

Effect of Agrin on the Distribution of Acetylcholine Receptors and Sodium Channels on Adult Skeletal Muscle Fibers in Culture

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Abstract. We used the loose patch voltage clamp technique and rhodamine-conjugated α -bungarotoxin to study the regulation of Na channel (NaCh) and acetylcholine receptor (AChR) distribution on dissociated adult skeletal muscle fibers in culture. The aggregate of AChRs and NaChs normally found in the postsynaptic membrane of these cells gradually fragmented and dispersed from the synaptic region after several days in culture. This dispersal was the result of the collagenase treatment used to dissociate the cells, suggesting that a factor associated with the extracellular matrix was responsible for maintaining the high concentration of AChRs and NaChs at the neuromuscular junction. We tested whether the basal lamina protein agrin, which has been shown to induce the aggregation of AChRs on em-

bryonic myotubes, could similarly influence the distribution of NaChs. By following identified fibers, we found that agrin accelerated both the fragmentation of the endplate AChR cluster into smaller patches as well as the appearance of new AChR clusters away from the endplate. AChR patches which were fragments of the original endplate retained a high density of NaChs, but no new NaCh hotspots were found elsewhere on the fiber, including sites of newly formed AChR clusters. The results are consistent with the hypothesis that extracellular signals regulate the distribution of AChRs and NaChs on skeletal muscle fibers. While agrin probably serves this function for the AChR, it does not appear to play a role in the regulation of the NaCh distribution.

THE postsynaptic specialization at the neuromuscular junction is a highly organized and complex structure. The most prominent component of this specialization is the acetylcholine receptor (AChR)¹, which has been shown to be concentrated about 1,000-fold compared with the extrajunctional membrane (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1983). In addition, several cytoskeletal and basal lamina proteins have been shown to be concentrated in this region (see reviews by Salpeter and Loring, 1985; Bloch, 1989). These include the cytoskeletal 43-kD protein, which is closely associated with the AChR (Froehner et al., 1981), as well as a heparan sulfate proteoglycan (Anderson and Fambrough, 1983; Bayne et al., 1984), acetylcholinesterase (Hall and Kelly, 1971; Massoulié and Bon, 1982), and a synaptic form of laminin (Hunter et al., 1989) in the basal lamina.

Aside from the AChR, the voltage-activated sodium channel (NaCh) is the only other major integral membrane protein known to be enriched in the postsynaptic membrane (Nastuk and Alexander, 1973; Thesleff et al., 1974; Betz et al., 1984). NaCh densities on adult skeletal muscle fibers were shown, by using loose patch clamp recording, to be

concentrated 10- to 20-fold at the neuromuscular junction (Beam et al., 1985; Caldwell et al., 1986; Roberts, 1987). This was recently confirmed at the light microscopic (Haimovich et al., 1987) and ultrastructural level (Flucher and Daniels, 1989) using antibodies to the NaCh. It was found that, while the 43-kD protein and AChR are both enriched at the crests of the postjunctional folds, the NaCh and the cytoskeletal protein ankyrin are found in the postjunctional troughs. Like the synaptic density of AChRs, the density of NaChs remains high at the neuromuscular junction even after denervation of the muscle (Caldwell and Milton, 1988).

The mechanisms directing the organization of the postsynaptic specialization have been the subject of considerable research. The formation of AChR clusters on embryonic myotubes is one of the earliest signs of synaptogenesis on muscle cells, occurring around the time of initial neurite-myotube contact (Bevan and Steinbach, 1977; Braithwaite and Harris, 1979; Lupa and Hall, 1989). Studies of nerve-muscle co-cultures demonstrated that neurites induce the formation of AChR patches at points where they contact myotubes (Anderson and Cohen, 1977; Frank and Fischbach, 1979). This simple but important observation led several groups to attempt to isolate from neural tissue factors capable of inducing the formation of AChR clusters on embryonic myotubes in culture. Several factors that could increase AChR expression (Neugebauer et al., 1985; Usdin and Fischbach,

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; NaCh, sodium channel; FDB, flexor digitorum brevis; rho-Butx, rhodamine-conjugated α -bungarotoxin.

1986; New and Mudge, 1986; Fontaine et al., 1986; Harris et al., 1988) or clustering (Christian et al., 1978; Podleski et al., 1978; Vyskocil and Syrový, 1979; Olek et al., 1983) were isolated from embryonic brain extract. A protein or family of proteins, termed agrin, has been isolated from an extract of *Torpedo* electric organ and shown to induce the formation of AChR clusters on myotubes in culture (Rubin and McMahan, 1982; Nitkin et al., 1987). Antigenically similar proteins are present on embryonic myotubes (Fallon and Gelfman, 1989), in adult synaptic basal lamina (Fallon et al., 1985; Reist et al., 1987), and in motor neurons (Magill-Solc and McMahan, 1988). This has led to the hypothesis that deposition of agrin at sites of nerve-muscle contact plays a primary role in the development of the post-synaptic specialization (Magill-Solc and McMahan, 1988; Wallace, 1989; Fallon and Gelfman, 1989).

In contrast to our knowledge of AChR clustering, little is known about how or when the synaptic concentration of NaChs develops. In one study, Angelides (1986) reported that NaChs became localized and immobilized to sites of neurite-induced AChR clusters in co-cultures of chick myotubes and spinal cord neurons. Because of the similarity in distribution of AChRs and NaChs on adult muscle fibers, and since agrin has been shown to influence the distribution not only of the AChR, but also of basal lamina and cytoskeletal proteins (Wallace, 1989; Nitkin and Rothschild, 1990), we tested the effect of agrin on NaCh distribution. We chose to use dissociated adult muscle fibers for this study because they have a significantly higher density of NaChs than embryonic myotubes. Our results reveal that agrin induces a fourfold increase in the number of AChR patches on cultured adult muscle fibers, similar to its effect on embryonic myotubes. However, agrin had little effect on the distribution of NaChs, and we conclude that this form of agrin does not play a role in the induction of the synaptic NaCh concentration. The results suggest that the localization of AChRs and NaChs is regulated through different processes.

Materials and Methods

Cell Culture

Flexor digitorum brevis (FDB) muscles from adult rats and mice were dissociated into single fibers essentially as described previously (Bekoff and Betz, 1977; Milton and Caldwell, 1990). Muscles were placed in a tube containing DMEM with 2.5 mg/ml collagenase type B (Boehringer-Mannheim GmbH, Mannheim, Germany) and 1 mg/ml BSA (Sigma Chemical Co., St. Louis, MO). After incubation at 36°C on a rotating wheel for 1–2 h, the muscles were gently triturated using Pasteur pipettes of decreasing tip diameter. FDB muscles were denervated before dissociation by cutting the sciatic nerve through an incision in the mid thigh.

The cell suspension was dropped onto 22 × 22-mm glass coverslips that had been coated with Matrigel (Collaborative Research Inc., Lexington, MA). The culture medium consisted of DME plus 7% heat-inactivated horse serum, 2% FBS, 1% *L*-glutamine, and 1% pen/strep, and was changed every two to three days. The muscles were incubated at 36°C, with 95% O₂ and 5% CO₂. Cytosine arabinoside was added on day 2 for 24–48 h to inhibit proliferation of dividing cells. All culture reagents were purchased from Gibco Laboratories (Grand Island, NY).

Intact FDB muscles were organ cultured in 35-mm plastic Petri dishes containing cured Sylgard resin; these dishes were sterilized with 70% EtOH and ultraviolet irradiation before use. Muscles were dissected into sterile Ringer solution (146 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 11 mM glucose, 2 mM Na₂Pipes, pH 7.3) and either placed directly into culture or treated with collagenase before culturing. Muscles were pinned at a loose resting position and covered with a culture medium consisting of

DME plus 3% heat-inactivated horse serum, 1% FBS, 1% *L*-glutamine, and 1% pen/strep. The medium was changed daily.

Loose Patch Clamp Recording

Coverslips were transferred to the stage of an inverted Nikon Diaphot microscope and bathed in Ringer solution at room temperature (21–23°C). Loose patch voltage clamp recording was accomplished with lightly fire-polished borosilicate electrodes of 6–12 μm tip diameter (200–400 KΩ resistance) filled with filtered Ringer solution. The voltage clamp circuit used has been described in Almers et al., 1983. An inchworm piezoelectric controller (Burleigh Instruments, Fishers, NY) was used to aid positioning and movement of the electrode. The electrode was advanced in 2 μm steps until contact with the muscle fiber produced a seal resistance one to two times the electrode resistance. Slight suction (5–15 mm Hg) was usually applied to enhance the electrical isolation. Care was taken to avoid production of blebs inside the electrode (Milton and Caldwell, 1990). All recordings were done with a constant potential applied through the patch electrode that made the membrane potential 70 mV more negative than the resting potential; this potential was applied for 1–2 min before recording to reduce slow inactivation of Na channels (Almers et al., 1983). Na currents were either photographed directly from the oscilloscope using a Polaroid C-5C camera, or digitized with a DEC 11/73 computer, and printed on a DataGeneral Plotter (Hewlett-Packard Co., Palo Alto, CA). Na currents were converted to current density by assuming that the area of membrane voltage clamped was equal to the area calculated from the pipette tip diameter; this calculation does not take into account membrane folding under the pipette.

Threshold for NaCh activation was recorded in order to estimate resting membrane potential of the muscle fibers. Assuming that the threshold membrane potential for NaCh activation is ~–50 mV (Pappone, 1980; Gonoï et al., 1989), the resting membrane potential was estimated to be –50 to –70 mV, and fibers with a resting potential less than –45 mV were not used. The holding potential in these experiments was thus equal to the steady applied potential (–70 mV) plus the resting membrane potential (–50 to –70 mV), or –120 to –140 mV.

We classified NaCh “hotspots” in two ways. For most of this work, NaCh hotspots were identified on single fibers as recordings where current density was more than twice the mean level on that fiber at sites without an AChR cluster. While it might be more accurate to call some of these clusters “warmspots” rather than “hotspots”, this low criterion value was chosen to ensure that we did not ignore incipient or newly formed aggregates. As a second method of classification, we examined the distribution of Na current densities in several populations of recordings. The distribution of Na current densities measured on cultured FDB fibers at sites with no apparent AChR cluster approximated a normal distribution (data not shown). This was taken as the basal level of NaCh density in the membrane, and hotspots were designated as any Na current density recording greater than the mean current density plus 2.5 times the standard deviation (which includes 99% of the normal distribution). The results obtained from these two methods were closely similar; a few of the NaCh hotspots may thus actually be part of the normal population. It should be noted that although some of the measurements were as close as 5 μm apart, we did not make enough recordings to obtain information on the size or shape of the hotspots.

Agrin

Agrin was prepared and generously provided by Dr. Justin Fallon of the Worcester Foundation (Worcester Foundation, Shrewsbury, MA). The agrin preparation used was an extract of *Torpedo* extracellular matrix, eluted from a column of Cibacron Blue 3GA-agarose, with an activity of about 1 U/μl (Nitkin et al., 1987). One unit of activity is defined as the amount of agrin needed to induce half-maximal aggregation of AChRs on chick myotubes after 24 h (Godfrey et al., 1984). Agrin was usually added directly to the medium at a concentration of 5–7 U/ml. For direct application to endplates, 100 μl of a 6–10 U/ml agrin solution (in DME) was pulled into the tip of a loose patch electrode (tip diameter 15–20 μm) by suction. The electrode was maneuvered directly over an endplate and slight positive pressure was applied, ejecting the agrin solution at a rate of ~3 μl/min, usually for 5–10 min.

Immunocytochemistry

mAb 210, which binds to an extracellular portion of the AChR (Ratnam et al., 1986), was generously provided by Dr. J. Lindstrom, Salk Institute (La Jolla, CA). Polyclonal secondary antibodies were purchased from Cappel

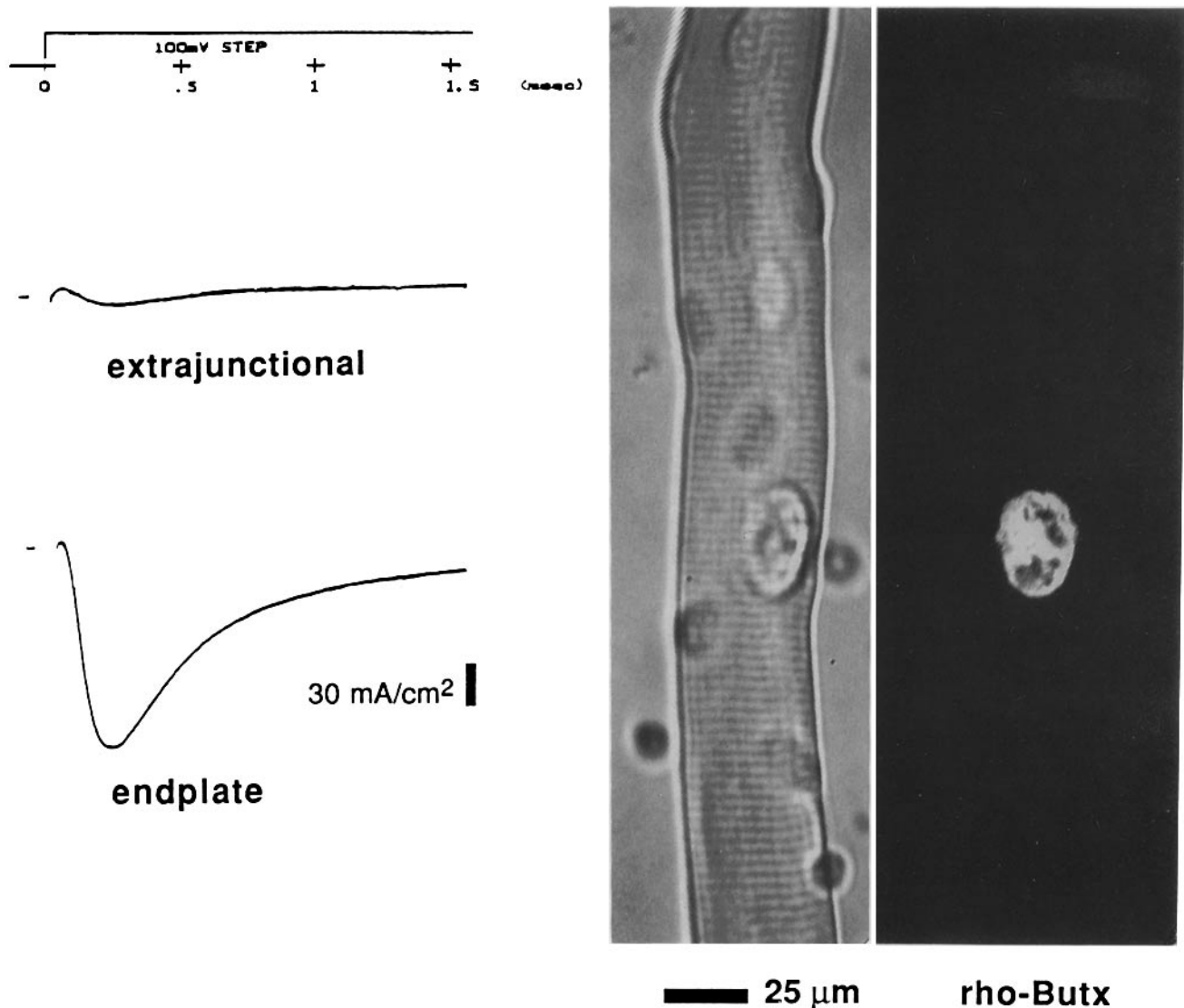


Figure 1. Example of preparation and technique used to study distribution of AChRs and NaChs on skeletal muscle fibers. (*Right*) Acutely dissociated muscle cell from an adult mouse FDB muscle. On the left is a Hoffman phase contrast image of a single fiber. On the right is the fluorescence image after labeling with rho-Butx to visualize AChRs. A single dense patch of AChRs is found at the endplate region of the cell. (*Left*) Na current recordings made at the endplate and extrajunctional areas (200 μm from endplate), using the loose patch voltage clamp. An electrode with a 7.5- μm -diam tip was used, and the maximum Na current was obtained with a + 100 mV applied step. Na current density was $\sim 130 \text{ mA/cm}^2$ at the endplate, 7 mA/cm^2 in the extrajunctional membrane.

Laboratories (Malvern, PA). Rhodamine-conjugated alpha-bungarotoxin (rho-Butx) was prepared according to Ravdin and Axelrod (1977).

Live myofibers were labeled with rho-Butx and/or FITC-mAb 210, diluted in culture medium, by incubation for 1–2 h in a 36°C incubator. Myofibers were fixed in ice-cold 3.0% paraformaldehyde in PBS with 0.1% saponin, rinsed two to three times with PBS or Ringer, and treated with 0.1 M glycine for 30 min. Fixed cultures were incubated with PBS/7% FBS/3% BSA for 10–20 min to inhibit nonspecific antibody binding. Myofibers were incubated with primary antibody overnight at 4°C, washed with PBS or Ringer, labeled for 1 h with secondary antibody at room temperature, and rinsed several more times with PBS or Ringer. Coverslips were mounted on glass slides with a glycerol-based solution containing p-phenylenediamine (Platt and Michael, 1983). Preparations were viewed and photographed with a microscope (Axiophot; Zeiss, Oberkochen, Germany) under epifluorescent illumination, and photographed with TMAX-p3200 film (Eastman Kodak Co., Rochester, NY).

Video Microscopy

A silicon-intensified camera (model 66X-Dage-MTI Inc., Wabash, WI) was

used to capture video images from a Nikon Diaphot inverted microscope (Nikon Inc., Garden City, NJ) equipped with a 100 W Hg source for epifluorescence. Images were taken through a 40 \times , 0.65 NA or a 20 \times , 0.4 NA objective; a Nikon continuous 0.75–2.3 zoom lens was placed between the microscope and the camera. Images were digitized with 12-bit resolution (256 grey levels), viewed on a Sony Trinitron color video monitor, and stored as a 512 \times 480 pixel array in a Silicon Graphics Iris computer. Further image processing was accomplished with a software package from G. W. Hannaway (Boulder, CO). Hard copies were produced on a Kodak XL 7700 color printer.

To follow surface AChRs over several days, cells were labeled with 60 nM rho-Butx in a 36°C incubator for 1–2 h. Increasing either the concentration of rho-Butx or the incubation time did not increase fluorescence intensity, suggesting that this concentration of rho-Butx was sufficient to saturate the surface AChRs. Averaged images (8–36 samples) of cells were captured using low-level epifluorescence (6–13% light intensity) with neutral density filters for short periods of time (0.2–1.0 s). Cells were relocated by using stage calibrations and referring to low-magnification phase photographs. An off-focus image of an empty field was subtracted from most images to reduce background, and most images were contrast enhanced using a linear grey scale expansion.

Statistics

Values are expressed as mean \pm SEM. Statistical significance was tested using students' *t* test or the χ^2 test for comparison of ratios.

Results

It was necessary to use the loose patch clamp technique for assaying NaCh density because antibodies and other molecular probes lack the sensitivity to detect NaChs in extrajunctional membrane. We estimate that the limit of sensitivity for our recordings corresponds to ~ 10 Na channels/ μm^2 , and since it is a quantitative technique, small differences can be easily measured. We chose to use adult dissociated muscle fibers for this study, instead of embryonic myotubes, because myotubes possess a sodium current density of around 1 mA/cm² (Milton and Caldwell, unpublished data), which is near the limit of resolution for the loose patch technique, while the Na current density in adult extrajunctional muscle membrane is ~ 10 -fold higher. The presence of the endplate, with its high concentration of AChRs and NaChs, was not crucial to this study, but allowed us to gain insight into the maintenance of the postsynaptic specialization.

Dissociated FDB muscle fibers survived well in culture for 1–2 wk, with most fibers retaining cross-striations and the ability to contract in response to electrical stimulation. However, most fibers also underwent gradual morphological and physiological changes. The cell membrane was often distended by bulging nuclei, suggesting movement of nuclei or loss of cell volume. Some fibers appeared to dedifferentiate, losing striations and exhibiting focal adhesion feet, similar to cultured myotubes (Bekoff and Betz, 1977; Gillespie and Ribchester, 1988; Hinterberger and Barald, 1990). Some fibers exhibited spontaneous fibrillation in culture, leading to detachment from the substrate and eventual cell death. Several changes also occurred at the endplate. Immediately after being dissociated, FDB fibers possessed a single dense patch of AChRs located at the endplate site, where NaChs were also concentrated about 20-fold (Fig. 1). Only minor changes occurred over the next two days, but by 3–4 d *in vitro* the AChR patch at the endplate had clearly begun to change shape, becoming somewhat fragmented and diffuse (Fig. 2). After 5–6 d in culture, the AChR distribution at the endplate was irregular and more dispersed, with some bright clusters and areas of speckled microclusters. After approximately one week in culture it was often difficult to identify the original endplate area and many fibers developed extrajunctional AChR patches.

A similar redistribution of NaChs could be monitored by applying the loose patch clamp technique to cultured FDB cells. As shown in Fig. 3, no significant change in Na current density was found over the first two days in culture. However, from day 3 onward a gradual reduction in Na current density was measured at the endplates of cultured FDB fibers; this reduction closely paralleled the breakup and fading of the AChR cluster at these endplates.

Muscle cells which had been denervated before culturing underwent changes identical to those shown in Figs. 2 and 3, but the process was accelerated by 24–36 h. Also, pre-denervated fibers seemed to acquire extrajunctional AChR clusters more readily, making them advantageous for this study (see also Bekoff and Betz, 1977; Ko et al., 1977). Most

of the experiments described in the following sections thus used FDB muscle cells denervated previously for 5–15 d.

To test whether the dissipation of AChRs and NaChs at the endplate was because of culture conditions or the collagenase treatment, FDB muscles were placed in organ culture for 3–7 d and assayed for AChR and NaCh distribution. Muscles which were kept in organ culture without collagenase pre-treatment retained their endplate concentrations of AChR and NaCh. In contrast, those muscles which were collagenase treated (but not dissociated) before being placed in culture exhibited faded rho-Butx fluorescence and reduced NaCh density at the endplate. Sodium current density at endplates of untreated FDB muscles organ cultured for 3–7 d was 81.1 ± 3.6 mA/cm² (nine fibers), while the corresponding Na current density at collagenase-treated, organ-cultured endplates was 28.1 ± 1.9 mA/cm² (10 fibers), a reduction that was highly significant ($p < 0.001$). Thus, treatment with collagenase, rather than culture conditions, was responsible for the dispersal of endplate AChRs.

Sodium Channel Density at Sites of AChR Clusters

The previous observations implied that NaCh and AChR distributions might be regulated by the same process. We were thus interested in testing whether NaCh density was increased at sites of AChR clusters that developed in culture. Na current densities were mapped on eight FDB fibers which had developed several clusters of AChRs on their surface. The mean Na current density at areas of low AChR density, where no AChR cluster was apparent, was taken as the basal level of Na current density on the muscle cells. NaCh "hotspots" were defined as any recording where the Na current density was more than twice this basal level (see Materials and Methods).

An example of a fiber with its AChR clusters and corresponding Na current density distribution is shown in Fig. 4. Only one of four AChR clusters on this muscle cell exhibited a Na current density high enough to be considered a NaCh hotspot. Overall, 50% of the recordings (11/22) made at AChR clusters on eight fibers demonstrated NaCh hotspots, with 7.7% of the recordings (2/26) made at regions of low AChR density also exhibiting NaCh hotspots. Thus, the probability of finding a NaCh hotspot was significantly higher at sites of AChR clusters than elsewhere on the muscle fiber.

These experiments suggested two possible mechanisms by which the distribution of NaChs could be regulated on muscle cells. First, there could be a tendency for NaChs to aggregate at any site of high AChR density. Second, the NaCh could be immobilized only at certain areas, such as the adult endplate, by a basal lamina or cytoskeletal protein which may also act on the AChR. An obvious candidate for this protein would be agrin, which has been shown to induce clustering of AChRs on embryonic myotubes (Rubin and McMahan, 1982; Nitkin et al., 1987). Therefore, we tested agrin for its effect on the distribution of AChRs and NaChs on adult muscle cells.

Effects of Agrin on AChRs and NaChs on Cultured FDB Cells

FDB muscle fibers were dissociated onto coverslips and cultured overnight. After ~ 18 h in culture, agrin (3–7 U/ml) was

rho-Butx

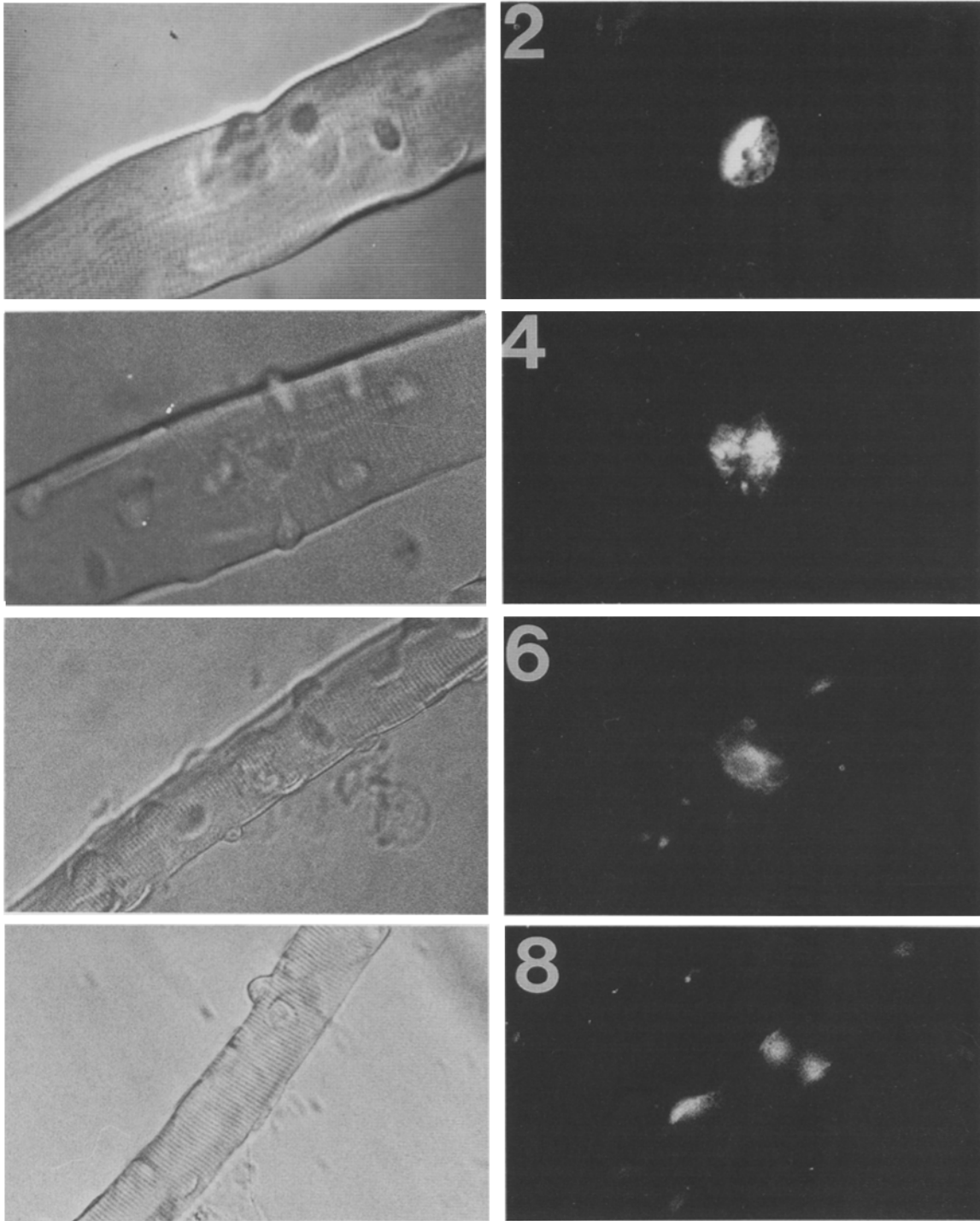


Figure 2. Examples of FDB muscle fibers kept in culture for up to eight days. On the left are Hoffman phase photographs of cells; on the right are the fluorescence photographs of the same cells after labeling the AChRs with rho-Butx. Numbers in the fluorescence panels refer to the number of days in culture. Note the gradual fragmentation and dissipation of the AChR cluster at the endplate, as well as the appearance of several AChR patches over the surface of the cultured cells. Bar: (Days 2-4) 45 μm ; (Days 6-8) 80 μm .

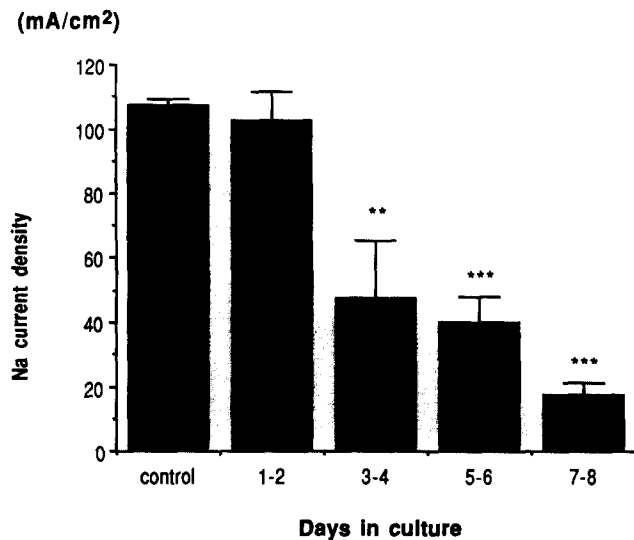


Figure 3. Na current density at endplates of FDB muscle fibers in culture. Each bar represents the mean \pm SEM of recordings from 10–25 cells. For the first 1–2 d of culture the Na current density at the endplate region remained similar to the density on acutely dissociated muscle cells (*control*). However, from day 3 onward there was a gradual reduction in the Na current density recorded at the endplate. **, $p < 0.01$; ***, $p < 0.001$.

added to the medium. One to two days later, the cells were labeled with rho-Butx and the distribution of AChRs on the cell surface was examined. As illustrated in Fig. 5, agrin induced an increase in the number of AChR aggregates on FDB fibers, when compared to untreated control fibers. The AChR clusters were found both on the top and bottom of the

fibers, and most were 5–10 μm in diameter, though many small microclusters were also visible. It was often difficult to visualize the original endplate region on agrin-treated muscle cells. When the effect of agrin was quantified, it was found that agrin caused an approximately fourfold increase in the number of AChR clusters per fiber (Fig. 6). However, the effect of agrin varied from fiber to fiber, inducing 1–20 AChR patches per cell. Control fibers generally exhibited one to two AChR clusters, one of them being the original endplate, while agrin-treated fibers had on average six to eight clusters.

The effect of agrin on the distribution of NaChs was less dramatic. Na currents were first recorded from the extrajunctional regions, devoid of AChR clusters, of a large number of control muscle fibers that had been in culture (without agrin) for 2–3 d. This was done to determine the normal variability of NaCh density on these cells, so that a valid comparison could be made with agrin-treated fibers. The current densities recorded fit a normal distribution, with a mean of 11.3 ± 0.7 mA/cm². Three of 49 (6.2%) of the recordings qualified as NaCh hotspots. Fig. 7 shows the range of Na current densities measured on fibers cultured with agrin added to the medium. Recordings made at regions of low AChR density on agrin-treated fibers gave a mean Na current density of 12.3 ± 1.6 mA/cm², which was not different from control fibers. The percentage of recordings revealing NaCh hotspots (11%; 5/46) was slightly but insignificantly greater than the percentage obtained from control fibers. Thus, agrin did not increase average NaCh density, nor did it induce additional hotspots of NaCh in this portion of the muscle cell membrane. Recordings of Na current made at AChR patches ranged from 0 to 53.4 mA/cm², with a mean of 18.9 ± 1.9 mA/cm². Mean current density

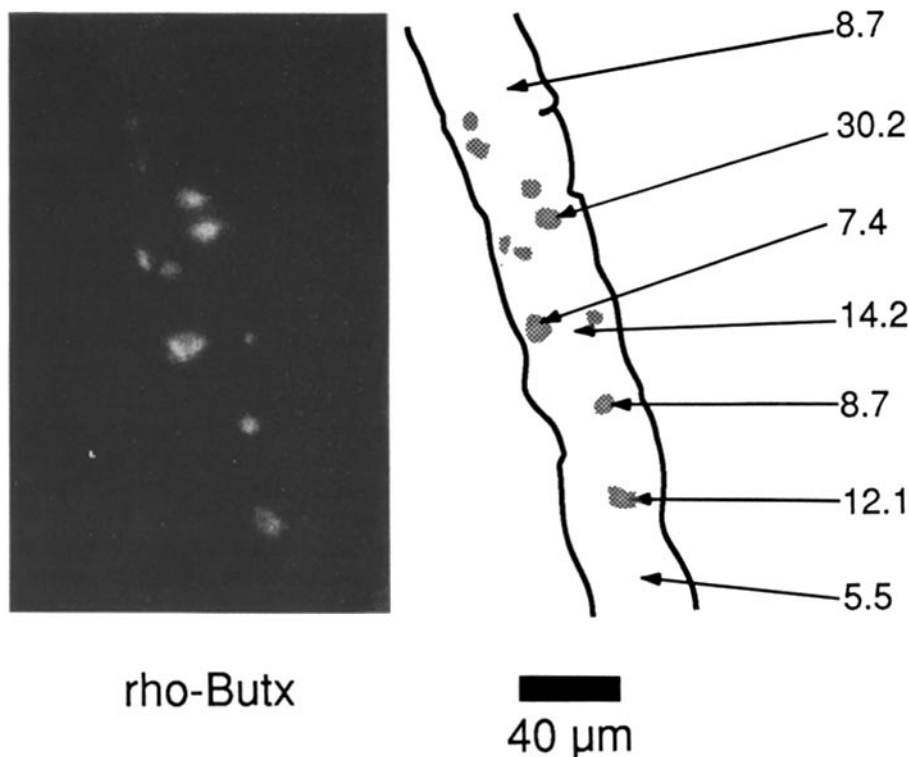
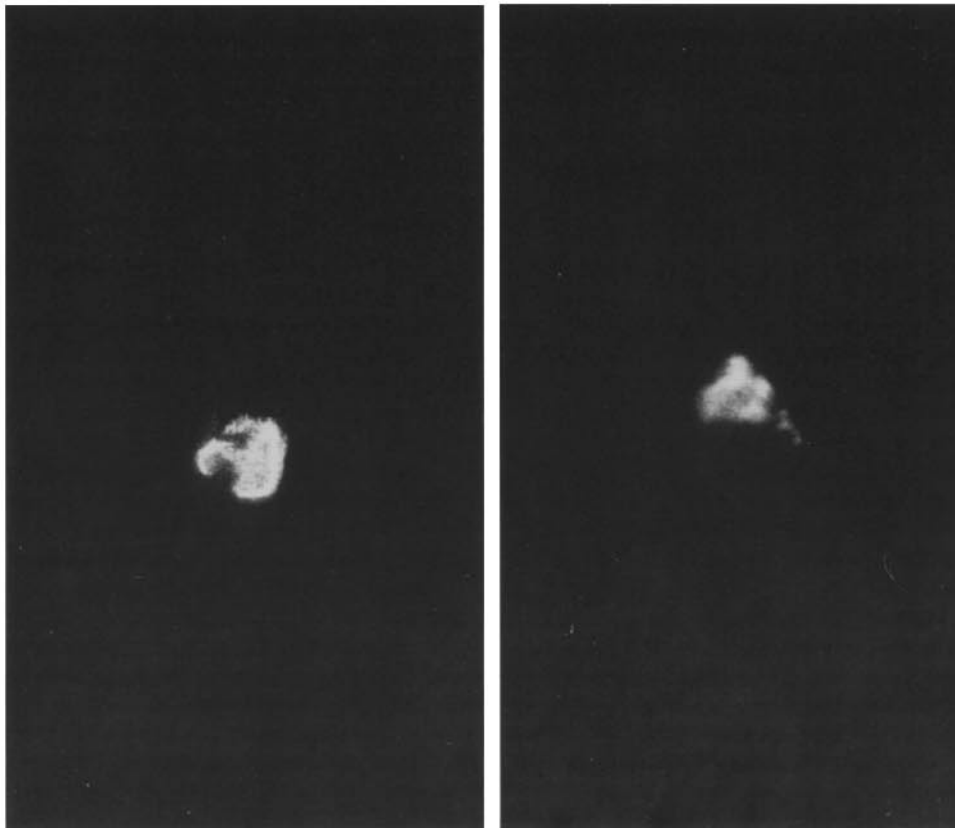
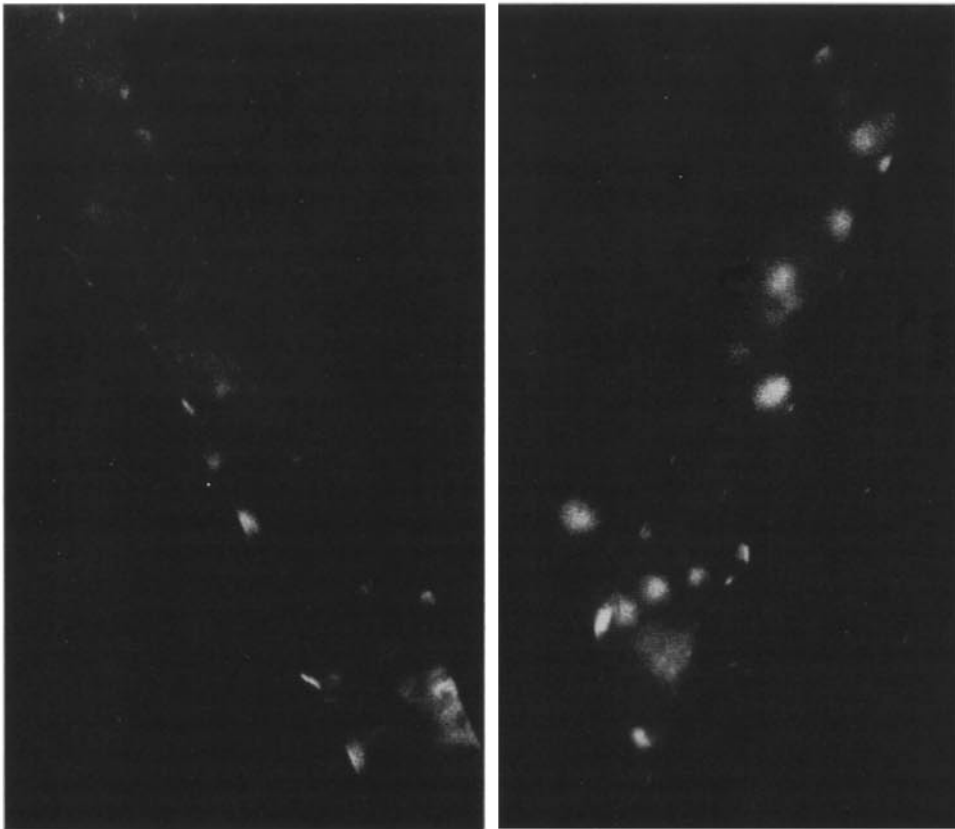


Figure 4. FDB muscle fiber, previously denervated for 13 d, then kept in culture for 5 d. The cell was labeled with rho-Butx to visualize AChRs, and the loose patch clamp technique was used to record Na current densities at various points on the cell surface. The mean Na current density at sites of low AChR density (no apparent AChR cluster) was 9.2 ± 1.8 mA/cm². Only one recording on this cell (AChR cluster, 30.2 mA/cm²) was greater than twice this mean, thus qualifying as a NaCh hotspot.

Control



Agrin



— 35 μm

Figure 5. Distribution of AChRs on FDB muscle cells cultured for 2 (top) or 3 (bottom) d. Fibers on the left (control) were cultured in normal culture medium, while fibers on the right (agrin), were cultured in the presence of 7 U/ml agrin, added on day 1. Agrin-treated muscle cells exhibited numerous AChR clusters over the length of the fibers.

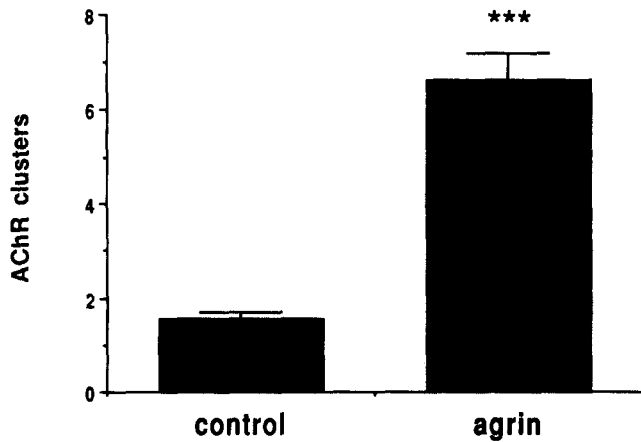


Figure 6. Number of AChR clusters per fiber in 2–3 d cultures of FDB muscle cells. Cells were pre-denervated for 3–13 d, then cultured either in normal culture medium (*control*) or in culture medium with agrin added on day 1 (*agrin*). Control bar represents the mean \pm SEM of four cultures, 37 fibers; agrin bar is for six cultures, 54 fibers. ***, $p < 0.001$.

at NaCh hotspots was 30.5 ± 2.8 mA/cm² ($n = 18$), while the current density at non-hotspot sites was 10.6 ± 1.3 mA/cm² ($n = 26$). The percentage of AChR clusters with a NaCh hotspot was 41%, comparable to that found on muscle cells after a week in culture without agrin. While the probability of locating a NaCh hotspot was significantly higher at

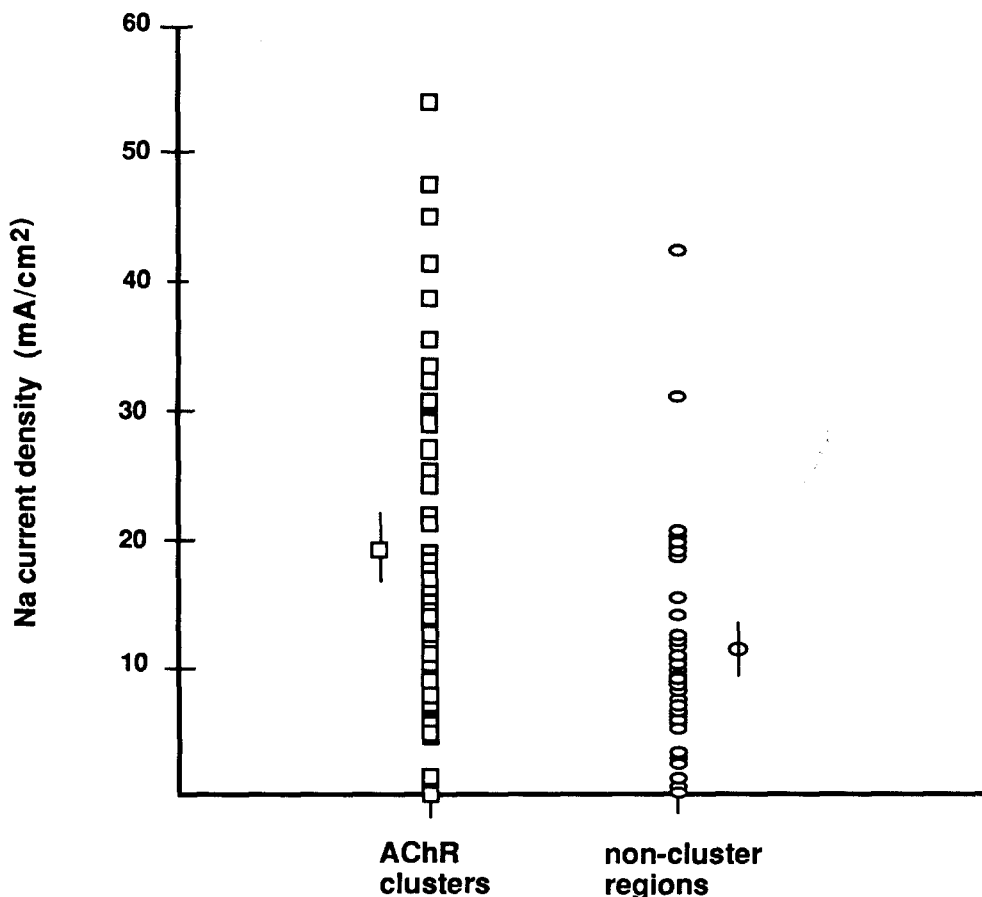


Figure 7. Range of Na current density measurements made on FDB muscle fibers cultured for 2–3 d, 1–2 d in the presence of agrin. Each symbol represents the Na current density at one point on a single cell (15 cells total). Left column (\square), recordings at sites of AChR clusters (44 recordings); right column (\circ), recordings from areas devoid of AChR clusters (46 recordings). Adjacent symbols are the mean \pm SEM for each category.

sites of AChR aggregation, more than half of the AChR clusters did not exhibit a high concentration of NaChs.

Several possible interpretations could explain why only about half of the AChR clusters possessed a high density of NaChs. The aggregation of NaChs may be a slower process than AChR clustering; in that case only older AChR clusters would acquire enough NaChs to constitute a hotspot. Alternatively, it may be that the AChR clusters with a NaCh hotspot were fragments of the original endplate, while the other AChR clusters were new clusters induced by agrin and did not acquire a high NaCh density. To examine these possibilities, we used low light level video microscopy to follow identified muscle fibers through the entire period of agrin-induced AChR and NaCh redistribution.

Redistribution of AChRs and NaChs on Identified FDB Muscle Cells

FDB cells were dissociated and cultured overnight. After about 18 h in culture, the cells were labeled with rho-Butx and fluorescence images of the AChR distribution on several fibers were stored digitally. Agrin was then added to the medium, and the fibers were photographed three to five times over the next 48 h to monitor changes in the distribution of AChRs on the surface of these cells. At the end of the experiment the cells were labeled once more with an FITC-conjugated antibody against the AChR (mAb 210) to reveal the distribution of the total AChR population, original plus newly inserted, on the surface of the muscle cells. Control fibers cultured without agrin were also followed this way.

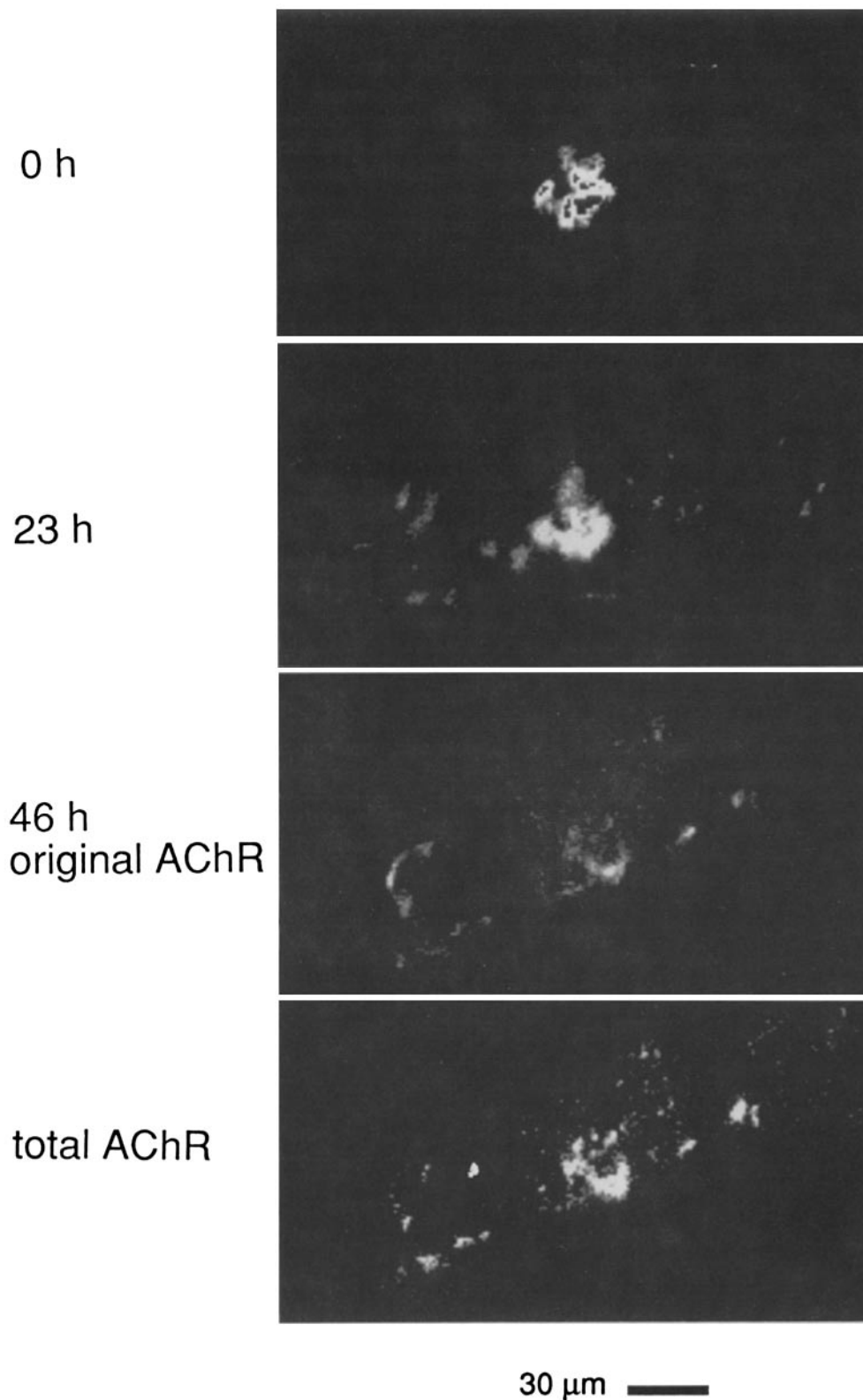


Figure 8. Fluorescence images of a single cell cultured in the presence of agrin. The muscle was denervated for 6 d before dissociation and culturing. Times to the left refer to the time after addition of agrin; 0 h actually represents about 18 h in vitro. The first three images show AChRs labeled at 0 h with rho-Butx, and are thus receptors that were present before adding agrin to the medium. Note the gradual breakup of the endplate AChR cluster, and the appearance of new AChR clusters elsewhere on the cell. The bottom image was taken after labeling the cell with FITC-mAb 210, to label the entire population of AChRs (original plus newly inserted) present after 46 h in agrin medium. Most AChR patches are composed of both newly inserted AChRs as well as AChRs present before adding agrin.

A sequence of images from one such fiber is presented in Fig. 8. The first image shows a single dense patch of AChRs localized at the endplate region. 23 h after the addition of agrin the endplate had begun to break up, and several new clusters of AChRs had appeared along the length of the cell. After 46 h the endplate had developed a region of low AChR

density, and some internal fluorescence resembling endocytic vesicles or small vacuoles was apparent. Although many extrajunctional AChR clusters remained, several patches present at 23 h had disappeared by 46 h. As these results illustrate, changes in the distribution of AChRs were usually produced by the appearance and disappearance of AChR

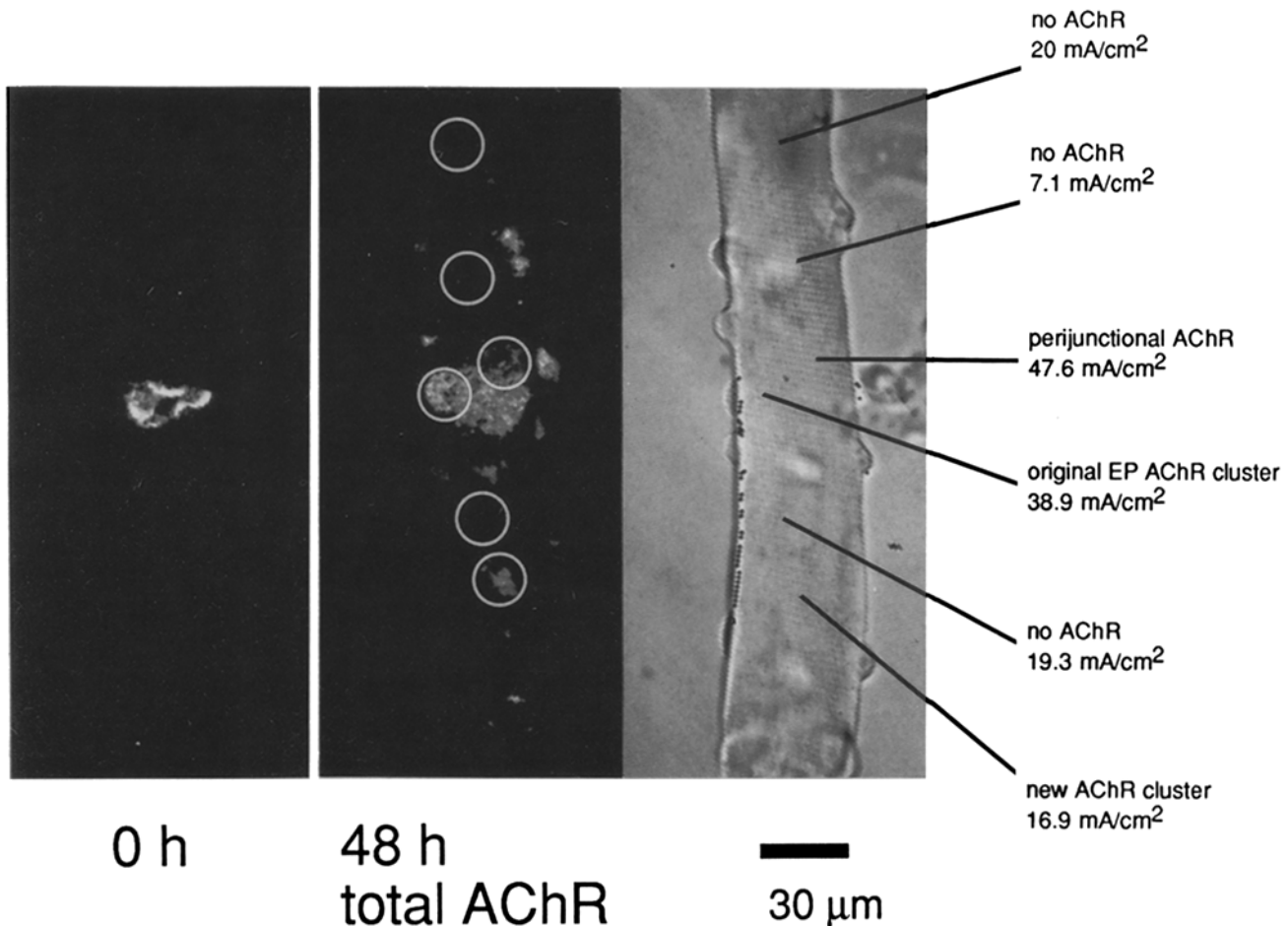


Figure 9. Loose patch clamp recording of Na currents from an FDB muscle cell followed through 48 h of culture in the presence of agrin. This cell was denervated for 7 d before dissociation and culture. Changes in the distribution of AChRs were monitored over 2 d and the Na current density was mapped at various points of interest on the fiber (*circled areas*). On this cell, the mean Na current density at areas of low AChR density was 15.1 mA/cm². Two NaCh hotspots were found: one at a part of the original endplate region which had retained its high concentration of AChRs, and another at a faint AChR cluster in a perijunctional area. In contrast, the Na current density at a new AChR cluster induced by agrin did not reveal a NaCh hotspot.

patches, and the movement of AChR clusters was generally restricted to 5–10 μm. When the culture was labeled with the FITC-conjugated anti-AChR mAb, it was found that nearly all clusters were composed of both original and newly inserted receptors. Overall, 85% of the AChR clusters (87/102) on 12 agrin-treated muscle fibers were composed at least partly of AChRs present before the addition of agrin. This demonstrates that, as for embryonic myotubes (Godfrey et al., 1984; Wallace, 1988), agrin induces aggregation of AChRs present in the membrane of adult FDB fibers.

At this point we knew the history of every point on the muscle cell, particularly which clusters were part of the original endplate region and which were new AChR clusters induced by agrin. We could then use the loose patch clamp technique to record Na currents at areas of interest. A typical fiber for which this was done is shown in Fig. 9. Recordings made on this fiber at areas devoid of AChR clusters ranged from 7.1 to 20.0 mA/cm², with a mean of 15.3 mA/cm². Patch clamping a part of the original endplate region that had retained its high AChR density revealed a NaCh hotspot with a current density of 38.9 mA/cm². A recording made in a perijunctional area into which AChRs had either diffused or been inserted gave a Na current density of 47.6 mA/cm²,

another hotspot. However, the Na current density at a new AChR cluster induced by agrin ~60 μm from the endplate was 16.9 mA/cm², not significantly different from current density in areas without an AChR cluster.

Similar results were obtained from eight fibers followed for 48 h in the presence of agrin (Fig. 10). There was a clear difference in Na current densities recorded from the original endplate region as compared to extrajunctional regions. The highest probability of finding a NaCh hotspot was at the original endplate site when it had maintained its high AChR density (80%; 8/10). Surprisingly, even parts of the original endplate region which had lost most of their AChRs usually retained their high NaCh density (67%; 2/3). In contrast, there was a low probability of finding a NaCh hotspot in the extrajunctional area, even when recording at an AChR cluster (7.3%; 1/14). We detected NaCh hotspots in the extrajunctional regions devoid of AChR clusters at only 8% (2/25) of the recordings, a figure not significantly different from control untreated fibers. We conclude from these experiments that NaCh hotspots are retained at AChR clusters formed from the fragmented endplate, but that new NaCh hotspots are not induced by agrin, either at sites of new AChR clusters or elsewhere on the muscle cell.

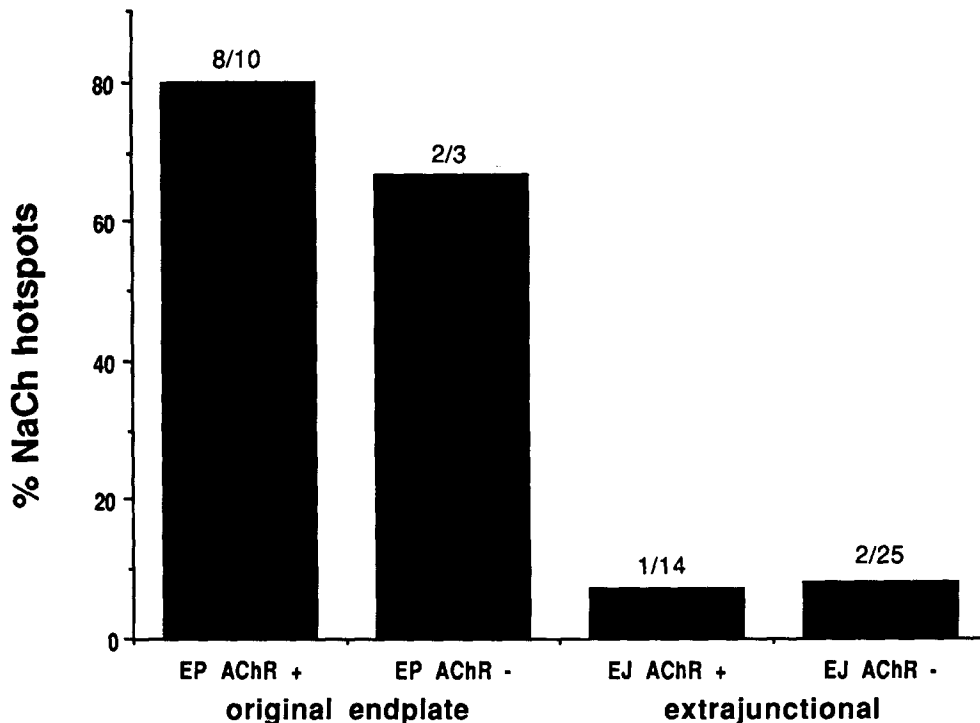


Figure 10. Summary of eight FDB fibers followed visually for 48 h in the presence of agrin, then recorded from in order to map NaCh distribution. Recordings were segregated into two main categories: those made at the *original endplate* region and those made in the *extrajunctional* membrane. These categories were further separated into measurements taken at sites of AChR clusters (*AChR+*) and at sites of low AChR density (*AChR-*). The bars show the percentage of recordings in each of these categories that gave NaCh hotspots. There was a significantly higher probability of finding a NaCh hotspot at the original endplate region, when compared to the extrajunctional membrane. Even sites of AChR patches found in the extrajunctional membrane demonstrated a low probability (7.3%) of co-localizing with a NaCh hotspot.

Focal Application of Agrin to the Endplate

Agrin has been shown to be highly concentrated in the synaptic cleft at neuromuscular junctions (Fallon et al., 1985; Reist et al., 1987). Our working hypothesis is that fragmentation of endplates on cultured FDB muscle fibers occurs because of enzymatic destruction of one or more basal lamina molecules, including agrin. We therefore tested whether focal application of agrin at the endplate would prevent the breakup of the endplate in cultured cells. To do this we filled a loose patch pipette with agrin (10 U/ml) and applied weak positive pressure to puff the agrin solution directly onto endplates of freshly dissociated FDB fibers. When these cells were labeled with rho-Butx four to seven days later, the AChR distribution appeared identical to cultured control fibers that had not been puffed with agrin. The AChR labeling at the endplate was fragmented and weak, and some fibers had developed extrajunctional patches of AChRs. Furthermore, the Na current density at agrin-puffed endplates decreased in culture, similar to untreated cultured fibers. In fact, Na current density at agrin-puffed endplates (19.8 ± 7.0 mA/cm²; eight fibers) was less than that found at control endplates (37.9 ± 13.4 mA/cm²; eight fibers), although this difference was not statistically significant ($p > 0.1$).

Discussion

The main objective of this work was to test whether the basal lamina protein agrin could induce the formation of NaCh hotspots on skeletal muscle fibers, similar to its effect on the AChR. The results clearly showed a redistribution of endplate AChRs and formation of new AChR clusters on adult FDB cells 24–48 h after the addition of agrin to the culture medium. In contrast, agrin appeared to have little effect on

the distribution of NaChs. AChR patches which were fragments of the original endplate retained a high density of NaChs. New NaCh hotspots did not develop at newly formed AChR clusters, nor elsewhere on the cell. We conclude from these experiments that agrin does not play a direct role in the aggregation of NaChs at neuromuscular endplates. The results imply that different processes are regulating the distribution of AChRs and NaChs on muscle cells.

These experiments also tested the effect of agrin on adult muscle fibers; all previous work with agrin had been done on embryonic myotubes. The results show adult mammalian muscle fibers to be remarkably receptive to the action of agrin, particularly if the muscle was denervated before culturing. However, agrin was not able to prevent the disruption of AChR and NaCh clusters at endplates of cultured FDB muscle fibers, and instead accelerated this process. Binding of agrin is apparently not sufficient to prevent the fragmentation of the endplate on collagenase-treated cells, suggesting that other extracellular factors, possibly agrin-binding proteins, are important for maintenance of the postsynaptic specialization (see also Flucher and Daniels, 1989).

Changes in AChR and NaCh Distribution on Cultured FDB Muscle Cells

Single muscle fibers dissociated from adult FDB muscles were normal in appearance and physiology, with prominent cross striations, peripheral nuclei, and a defined oval endplate region. However, as previously described for rat diaphragm (Bloch et al., 1986), collagenase digestion disrupted the junctional receptor aggregate so that the internal endplate fine structure became blurred and disorganized. The present study shows that this disorganization continues if the cells are kept in culture. After 2–3 d *in vitro* the lateral

boundaries of the endplate AChR patch began to break down, and the intensity of rho-Butx labeling at the endplate was reduced. On fibers that had been cultured a week or longer the endplate was usually difficult to identify, and several patches of AChRs were often visible (see also Bekoff and Betz, 1977). Measurement of NaCh density on cultured muscle cells revealed parallel changes in NaCh distribution at the endplate, with a few extrajunctional NaCh hotspots.

What caused the fragmentation of mature endplates on adult muscle fibers? Culture conditions were not responsible, since organ culture of FDB muscles without collagenase treatment did not produce fragmentation of the endplate. This suggested that the collagenase treatment was the cause of these changes, possibly through removal of one or more extracellular molecules. It has previously been shown that collagenase digestion of muscles diminishes acetylcholinesterase levels (Hall and Kelly, 1971; Betz and Sakmann, 1973; Bloch et al., 1986). Using immunocytochemical methods, we found that binding of the *Dolichus biflorus* agglutinin, shown to be highly enriched in synaptic basal lamina (Sanes and Cheney, 1982; Ribera et al., 1987), is nearly absent from collagenase-treated muscle fibers (data not shown). On the other hand, a significant amount of laminin staining remains after collagenase dissociation (Bischoff, 1986; Lupa and Caldwell, unpublished data). Thus, at least part of the synaptic basal lamina is removed by collagenase treatment. The parallel dispersal of both AChRs and NaChs from collagenase-treated endplates suggests that a basal lamina or extracellular matrix protein is necessary for maintaining the high density of these proteins at the synapse. It appears, however, that agrin alone is not sufficient to serve this function. The results also suggest that cytoskeletal proteins, such as ankyrin and the 43-kD protein, depend on some extracellular co-factor to anchor the AChR and NaCh in place.

The instability of the endplate after collagenase treatment is reminiscent of the dispersal of AChRs from the endplate after denervation of neonatal muscle (Slater, 1982). Similarly, denervation of adult frog cardiac ganglion cells induces AChR clusters to break up into smaller patches distributed over a larger surface area on the neuronal soma (Sargent and Pang, 1988). These results imply a persistent influence of the nerve on AChR distribution in neonatal muscle and frog cardiac ganglia. Our results on collagenase-treated muscles suggest that an extracellular matrix or basal lamina protein may be required to maintain the high density of receptors. This protein may be secreted by nerve terminals at embryonic and neonatal neuromuscular junctions, while the same or a different molecule may become stably incorporated in the basal lamina 1–2 wk after birth. Alternatively, denervated cardiac ganglion cells and neonatal muscle may secrete a collagenase which could release this factor from the basal lamina, thus mimicking the effects that we have observed in vitro.

Effect of Agrin on Adult Muscle Fibers

Using cultured chick myotubes, Wallace (1989) and Nitkin and Rothschild (1990) showed that agrin can trigger the formation of specializations containing at least seven components of the postsynaptic apparatus within 24 h of application. It is possible that formation of NaCh hotspots is a much slower process, requiring more than 48 h to occur. The mobility of NaChs in mouse muscle cell membrane has been

shown to be relatively low, with a diffusion coefficient near 3×10^{-10} cm²/s (Angelides, 1986). However, even this low mobility would allow NaChs to diffuse at least 140 μ m within a 48-h period, which should be sufficient movement for aggregation to occur. If agrin plays a role in the induction of NaCh hotspots, it must be to initiate a different, more prolonged process from the one leading to AChR accumulation.

Angelides (1986) reported that NaChs were concentrated at sites of neurite-induced AChR clusters on embryonic myotubes in culture; these results suggest that aggregation of NaChs is an early event in neuromuscular synaptogenesis. However, preliminary experiments on developing muscles from postnatal rodents suggest that NaChs cluster late in development, several weeks after birth (Lupa et al., 1991). The absence of any effect of agrin on NaCh distribution, demonstrated in this paper, would be consistent with a different mechanism for NaCh clustering than the one for AChR clustering, which is presumably initiated by agrin early in development.

mAbs directed against agrin immunoprecipitate four polypeptides from extracts of *Torpedo* electric organ, with molecular masses of 150, 135, 95, and 70 kD (Nitkin et al., 1987). All of the AChR-aggregating activity is possessed by the 150- and 95-kD proteins. Immunologically, chemically, and functionally similar molecules are also present in the extracellular matrix of several other tissues in *Torpedo*, including Schwann cell sheaths, smooth and cardiac muscle cells, and epithelial basement membrane (Reist et al., 1987; Godfrey et al., 1988). All of this information supports the idea that the *Torpedo* agrin used in the present study is one member of a family of related molecules which may play different roles in different tissues or at different developmental times. It is quite possible, for example, that one form of agrin induces AChR clustering early in synaptogenesis, while another form is responsible for stabilizing the postsynaptic specialization and inducing an accumulation of NaChs at the synapse.

Agrin induces AChR clustering on embryonic myotubes largely through lateral migration of receptors already in the plasma membrane (Godfrey et al., 1984; Wallace, 1988), similar to nerve-induced AChR clustering on myotubes in vivo (Ziskind-Conhaim et al., 1984) and in vitro (Anderson and Cohen, 1977). The present results extend this finding to adult mammalian muscle cells in culture, since 85% of the AChR clusters formed by agrin were composed at least partly of receptors present in the membrane before the addition of agrin. Surprisingly, the ability of agrin to redistribute AChRs on the cell surface extended even to AChRs present at the endplate. Thus, agrin induced not only the formation of new AChR clusters, but also the fragmentation of the endplate AChR into smaller receptor patches. This result demonstrates the importance of basal lamina proteins, particularly agrin, in determining the distribution of AChRs on the cell surface. It would be interesting to disrupt the cytoskeleton in organ-cultured muscles, possibly through pharmacological means, to test whether endplate stability could be maintained by the basal lamina alone.

The redistribution of endplate AChRs by agrin led us to postulate that receptors for agrin were located over most of the cell surface, possibly because the muscles had been denervated before culture. However, when innervated mus-

cles were dissociated and cultured in the presence of agrin for 2–3 d a similar induction of new AChR clusters and dissociation of endplate AChRs was observed. Even when agrin was locally applied to the endplate area of muscle fibers in culture, no preservation of the endplate AChR or NaCh densities was obtained. Since agrin was applied focally for only a short time (10 min), this result may indicate that a necessary binding site for agrin in the extracellular matrix was removed by the collagenase treatment. A similar argument was proposed to explain the fast reversibility of agrin effects on embryonic myotubes (Wallace, 1988). Assuming that agrin bound to its receptor at the endplate region of the cells, the existence of a high AChR density, cytoskeletal specialization, and cluster of synaptic nuclei offered no advantage to this region with regard to agrin-induced AChR aggregation. This suggests that other factors, possibly the extracellular matrix or Schwann cells (Chapron and Koenig, 1989), are important for maintaining the integrity of the postsynaptic specialization.

In conclusion, we have found that agrin does not induce new hotspots of NaChs either at sites of new AChR patches or elsewhere on muscle cells. The results are consistent with the hypothesis that extracellular signals serve to regulate the distribution of AChRs and NaChs at the neuromuscular junction. While agrin may function in this role for the AChR, it appears not to play a role in the determination of the NaCh distribution.

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