ChE (17) in culture homogenates and medium 24 h after inactivating all ChE in the cultures with diisopropylpyrophosphate (64) and then incubating in MSF-treated culture medium with or without electric organ extract. Therefore, the effects of electric organ extracts on the number of aggregates of AChRs, AChE, and BuChE are due to changes in distribution of these components and not to overall changes in myotube size, shape, AChR content, or ChE content.

Preparation of Electric Organ Extracts

Extracts containing AChR/AChE-aggregating activity were prepared as previously described (23, 50). In brief, electric organs of large Torpedo californica (40-70 cm across, supplied by Pacific Bio-Marine Laboratories, Inc., Venice, CA) were removed, diced, and frozen in liquid nitrogen. The tissue was pulverized and homogenized in a Waring blender in 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. The homogenate was centrifuged (17,700 g for 30 min) and the pellet was homogenized in 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 (30,000 g for 30 min), and the pellet was resuspended with a Teflon-glass homogenizer in 10 mM Tris-Cl, pH 7.5, containing 3% (vol/vol) Triton X-100 and then stirred for 30 min. The suspension was centrifuged (30,000 g for 30 min), the pellet was reextracted with the buffered Triton, and then the pellet was twice homogenized and centrifuged (30,000 g for 30 min) in saline buffer containing 150 mM NaCl and 10 mM Tris-Cl, pH 7.5. The final pellet was resuspended in 150 mM NaCl, 10 mM Tris-Cl, pH 7.5, to give the "insoluble fraction."

To solubilize the aggregating factor the insoluble fraction was centrifuged

(30,000 g for 30 min) and the pellet was resuspended in buffer containing 50 mM NaCl, 0.01% NaN₃, and 50 mM sodium citrate, pH 5.5. This suspension was stirred overnight at 4°C, then centrifuged (48,000 g for 30 min). The clear supernatant is referred to as the pH 5.5 extract.

The pH 5.5 extract was concentrated by vacuum dialysis against buffer containing 500 mM NaCl, 5% (vol/vol) glycerol, 50 mM sodium citrate, pH 5.5, cleared by centrifugation (48,000 g for 30 min), and applied to a column of Biogel A 1.5m (Bio-Rad Laboratories, Richmond, CA) equilibrated and eluted with the same buffer. The most active fractions were combined to give the "agarose pool."

Results

Formation of AChR, ChE, and AChR/ChE Patches

To determine whether or not AChRs and AChE accumulated at the same sites on the myotubes, AChRs were labeled with rhodamine-conjugated α-bungarotoxin, and sites of ChE activity were stained histochemically. Our previous studies (69) demonstrated that ChE patches stained in this way contained AChE. As illustrated in Fig. 1, a and b, myotubes cultured for 6 d had a small number of labeled patches. Treating the cultures with the basal lamina–containing insoluble fraction of Torpedo electric organ (23) increased the number of AChR and ChE patches (Fig. 1, c and d). A striking feature of cultures stained in this way, especially of those treated with electric organ extract, was the frequent superposition of AChRs and ChE.

To quantitate the effect of the electric organ extract, the patches that stained for AChRs, ChE, and both AChRs and

ChE were counted on 400-μm long segments of 10–20 myotubes in each culture dish. There was considerable variability from plating to plating in the numbers of ChE, AChR, and AChR/ChE patches on both control and extract-treated myotubes, but much less among myotubes from cultures plated at the same time. This is illustrated in Table I, which gives data from experiments made on three different sets of cultures. In all three experiments the electric organ extract caused the total number of labeled patches per myotube segment to increase by approximately eight. In each case there was little change in the number of ChE spots. The changes in AChR and AChR/ChE patches, however, were more variable. In some experiments, such as the one illustrated in Table I*A*, electric organ extracts induced primarily co-patches, in others (Table I*B*) they induced approximately equal numbers of AChR patches and co-patches, and in still others (Table I*C*) the increase was primarily in AChR patches. The results from many such experiments are summarized in Table I*D*. On average, myotubes in control cultures had a total of 2.7 labeled patches per 400-μm segment of myotube; δ46% were ChE patches, 25% AChR patches, and 29% AChR/ChE co-patches. Treating the myotubes with electric organ extract induced on average 4.4 AChR/ChE co-patches per myotube segment (a 6.5-fold increase) and 2.6 AChR patches (a 4.7-fold increase) but caused little change in the number of ChE patches. Thus, the predominant effect of the electric organ extract was to induce the formation of patches containing both AChRs and ChE.
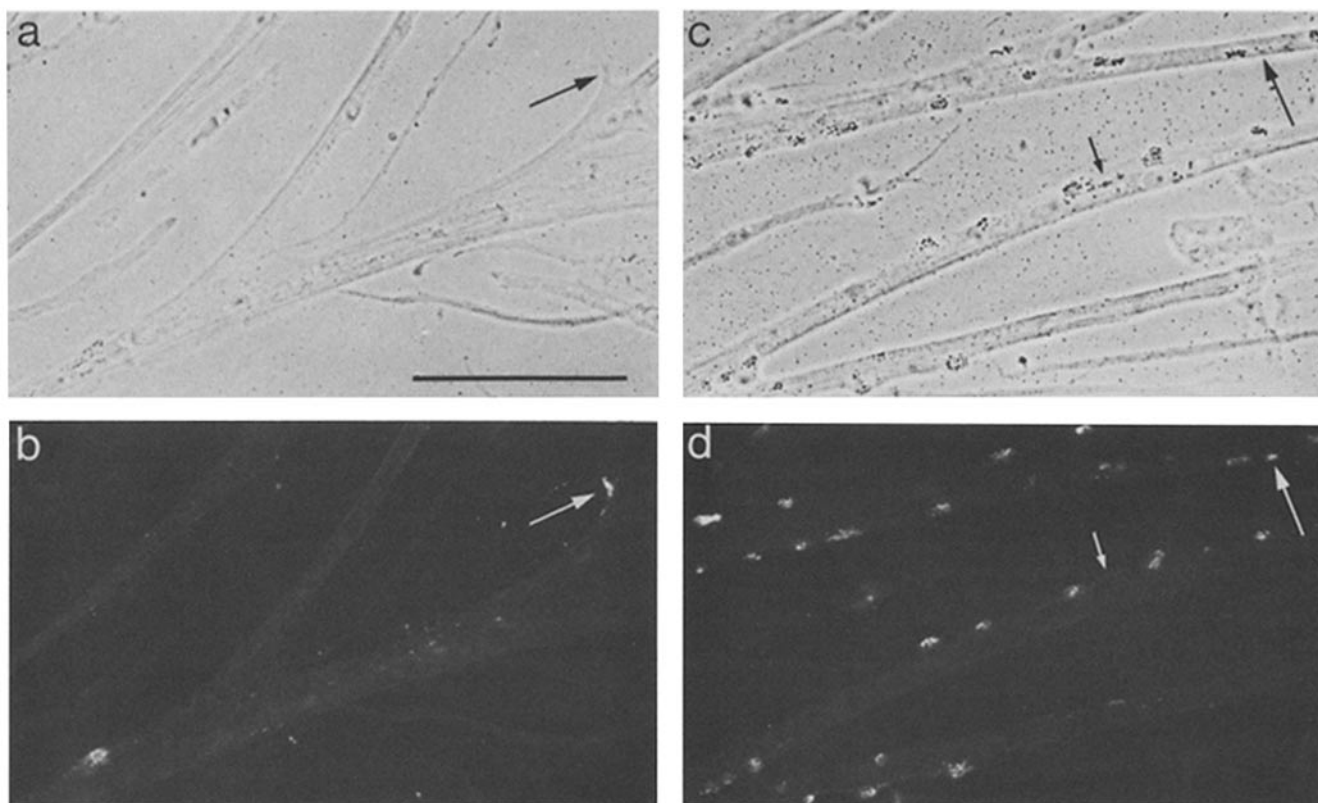

Figure 1. Electric organ extract induces patches rich in ChE and AChRs to form on cultured myotubes. Cultures were stained histochemically for sites of ChE activity, and AChRs were labeled with rhodamine-α-bungarotoxin. (*a* and *b*) Control culture; (*c* and *d*) extract-treated culture. (*a* and *c*) Phase-contrast micrographs. Patches of granular reaction product mark sites of ChE activity. (*b* and *d*) Fluorescence micrographs of the same fields as in *a* and *c*, showing distribution of AChRs. Most patches contain both AChRs and ChE reaction product, although in some patches only AChRs (long arrows) or ChE (short arrows) is detected. Bar, 100 μm.

Table I. AChR, ChE and AChR/ChE Patches on Control and Electric Organ Extract-treated Myotubes

| Treatment | Aggregates/myotube segment | | | |
	Total	ChE	AChR	AChR/ChE
A Control	1.3	0.3 ± 0.2	0.2 ± 0.1	0.8 ± 0.1
Extract	9.4	0.03 ± 0.06	0.5 ± 0.3	8.9 ± 0.1
B Control	3.4	1.4 ± 0.3	1.0 ± 0.1	1.0 ± 0.2
Extract	11.7	0.7 ± 0.2	5.3 ± 1.7	5.7 ± 1.4
C Control	2.4	0.6 ± 0.2	1.2 ± 0.9	0.6 ± 0
Extract	10.5	0.8 ± 0.4	7.4 ± 1.1	2.3 ± 1.4
D Control	2.7	1.2	0.7	0.8
Extract	9.5	1.1	3.3	5.2

Values given are the mean number of patches per 400-μm-long myotube segment. (*A–C*) Experiments made on sets of cultures from three different platings. Data expressed as mean ± SEM for triplicate cultures. (*D*) Means of 39 experiments, each done in triplicate.
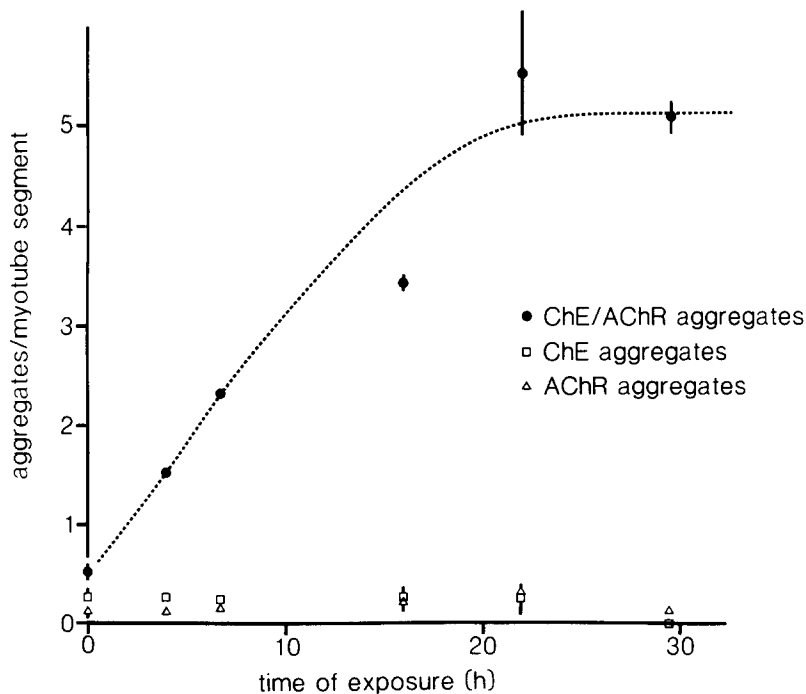

Figure 2. Time course of appearance of ChE, AChR, and ChE/AChR aggregates. Myotube cultures were incubated for the indicated length of time with an extract of electric organ (50 μg agarose pool/dish), stained histochemically for sites of ChE activity and with rhodamine-α-bungarotoxin for AChRs, and the mean number of ChE, AChR, and ChE/AChR aggregates per myotube segment was determined. Error bars, shown where they exceed the size of the data point, are ± SEM (*n* = 3).

Time Course of Appearance of Extract-induced Patches

Previous studies have shown that when extracts of *Torpedo* electric organ are added to cultured myotubes, the number of patches of AChRs increases gradually over the course of ~24 h and then remains constant (23). Fig. 2 compares the time course of appearance of AChR, ChE, and AChR/ChE patches on myotubes incubated for various times with an electric organ extract. The number of AChR/ChE co-patches began to increase within 4 h of adding the extract and reached a plateau by 24 h. In this set of cultures there was no significant change in the number of ChE or AChR patches on the myotubes. In other experiments, on cultures in which extracts induced both AChR and AChR/ChE patches, the formation of AChR and AChR/ChE patches followed similar time courses (data not shown). Thus, within the limits of the assay techniques, electric organ extract-induced co-patches form by the simultaneous accumulation of AChRs and ChE.

Accumulation of AChE and BuChE in Patches

To determine whether both AChE and BuChE were accumulating at electric organ extract-induced ChE patches, cultures were stained with acetylthiocholine as substrate in the presence of 10^{-4} M iso-OMPA, a selective inhibitor of BuChE, to label AChE aggregates, or with butyrylthiocholine as substrate in the presence of 10^{-5} M BW284c51, a selective inhibitor of AChE, to label BuChE aggregates. Neither inhibitor by itself completely prevented staining; however, when both inhibitors were added together no reaction product was seen with either substrate. There was no obvious difference in the distribution of reaction product on cultures stained for AChE or BuChE; in each case there was some diffuse staining as well as dense patches of reaction product. BuChE staining tended to be less intense than AChE staining. There was variability from plating to plating in the relative number of AChE and BuChE patches (Table II). In most cases the sum of AChE-stained patches and BuChE-stained patches ex-

Table II. Contribution of AChE and BuChE to ChE Aggregates

| Treatment | Aggregates/myotube segment | | | |
	ChE	AChE	BuChE	AChE + BuChE
Control*	4.0 ± 0.8	3.9 ± 0.7	0.8 ± 0.2	4.7
Extract*	11.3 ± 1.3	11.2 ± 2.9	4.4 ± 1.3	14.6
Control‡	100	79 (40–126)	26 (0–69)	105 (40–176)
Extract‡	100	89 (81–108)	32 (0–76)	122 (90–184)

Cultures were stained with acetylthiocholine as substrate with no inhibitor (ChE), acetylthiocholine in the presence of 10^{-4} M iso-OMPA (AChE), or butyrylthiocholine as substrate in the presence of 10^{-5} M BW284c51 (BuChE).

* Cultures were treated for 20 h with agarose pool. Results are the mean ± SEM number of aggregates per 400-μm myotube segment, assayed in triplicate.

‡ Combined results from cultures treated with either the basal lamina–containing insoluble fraction, the pH 5.5 extract, or the agarose pool. Data, expressed as a percentage of the total ChE patches, is the mean of seven or eight determinations, each in triplicate. The numbers in parentheses are the range of observed values.
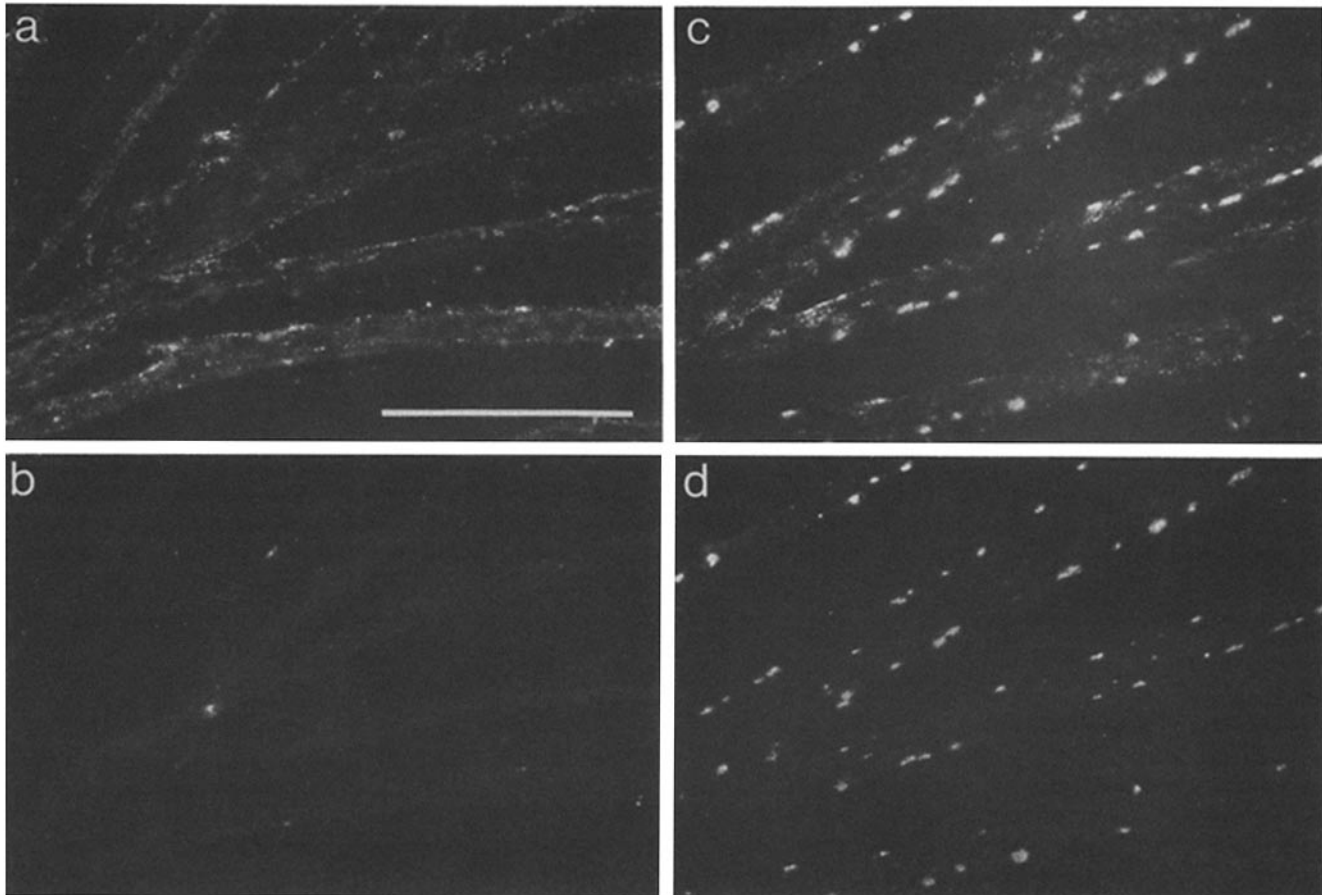

Figure 3. Patches containing both AChE and AChRs are induced by electric organ extract. Myotube cultures incubated in serum-free medium. AChRs were labeled with rhodamine-α-bungarotoxin and AChE with 1A2 anti-AChE followed by fluorescein-conjugated second antibody. Shown are fluorescence micrographs of a control culture (*a* and *b*) and an extract-treated culture (*c* and *d*) viewed with fluorescein optics (*a* and *c*) to reveal AChE staining and rhodamine optics (*b* and *d*) to demonstrate AChR distribution. Bar, 100 μm.

ceeded the total number of ChE-stained patches (as determined by staining with acetylthiocholine as substrate in the absence of either inhibitor), suggesting that at least some patches contained both AChE and BuChE.

Distribution of AChRs and AChE

To determine more accurately the relative distribution of AChRs and AChE, cultures were labeled with rhodamine-α-bungarotoxin as before and 1A2 anti-AChE, a monoclonal antibody against chick AChE (58), followed by fluorescein-conjugated second antibody. This procedure avoids any distortion of the apparent distribution of AChRs by the dense

ChE reaction product. For these experiments cultures were incubated in medium without horse serum or embryo extract and with 3 mg/ml bovine serum albumin. Fig. 3 shows cultures labeled in this way. On both control and extract-treated cultures the diffuse labeling on the myotube surface was much more conspicuous for AChE than for AChRs. As expected from the results of the histochemical and pharmacological studies, electric organ extracts caused a marked increase in the number of patches of AChRs and AChE. Most patches contained both AChRs and AChE; in some patches only one label or the other was detected. Examples of extract-treated myotubes are shown at higher power in Fig. 4, labeled
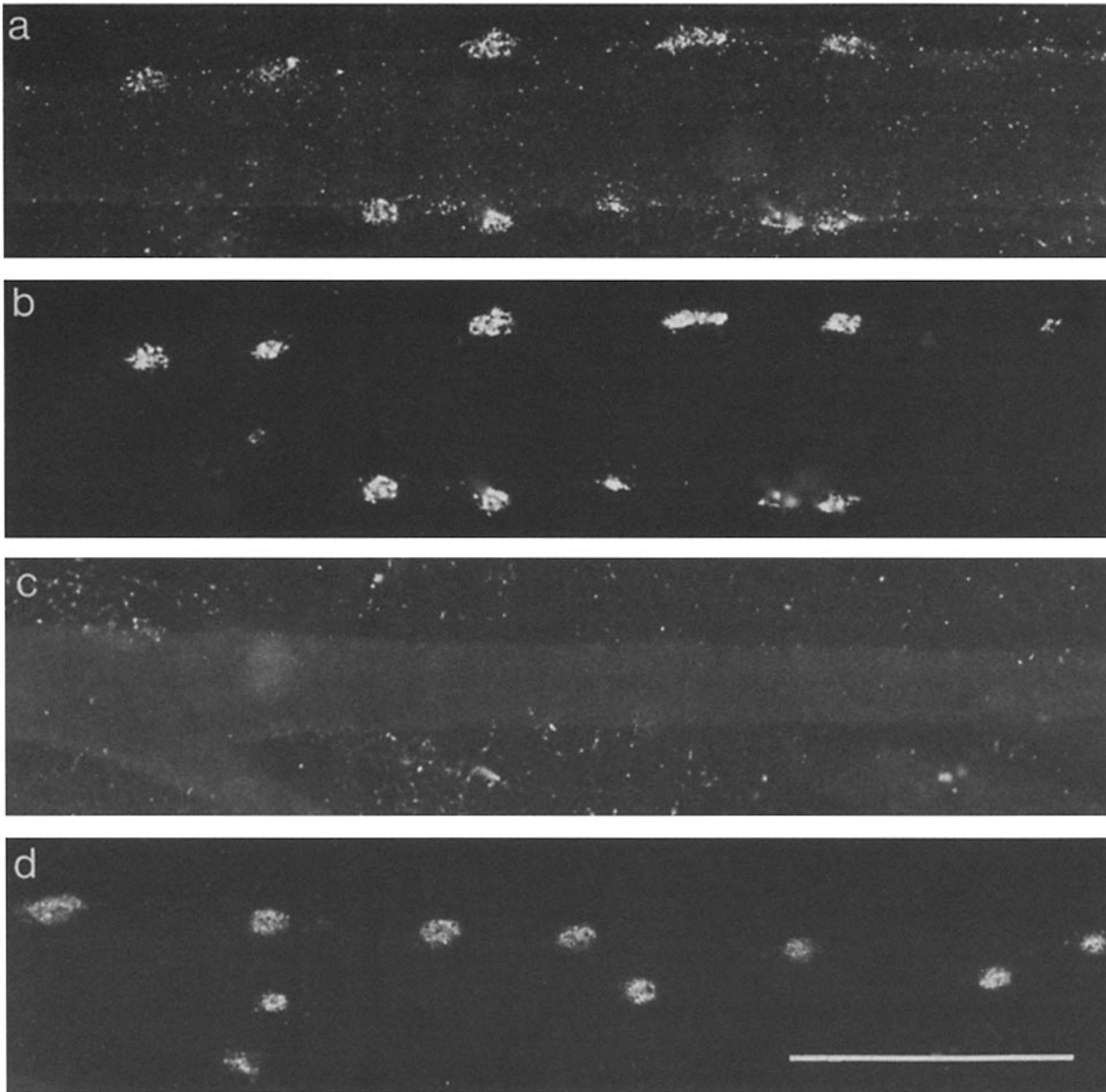
Figure 4. Co-localization of AChE and AChRs in extract-induced aggregates. Myotube cultures were stained with rhodamine-α-bungarotoxin and either 1A2 anti-AChE (*a* and *b*) or a nonspecific antibody (*c* and *d*) followed by fluorescein-conjugated second antibody. Fluorescence micrographs of myotubes viewed with fluorescein optics (*a* and *c*) or rhodamine optics (*b* and *d*). (*a* and *b*) Most extract-induced patches on this myotube contain both AChE (*a*) and AChRs (*b*). As illustrated here, the borders of the patches revealed by labeling AChE (*a*) were less distinct and the diffuse staining of the myotube surface was greater than when AChR distribution was visualized (*b*). (*c* and *d*) Myotube incubated with antibodies secreted by the parent myeloma SP3 (58). The absence of fluorescein staining (*c*) indicates that the labeling protocol detected specific anti-AChE binding and that there is no significant penetration of rhodamine fluorescence (*d*) when viewed with fluorescein optics. Bar, 50 μm.

with rhodamine-α-bungarotoxin and 1A2 anti-AChE (Fig. 4, *a* and *b*) or a control monoclonal antibody (Fig. 4, *c* and *d*). The lack of fluorescein labeling on myotubes in cultures treated with the control antibody indicates that the labeling with 1A2 anti-AChE is specific. As illustrated in Fig. 4, *a* and *b*, there was often a striking degree of overlap in the distribution of AChRs and AChE; however, even in co-patches the two labels were not always precisely co-extensive.

Formation of AChE/AChR Patches and BuChE/AChR Patches

To compare the effect of electric organ extracts on patches containing AChRs, AChE, and BuChE, eight sets of control and extract-treated cultures were stained for AChRs, using rhodamine-α-bungarotoxin, and for either AChE, using 1A2 anti-AChE, or BuChE, using the histochemical procedure with butyrylthiocholine as substrate in the presence of BW284c51. As shown in Table III, the incidence of both AChE and BuChE patches varied in the same way as was observed for ChE patches (see Table I); that is, treating myotube cultures with electric organ extracts caused a large increase in AChE/AChR and BuChE/AChR co-patches, a smaller increase in AChR patches, and a slight decrease in AChE and BuChE patches. Based on the numbers of AChR,

Table III. AChE/AChR and BuChE/AChR Patches on Control and Electric Organ Extract–treated Myotubes

| | Aggregates/myotube segment | | | |
	Total	ChE	AChR	ChE/AChR
AChE/AChR staining				
Treatment				
Control	4.6 ± 0.9	2.5 ± 0.8	0.5 ± 0.2	1.6 ± 0.4
Extract	14.3 ± 2.5	0.7 ± 0.2	2.7 ± 0.4	10.9 ± 2.4
BuChE/AChR staining				
Treatment				
Control	2.6 ± 0.5	1.4 ± 0.5	0.7 ± 0.1	0.5 ± 0.1
Extract	15.3 ± 1.7	0.5 ± 0.2	6.6 ± 1.3	8.2 ± 2.1

Data, expressed as patches per 400-μm-long myotube segments, are mean ± SEM of the results from eight experiments.
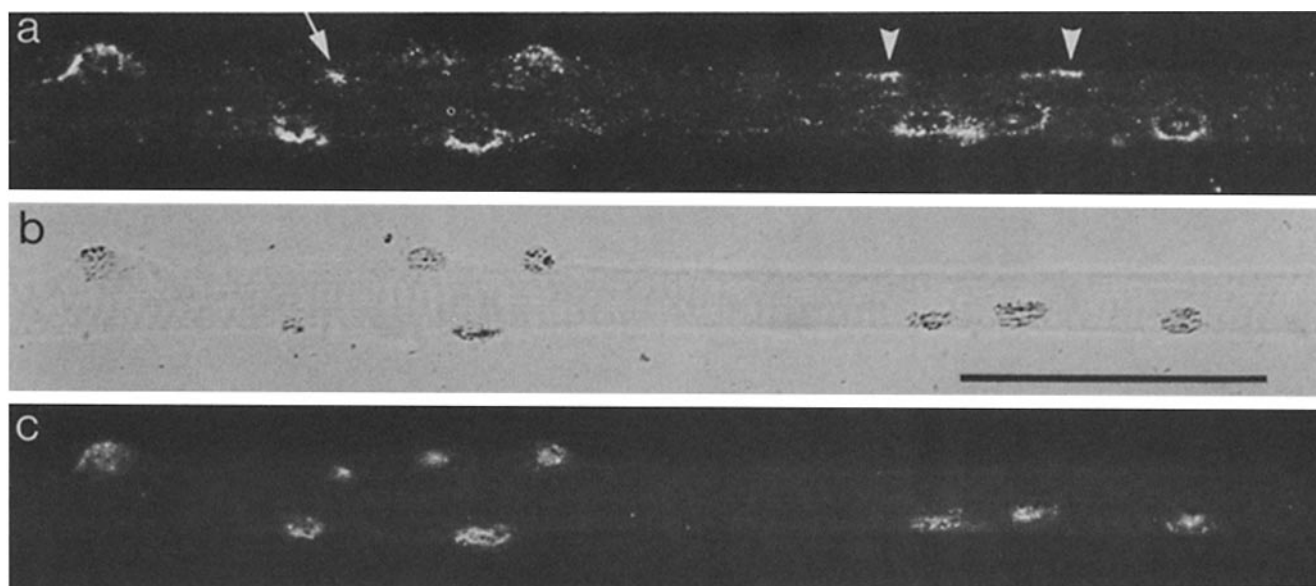
Figure 5. Co-localization of AChE, BuChE, and AChRs in extract-induced aggregates. The myotube was labeled with rhodamine-α-bungarotoxin, 1A2 anti-AChE followed by fluorescein-conjugated second antibody, and stained histochemically for BuChE activity. (a) Fluorescence micrograph with fluorescein optics to visualize AChE. (b) Phase micrograph showing patches of granular BuChE reaction product. (c) Fluorescence micrograph with rhodamine optics to show AChR distribution. Most patches contain all three labels; the fluorescent label is often partially obscured by the dense reaction product. In some patches only one (arrowheads) or two (arrow) of the labels can be detected. Bar, 50 μm.

Table IV. Occurrence of AChRs, AChE, and BuChE in Control and Extract-induced Aggregates

| | Aggregates/myotube segment | | | | | | | |
	Total	AChE	AChR	BuChE	AChE/AChR	AChE/BuChE	BuChE/AChR	AChE/BuChE/AChR
Treatment								
Control	7.3	2.7	0	1.6	0.6	2.0	0	0.4
Extract	12.2	0.7	0	0	4.0	0	0.3	7.2

Data is expressed as the mean number of aggregates per 400-μm-long myotube segment. AChE, BuChE, and AChR patches were counted on 20 myotube segments in each of two control cultures and two cultures treated for 24 h with agarose pool.

AChE/AChR, and BuChE/AChR patches detected on these sets of cultures, of the 15 patches on an average myotube segment in extract-treated cultures at least 4 or 5 patches, and perhaps as many as 8, must have contained AChRs, AChE, and BuChE. Thus, some 30 to 60% of the patches induced by electric organ extracts have high concentrations of all three of these components of the postsynaptic apparatus.

Co-localization of AChRs, AChE, and BuChE

In some experiments, the distribution of AChRs, AChE, and

BuChE was visualized on the same myotubes by labeling AChRs with rhodamine-α-bungarotoxin, AChE by 1A2 anti-AChE and fluorescein-conjugated second antibody, and BuChE by the histochemical stain for enzyme activity with butyrylthiocholine as substrate in the presence of BW284c51 (Fig. 5 and Table IV). Most patches on control myotubes in cultures stained in this way contained either AChE, BuChE, or both ChEs; only 5% of the patches contained AChE, BuChE, and AChRs. Treating myotubes with electric organ extracts caused an increase in the overall number of patches

staining for each component. As expected from the results of experiments in which AChE and BuChE were labeled in separate cultures, the electric organ extract caused a large increase in AChE/BuChE/AChR co-patches, which accounted for 59% of the patches on extract-treated myotubes. There was also an increase in AChE/AChR patches. The number of AChE, BuChE, and AChE/BuChE patches decreased. Only half of the increase in AChE/BuChE/AChR and AChE/AChR patches could have been due to the accumulation of AChRs at pre-existing ChE patches, and at least 22% of the AChE/BuChE/AChR patches on extract-treated myotubes must have arisen by the *de novo* co-accumulation of all three components. Thus, electric organ extracts induce the formation of patches that contain AChRs, AChE, and BuChE.

Co-purification of AChR-, AChE-, and BuChE-aggregating Activities

Our previous studies had demonstrated that AChR- and AChE-aggregating activities co-purified, suggesting that the same molecule was causing the formation of both types of aggregates (69). To confirm and extend these findings, the dose dependence for AChR-, AChE-, and BuChE-aggregating activity was examined at each step in our purification protocol (50). The basal lamina–containing insoluble fraction was extracted with pH 5.5 citrate buffer (pH 5.5 extract), the solubilized material was fractionated by gel filtration chromatography on agarose, and the most active fractions were pooled (agarose pool). As shown in Table V, the specific activity of all three aggregating factors was increased to the same extent by pH 5.5 extraction and gel filtration chromatography, steps that gave an overall purification of ~150-fold. Moreover, as illustrated in Fig. 6, at each step in the purification the dose dependence of aggregate formation was the same for AChRs, AChE, and BuChE.

Immunoprecipitation of AChR-, AChE-, and BuChE-aggregating Activities by Monoclonal Antibody 6D4

To provide further evidence that the same molecule causes the formation of aggregates of AChRs, AChE, and BuChE, ascites fluid containing monoclonal antibody 6D4 was titered by immunoprecipitation against all three aggregating activities. We have previously shown that 6D4, selected on the basis of its ability to bind to and inhibit AChR-aggregating activity, also binds to and inhibits ChE-aggregating activity (69) and recognizes molecules concentrated in the synaptic cleft at the neuromuscular junction (18). Fig. 7 shows that as the relative proportions of electric organ extract and 6D4 were varied, AChR-, AChE-, and BuChE-aggregating activities were reduced to a similar extent. Thus, all three aggregating activities are equally sensitive to immunoprecipitation by 6D4, as expected if they all reside in the same molecule.

Discussion

As the vertebrate skeletal neuromuscular junction differentiates, various molecules become concentrated under the nerve terminal in and on the muscle fiber, forming the postsynaptic apparatus. In this study, I have examined the

Table V. Co-purification of AChR-, AChE-, and BuChE-aggregating Activities

	Aggregating activity ($U/\mu g$ protein)		
	AChR	AChE	BuChE
Insoluble fraction	0.72	0.75	0.74
pH 5.5 extract	4.13	4.01	3.71
Agarose pool	114 ± 7.8	108 ± 6.4	107 ± 4.5

For each fraction AChR-, AChE-, and BuChE-aggregating activities were measured from dose-response curves (see Materials and Methods and Fig. 6). The data are expressed as the mean of two separate determinations for the insoluble fraction and pH 5.5 extract, and the mean ± SEM of three determinations for the agarose pool.
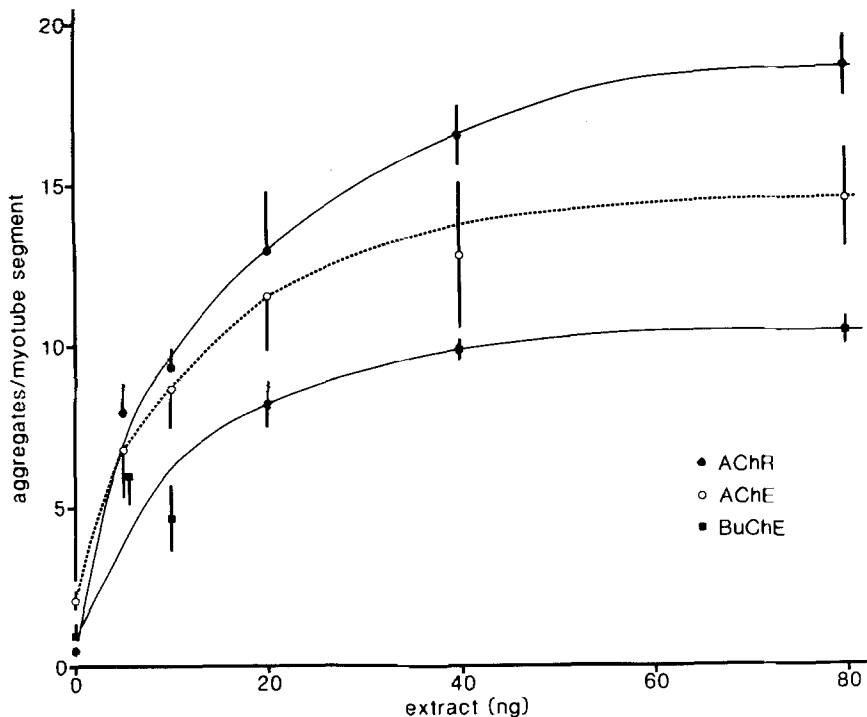

Figure 6. Dose dependence of the formation of AChE, BuChE, and AChR aggregates. Myotube cultures, treated for 24 h with the indicated amount of an agarose pool extract of electric organ, were labeled with rhodamine-α-bungarotoxin and 1A2 anti-AChE followed by fluorescein-conjugated second antibody, or with rhodamine-α-bungarotoxin and stained histochemically for BuChE activity. The mean number of aggregates per myotube segment was determined for each label; error bars show ± SEM ($n = 3$). To quantitate aggregating activity, curves were drawn through the points by eye, and 1 U of activity was taken as the amount of the extract required to give a half-maximal response. In the experiment illustrated, this was 9 ng/U for AChE-, 8.7 ng/U for BuChE-, and 9 ng/U for AChR-aggregating activity. Similar results were obtained for the insoluble fraction and the pH 5.5 extract (data not shown).
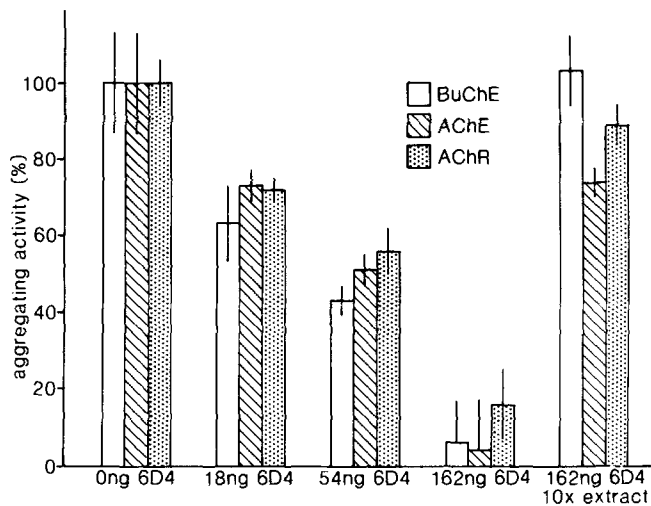
• AChR
○ AChE
■ BuChE

Figure 7. Immunoprecipitation of BuChE-, AChE-, and AChR-aggregating activities by monoclonal antibody 6D4. 120 ng of an agarose pool extract of electric organ (or 1,200 ng, as indicated) were mixed with the designated amount of 6D4 in 300 µl of minimum essential medium (containing 3 mg/ml BSA) and incubated for 2 h at 37°C. Immune complexes were precipitated by adding 400 µl of a suspension of Sepharose beads conjugated with goat anti-mouse IgG (sufficient to bind 4.6 µg mouse IgG [23]) and mixing for 1 h at room temperature. Aliquots (100 µl) of the resulting supernatant were added to each of six cultures, which were incubated overnight and stained for BuChE and AChRs (three dishes) or AChE and AChRs (three dishes). The mean number of BuChE, AChE, and AChR aggregates per myotube segment was determined for each set of cultures. As the relative amounts of extract and 6D4 were varied, all three aggregating activities were reduced by the same extent. Aggregating activity is expressed as the percentage of the mean number of aggregates per myotube segment in cultures receiving extract not treated with 6D4. Error bars are ± SEM ($n = 3$).

effects an extract of *Torpedo* electric organ had on the distribution of three components of the postsynaptic apparatus, AChRs, AChE, and BuChE, on chick myotubes in culture. The results indicate that a single factor in the electric organ extracts induces the formation of patches on the myotube surface in which all three of these components accumulate simultaneously.

Accumulation of AChR, AChE, and BuChE at Electric Organ Extract–induced Patches

Treating cultures of chick myotubes with electric organ extracts increased the number of patches from three- to sixfold; there was on average a large increase in the number of AChR/ChE co-patches, a smaller increase in AChR patches, and a small decrease in the number of ChE patches. The increase in the number of AChR/ChE co-patches was always greater than the decrease in ChE patches. Thus, although electric organ extracts may cause some accumulation of AChRs at pre-existing ChE patches, the primary effect of treating cultures with electric organ extracts was to increase the number of AChR/ChE co-patches by the *de novo* accumulation of both AChRs and ChE.

The electric organ extracts caused AChR/ChE aggregation in defined medium. Indeed, removing horse serum and embryo extract from the medium for the final 24 h of culture increased the intensity of ChE staining on the myotubes and reduced by twofold the amount of electric organ extract

required to elicit a given increase in patch number, as indicated by the increase in specific activity, as compared with assays of the same extracts done in normal medium (compare Table V with Table III in reference 69). Thus, the aggregating activity is not dependent on some factor in chick embryo extract or horse serum.

The ChE that accumulates at avian and rodent skeletal neuromuscular junctions in vivo includes both AChE and BuChE. Although BuChE accounts for only 1–10% of the total ChE activity in extracts of whole adult muscles (42, 43, 75), ~30% of the ChE at endplates in adult muscles is BuChE (29, 30, 34, 56). The level of BuChE activity is higher in extracts of embryonic and newborn muscles, accounting for 10–60% of the total ChE (10, 42), and, like AChE, in these muscles BuChE is found diffusely in extrajunctional regions of the myofiber as well as concentrated in discrete patches at the endplates (77). In our cultures of chick myotubes, pharmacological and immunohistochemical experiments demonstrated that ChE patches on both control and extract-treated myotubes included AChE and BuChE, and that 30 to 60% of the patches induced by electric organ extracts contained both AChRs, AChE, and BuChE. Thus the patches induced by electric organ extracts on myotubes in vitro resemble those at neuromuscular junctions in vivo in that they contain high concentrations of AChRs, AChE, and BuChE.

In adult muscles, AChE and BuChE are found on the surface of myofibers in both membrane-bound forms and in association with the extracellular matrix (45). At mature neuromuscular junctions, much of the ChE is associated with the synaptic basal lamina (47). The primary species of AChE at developing rat neuromuscular junctions is the 16S (A_{12}) form (77), a form generally considered to be associated with extracellular matrix, at least in adult muscle (45). However, 16S enzyme is detected on the surface of myofibers as early as embryonic day 14 (77) and on the surface of rat pheochromocytoma PC12 cells (27), neither of which have a well-developed sheath of basal lamina. Thus, it is not known whether ChE accumulating at newly formed neuromuscular junctions is associated with the cell membrane or with extracellular structures or both (77).

The staining procedures employed in the experiments reported here only detect AChRs accessible from the extracellular space; thus, the change in distribution caused by electric organ extracts involved AChRs in the myotube plasma membrane. Similarly, the experiments in which AChE was visualized using monoclonal antibodies were done under conditions where only AChE accessible from the extracellular space was labeled and so provide information about changes in the distribution of surface AChE. The technique used to detect BuChE activity is not selective for extracellular enzyme, although the reaction product that accumulated during the histochemical staining for BuChE appeared by light microscopy to be primarily on the surface of the myotubes. No information is available concerning the form of AChE or BuChE in control or extract-induced patches on chick myotubes, or whether these enzymes are membrane bound or associated with the extracellular matrix.

The time course of appearance of AChRs and ChE on factor-treated myotubes supports the hypothesis that these two components accumulate simultaneously, rather than AChRs accumulating at pre-existing ChE patches or vice versa. At developing neuromuscular junctions, both in vivo

and in vitro, the accumulation of AChRs occurs, at least in part, by the lateral migration within the plasma membrane of AChRs that were diffusely distributed over the myotube surface before innervation (1, 76), although it has recently been shown that cholinergic neurites also increase the rate of local insertion of new AChRs at sites of nerve-muscle contact in vitro (57). The accumulation of AChRs induced by electric organ extracts also has been shown to be due, at least in part, to the lateral migration of AChRs in the myofiber plasma membrane (23). Like AChRs, AChE is distributed diffusely over uninnervated myotubes in vivo and rapidly accumulates at sites of nerve-muscle contact (5, 20, 25, 77). The mechanism of AChE accumulation is not known. If AChE on the surface of uninnervated myofibers is associated with a stable sheath of extracellular matrix material, then accumulation by lateral migration would appear to be unlikely and some other mechanism involving enhanced local insertion or stabilization would be necessary. Alternatively, the initial accumulation observed in vivo might be due to lateral migration of membrane-bound ChE, the association of ChE with the basal lamina being a subsequent event in synapse formation.

Assembly of the Postsynaptic Apparatus

Several lines of evidence suggest that muscle cells contain all of the information necessary to construct a complete postsynaptic specialization and require only a signal to tell them where and when to assemble the components. For example, denervated and regenerating muscle fibers in vivo form patches that have a high concentration of AChRs and ChE (37, 46, 67). Similar patches are found, although only rarely, in aneural cultures of muscles from chick and rat (60, 64) and, much more frequently, in cultures of muscles from frog (49). Moreover, many of the patches found on chick myotubes and *Xenopus* myotomal muscle cells in aneural cultures also have ultrastructural specializations resembling those seen at neuromuscular junctions (8, 49, 72–74). Extracts from various sources cause AChR aggregation (4, 12, 13, 31, 32, 44, 53, 62), and many of the AChR patches formed in response to at least one of these extracts also have associated with them synapse-specific antigens, AChE, and invaginations in the plasma membrane that resemble junctional folds (64). Even positively charged latex beads can induce AChR aggregation and many morphological features that resemble postsynaptic specializations at sites where they contact muscle cells in culture (51). Thus, muscle cells appear competent to assemble a complete subsynaptic specialization in the absence of a synaptic partner. Moreover, this assembly can be triggered by several different signals, some clearly nonphysiological.

Monomolecular Induction of Postsynaptic Specializations at Regenerating Neuromuscular Junctions

What molecules trigger the differentiation of the postsynaptic apparatus on muscle cells in vivo? Is it known that components of the synaptic basal lamina can induce the formation of postsynaptic specializations on regenerating myofibers (2, 7, 47). My co-workers and I have previously shown that a factor in our basal lamina–containing extract of *Torpedo* electric organ causes aggregates of AChRs and AChE on chick myotubes in culture and that this factor is antigenically related to molecules concentrated in the synaptic cleft at the neuromuscular junction (18, 69). This led us to hypothesize that

molecules in the synaptic basal lamina that direct the formation of AChR aggregates on regenerating myofibers also cause aggregation of AChE (69). This report demonstrates that the effect of the electric organ factor is to induce patches in which three components of the subsynaptic apparatus at mature neuromuscular junctions, AChRs, AChE, and BuChE, are concentrated. Extrapolating from these results, I propose to extend our monomolecular-induction hypothesis (69) to include the formation of a complete postsynaptic specialization; that is, that a molecule in the synaptic basal lamina similar to the active component in our extracts of electric organ directs the assembly of all components of the postsynaptic apparatus on regenerating myofibers.

The formation of a mature postsynaptic apparatus in muscle cells, however, depends on other factors as well. A conspicuous feature of the patches of AChRs and AChE on denervated or cultured myofibers is the relatively low level of ChE. Under our normal culture conditions it requires 1–1.5 h of staining before reaction product can readily be detected, whereas similar protocols stain ChE at adult neuromuscular junctions intensely in a few minutes. Apparently, the accumulation of high levels of ChE requires an additional signal(s). The intensity of ChE staining on myofibers in culture was increased by omitting horse serum and chick embryo extract from the culture medium. Horse serum has been shown to decrease the level of ChE activity in cultures of chick myotubes, especially of the 20S (A_{12}) form (36, 68). Even under these conditions patches of ChE on cultured myotubes appeared to stain less intensely than adult neuromuscular junctions. Several studies, both in vivo and in vitro, indicate that muscle activity plays an important role in regulating the accumulation of AChE at both junctional and ectopic sites, at least in chick and rat muscles. For example, the loss of ChE at denervated adult neuromuscular junctions can be reversed if muscle fiber activity is restored by direct electrical stimulation or by innervation at an ectopic site (24, 41, 70). Likewise, blocking nerve-induced contractions in spinal cord-muscle co-cultures prevents the accumulation of AChE at newly formed synapses (38, 61). Thus, the information conveyed by the synaptic basal lamina specifies where the assembly of subsynaptic components is to take place and initiates this process, but the extent of accumulation of AChE can be regulated by other factors, including muscle activity. Other modifications in subsynaptic components that occur after synapses have formed, such as the change in receptor turnover and channel open time (see references 16 and 11), might also occur as a natural consequence of myofiber activity or maturation, and thus not require additional signals from the basal lamina.

Synaptic Differentiation during Embryogenesis

During normal embryogenesis the nerve releases a signal(s) that induces postsynaptic specializations on developing myofibers (see reference 16); during regeneration in adult muscles components of the synaptic basal lamina can trigger subsynaptic differentiation in the absence of the nerve terminal (7, 46). Similarly, contact with its target myotube appears to cause differentiation of developing nerve terminals, whereas regenerating nerve terminals differentiate when they contact synaptic basal lamina even in the absence of a muscle cell (65). Thus, synaptic basal lamina in the adult provides the same synaptogenic information as nerve terminals and my-

ofibers do during embryogenesis. In light of the observation that basal lamina is present at a very early stage of synapse formation (3, 11, 22, 28, 35, 40, 66, 71), it seems reasonable to propose that the growing nerve terminal initiates synapse formation by inserting into the myofiber's nascent basal lamina sheath a molecule identical to the component of the synaptic basal lamina in the adult that directs the differentiation of regenerating myofibers. Moreover, perhaps by virtue of its association with the basal lamina or by being modified by the myofiber, this same molecule might then also direct differentiation of the presynaptic nerve terminal, just as contact with positively charged beads in vitro not only induces postsynaptic specializations in myofibers (51) but also causes the formation of apparent presynaptic specializations in nerve terminals (9). Purification and further characterization of the electric organ AChR/ChE-aggregating factor should enable us to determine if this molecule directs assembly of a complete postsynaptic apparatus, if it acts at developing synapses in addition to regenerating ones, and if it regulates presynaptic differentiation as well.

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