

Acetylcholine Receptor Clustering and Nuclear Movement in Muscle Fibers in Culture

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Abstract. We have studied the formation of acetylcholine receptor (AChR) clusters and the behavior of myonuclei in rat and chick skeletal muscle cells grown in cell culture. These cells were treated with a factor derived from *Torpedo* electric tissue extracellular matrix, which causes a large increase in their number of AChR clusters. We found that these clusters were located preferentially in membrane regions above myonuclei. This cluster-nucleus colocalization is explained by our finding that most of the nuclei near clusters re-

main relatively stationary, while most of those away from clusters are able to translocate throughout the myotube. In some cases, clusters clearly formed first, then nuclei migrated underneath and became immobilized. If clustered AChRs later dispersed, their associated nuclei resumed moving. These results suggest that AChR clustering initiates an extensive cytoskeletal rearrangement that causes the subcluster localization of organelles, potentially providing a stable source of newly synthesized AChRs for insertion into the cluster.

THE vertebrate neuromuscular junction is a highly specialized region of muscle characterized by high concentrations of acetylcholine receptors (AChRs),¹ acetylcholinesterase and other basal lamina components, postsynaptic folds, and a variety of postsynaptic cytoskeletal elements (6, 7, 26, 27, 33, 37, 38, 39, 47; reviewed in reference 25). Most evidence suggests that this specialized region develops after the initial interactions between the motor neuron growth cone and the muscle membrane, presumably in response to one or more factors released at the nerve terminal (13, 45).

AChR clustering at new synapses has been extensively studied, particularly in cell cultures derived from embryonic neurons and muscle cells. Muscle fibers appear to have a uniform distribution of AChRs before synapse formation, but, within hours of the onset of synaptic transmission, AChRs cluster at synaptic sites (2, 23). During innervation of embryonic rat muscle (49) and during synapse formation in neuron-myocyte cultures derived from *Xenopus* embryos (1, 29), many of the AChRs that localize to synaptic sites existed elsewhere on the muscle membrane before innervation. In these cases, therefore, AChR redistribution is an important part of new cluster formation. In neuron-muscle cultures derived from chick embryos, insertion of newly synthesized AChRs into the synaptic region may make a significant contribution (42).

Sometime during the normal development of embryonic muscle, the junctional AChR cluster becomes stabilized, apparently by a component of the muscle's basal lamina (9), and the density of AChRs in this region then changes little, even after denervation (24). In muscle cell cultures as well, once an AChR cluster has formed, its concentration of

AChRs generally remains approximately constant (23). Yet, individual AChRs are internalized and degraded, with a half-time of ~24 h (14). There are two ways in which the cell could replace these degraded AChRs and maintain clusters once they have formed. First, AChRs might be synthesized at points throughout the muscle's cytoplasm and be inserted randomly into the membrane. These new AChRs would then diffuse randomly in the membrane until they reached the cluster, where they would become immobilized. Alternatively, AChRs might be preferentially produced or at least inserted only near clusters. In adult muscle, the junctional region occupies a very small percentage of the fiber's surface, and the latter mechanism would seem to be the more economical one.

Support for the local synthesis and insertion mechanism comes from the recent observations by Merlie and Sanes (34) that greater quantities of mRNA for two subunits of the AChR are present in the junctional region of adult rat muscle than in extrajunctional regions. These results imply that there must be a stable subjunctional source of genetic and protein synthetic machinery. Indeed, it has been known for some time that myonuclei from the center of the muscle fiber assume a subsarcolemmal position and accumulate under the nerve terminal after synapse formation (28, 35). In addition, Fischbach and Cohen (21) noted that high concentrations of AChRs were found near myonuclei on cultured chick myotubes. Also, in cultured myotubes, more coated vesicles containing AChRs on their way to the cell surface were found beneath membrane regions containing AChR clusters (10).

We have investigated the process of AChR clustering on rat and chick muscle fibers growing in cell culture. To facilitate these experiments, we have treated these cells with an extract derived from the extracellular matrix of *Torpedo* electric tissue (46). This extract contains a factor that markedly in-

1. Abbreviation used in this paper: AChR(s), acetylcholine receptor(s).

creases AChR clustering. We show here that these clusters become associated with myonuclei because a subset of myonuclei stop their normal migration through the cell and remain beneath the clusters. This colocalization seems likely to arise through cytoskeletal rearrangements that accompany cluster formation.

A portion of this work has been reported in abstract form (17, 18).²

Materials and Methods

Cell Culture

The hind limb muscles of 11–12-d-old chicken embryos or of 20–21-d-old rat embryos were dissociated enzymatically and grown according to standard procedures (20, 43). Chicken cells were maintained in a medium consisting of Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) with 10% horse serum (Hyclone Laboratories, Logan, UT) and 5% chicken embryo extract. Rat cells were grown in Dulbecco's modified Eagle's medium (Gibco) with 10% horse serum, 2% chicken embryo extract, and 33 mM additional glucose. Most of the fibroblasts were removed by adding 10^{-5} M cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) to the medium for 48 h beginning 3 d after plating. Myotubes were used at day 7 or 8.

Cells were plated on 12-mm collagen-coated glass coverslips for autoradiography and statistical studies. For continuous observation by cinematography, cells were grown and viewed in 35-mm collagen-coated plastic dishes that were gassed with 5% CO₂/95% O₂ and sealed with vacuum grease before filming. For sequential studies on individual cells, cells were plated on collagen-coated glass coverslips previously cemented with Sylgard (Dow Corning Corp., Midland, MI) to 20-mm diameter openings made in 35-mm dishes.

AChR Clustering Factor

An extract of *Torpedo* electric tissue extracellular matrix containing a factor that increases the number of AChR clusters on muscle cells was prepared by procedures similar to those published previously (46). Briefly, tissue was homogenized in ice-cold 150 mM NaCl/20 mM Tris, pH 7.4, containing EGTA, EDTA, *N*-ethylmaleimide, Trasylol, and phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors and centrifuged at 30,000 *g* for 90 min. The pellet was resuspended in 2 M MgCl₂/20 mM Tris, pH 7.4, containing *N*-ethylmaleimide, Trasylol, and PMSF, and stirred for 30 min at 4°C. After centrifugation as above, the supernatant was dialysed against MEM, centrifuged, and the final supernatant stored at 4°C (or at –80° for longer storage periods). Cells were treated with 25–50 μ l (10–20 μ g) of this extract.

Determination of Cluster Number

For experiments concerning the effects of the *Torpedo* clustering factor, cells were grown on glass coverslips and labeled with tetramethylrhodamine-conjugated alpha-bungarotoxin (prepared by the method of Ravdin and Axelrod; 40) in 2% horse serum in MEM for 90 min at 37°C. Clusters were defined as patches of bright rhodamine alpha-bungarotoxin labeling with a total area equal to or greater than 25 μ m². We also quantified the number of AChR microaggregates, which we defined as areas of bright rhodamine alpha-bungarotoxin fluorescence between 2 and 25 μ m² in area.

For studies on the effects of colchicine and cytochalasin D on cluster number, cells grown on glass coverslips were labeled for 75 min at 37°C with 5 nM [¹²⁵I]-alpha-bungarotoxin (Amersham Corp., Arlington Heights, IL) in MEM containing 2% horse serum. The cells were washed extensively and fixed for 30 min in 1% glutaraldehyde/1% paraformaldehyde in 90 mM phosphate buffer, pH 7.0. The coverslips were attached to glass slides, coated with Kodak NTB2 emulsion, and exposed for 4–6 d at 4° in a light-tight box containing dessicant. The autoradiograms were developed by standard techniques (41). AChR clusters were counted by microscopic examination of random fields in these autoradiograms as described previously (46). Clusters were defined as areas with a grain density at least three times higher

than that elsewhere on the myotubes and with a minimum area of ~ 25 μ m². The number of muscle cell segments in each field was also determined; the number of myotubes was not affected by treatment with the clustering factor.

Microscopy

A Zeiss ICM inverted microscope equipped for epifluorescence, with a 75-W xenon lamp, a BP 546/12 excitation filter, a 590 dichroic, an LP 590 barrier filter, a KG 1 heat (absorption) filter, and a 63 \times , 1.4 numerical aperture oil immersion lens was used. For sequential studies, cells were viewed using a Silicon Intensified Instrument Camera (No. 65-MK II; Dage-MTI Inc., Wabash, MI), displayed on a Hitachi 12" video monitor and recorded with a video cassette recorder (VC-9507; NEC Home Electronics [U.S.A.] Inc., Elk Grove Village, IL). Information on nuclear and cluster position and cluster shape was transferred from the monitor to clear acetate sheets that were superimposed to compare different time points.

Statistical Correlations

Muscle cultures on 12-mm coverslips were treated with *Torpedo* clustering factor for 0, 1, 3, 6, 12, 24, and 36 h. The cells were labeled with rhodamine alpha-bungarotoxin, and, for some experiments, nuclei were labeled with the fluorescent dye 4', 6-diamidino-2-phenylindol-2HCl (Sigma Chemical Co.) by treating fixed cells with a 0.01% solution made in MEM. At each time point, the cells were fixed with 2% paraformaldehyde/lysine (19) and the coverslips mounted on slides with UVinert mountant (Atomerger Chemetals Corp., Farmingdale, NY). Cells were randomly selected and photographed serially along their lengths with both phase optics and a double exposure of 4', 6-diamidino-2-phenylindol-2HCl and rhodamine fluorescence. The boundaries of each cell, its clusters, and its nuclei were traced on a digitizing tablet (GICO Corp., Rockville, MD) interfaced to a Digital Equipment Corp. (Marlboro, MA) system 2020. The tablet has a precision of 1 part in >10,000. These data were used to calculate the area and length of each muscle fiber and the area and center of area of each cluster and nucleus.

We were interested in testing if clusters are preferentially located in membrane regions near nuclei. For a statistical test of this possibility, we assumed that: (a) the probability of a randomly dropped cluster landing in any cell segment is proportional to the length of the segment relative to the total length of the cell, and (b) if *D* is the distance between any two nuclei, then, contingent on the cluster landing between the two nuclei, on the average it should be *D*/4 away from the nearer nucleus. (The midpoint between two nuclei, *D*/2, is the maximum distance a cluster can be from its nearest nucleus. Thus, the expected distance between a cluster and its nearest nucleus, the average distance for a randomly placed cluster, is *D*/4.) Measurement of distances between nuclei permitted the calculation of both the expected mean distance of a cluster from its nearest nucleus and the expected standard deviation of the mean cluster to nearest nucleus distance. For each cell a *Z* value was computed as the ratio of the difference between the actual and the expected mean distance divided by the expected standard deviation under the null hypothesis. The sum of the *Z*² values for each cell yielded a statistic with a chi-squared distribution (degrees of freedom equal to the number of cells) under the null hypothesis (15). All distances were measured center-to-center, and distances from all clusters to their nearest nuclei were determined.

Time-lapse Cinematography

Time-lapse cinematographic studies were done in a 37°C humidified room using a 16-mm Bolex camera connected to a time-lapse unit (Sage Instruments, Cambridge, MA). The camera was attached to a Zeiss inverted microscope equipped with a 16 \times objective. Plus-X reversal films were taken at 0.25 s exposures, 1 frame/10 s, for 4–6 h.

Sequential Studies

Cells were first labeled with rhodamine alpha-bungarotoxin, and the positions of AChR clusters were recorded. Individual cells, either untreated or treated with the *Torpedo* clustering factor, were followed for up to 72 h. During all observations, the microscope stage was kept between 33 and 37°C using infrared irradiation and space heaters, and, between time points, the cells were flushed with tissue culture medium kept at 37°C and at pH 7.4 before returning them to their normal tissue culture incubator. Marks on the substratum made with a diamond scribe gave absolute positional information, and the changes in cluster and nuclear location over time were

2. After this paper was submitted, another report of an association between nuclei and AChR clusters appeared (J. M. Bruner and S. Bursztajn, *Dev. Biol.* 115:35–43).

recorded on clear acetate sheets. Measurements of cluster to nuclear distance and nuclear movement were made directly from estimated center of area points on these tracings.

Drug Treatments

For studies on cluster number, cells were exposed for 9 h to 0.4 $\mu\text{g}/\text{ml}$ of cytochalasin D (stock solution was 2 mg/ml in dimethylsulfoxide; Sigma Chemical Co.; this vehicle itself had no effect on clusters when used at comparable concentrations) or for 6 h to 10–50 μM colchicine (Sigma Chemical Co.). Much longer treatment with colchicine resulted in marked rounding up of the cells. To study the effects of these agents on cluster formation, cells were treated simultaneously with clustering factor. To examine their effects on cluster maintenance, cells were pretreated for 24 h with clustering factor. For studies on rates of nuclear movement, drug treatment followed a 24–36-h period of factor pretreatment.

Results

Cluster Formation

Previous studies (e.g., 46) have shown that the *Torpedo* clustering factor causes a large increase in number of clusters, acting, at least in part, by causing AChRs already in the muscle membrane to redistribute and aggregate. We examined 38 cells at various times after treatment with this factor. In support of these previous observations, there was approximately an eightfold increase in the number of actual clusters during this period. At the same time, the average number of AChR microaggregates per cell decreased markedly (Fig. 1). Consequently, the average size of all AChR patches (clusters plus microaggregates) increased noticeably, from 4 μm^2 initially to 18.3 μm^2 at 36 h.

We also examined the effects of cytochalasin D and colchicine on cluster formation and maintenance. In one set of experiments, rat muscle cells were treated simultaneously with clustering factor and either cytochalasin D or colchicine. Colchicine had no inhibitory effect on the increase in cluster number in spite of the fact that, after prolonged treatment, it caused marked shape changes in muscle cells (Table I). Cytochalasin D, however, caused a significant inhibition of de novo cluster formation.

In another set of experiments, cells were first treated with clustering factor for 24 h and then treated for 9 h with

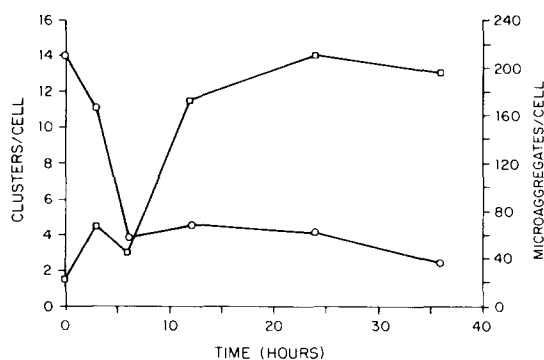


Figure 1. Average number of AChR clusters and microaggregates per cell during clustering factor treatment. 38 chick muscle cells were incubated with clustering factor for varying lengths of time, labeled with rhodamine alpha-bungarotoxin, and fixed. Cluster (open square) and microaggregate (open circle) numbers were counted from photomicrographs of the cells viewed with fluorescent optics.

Table I. Effect of Colchicine and Cytochalasin D on AChR Cluster Number

Treatment	Average No. of clusters per cell segment
Control	0.81
Clustering factor (6 h)	1.69
Simultaneous clustering factor and colchicine (6 h)	1.88
Clustering factor (9 h)	2.01
Simultaneous clustering factor and cytochalasin D (9 h)	0.95
Clustering factor (33 h)	2.32
Clustering factor pretreatment; cytochalasin D	2.35
Clustering factor pretreatment; colchicine	2.27

AChRs on rat muscle cultures were labeled with ^{125}I -alpha-bungarotoxin, the cells were processed for autoradiography, and the average number of AChR clusters per cell segment was determined as described in Materials and Methods. To examine the effects of drugs on cluster formation, cells were treated simultaneously for either 6 h with clustering factor and colchicine or 9 h with clustering factor. To examine the effects of drugs on cluster stability, cells were pretreated with clustering factor for 24 h and then with either colchicine (for 6 h) or cytochalasin D (for 9 h). Four separate experiments were done with colchicine, and five were done with cytochalasin D. A minimum of 100 cell segments were counted for each condition in each experiment. Two-tailed *t* tests revealed a significant effect ($P = 0.001$) of cytochalasin D on cluster formation. Neither colchicine nor cytochalasin had a significant effect on cluster maintenance, nor did colchicine have a significant effect on cluster formation.

cytochalasin D or colchicine. Under these circumstances, neither disrupting agent had a major effect on cluster number (Table I). That is, neither compound appeared to disperse existing clusters preferentially. This was confirmed in several experiments in which individual clusters were labeled with rhodamine alpha-bungarotoxin and examined periodically under fluorescence optics, using the silicon intensified instrument camera and a VCR to record their positions (data not shown).

Nucleus-Cluster Association

We wished to see if AChR clusters were randomly distributed in the cell or if they were located preferentially near nuclei, as had been previously suggested (21). We measured the distances between the centers of clusters and their nearest nuclei 36 h after treating chick muscle cultures with *Torpedo* clustering factor. The distribution of these distances was compared with that expected if the clusters were randomly placed (Figs. 2 and 3). We found that 87.8% of the clusters were closer to their nearest nuclei than expected if they were distributed randomly in the cell. Moreover, 66.7% of the clusters were closer than half of their expected distance. Overall, our statistical test yielded a χ^2 of 38.48 ($P = 0.001$), strongly indicating that clusters are located preferentially in membrane regions above myonuclei. These statistics take into account the fact that the nuclei themselves are distributed nonuniformly in the cells.

We were also interested in the temporal development of the nucleus-cluster association. Calculations of statistical associations revealed that at all times before and after factor addition, both clusters and microaggregates were located preferentially near nuclei.

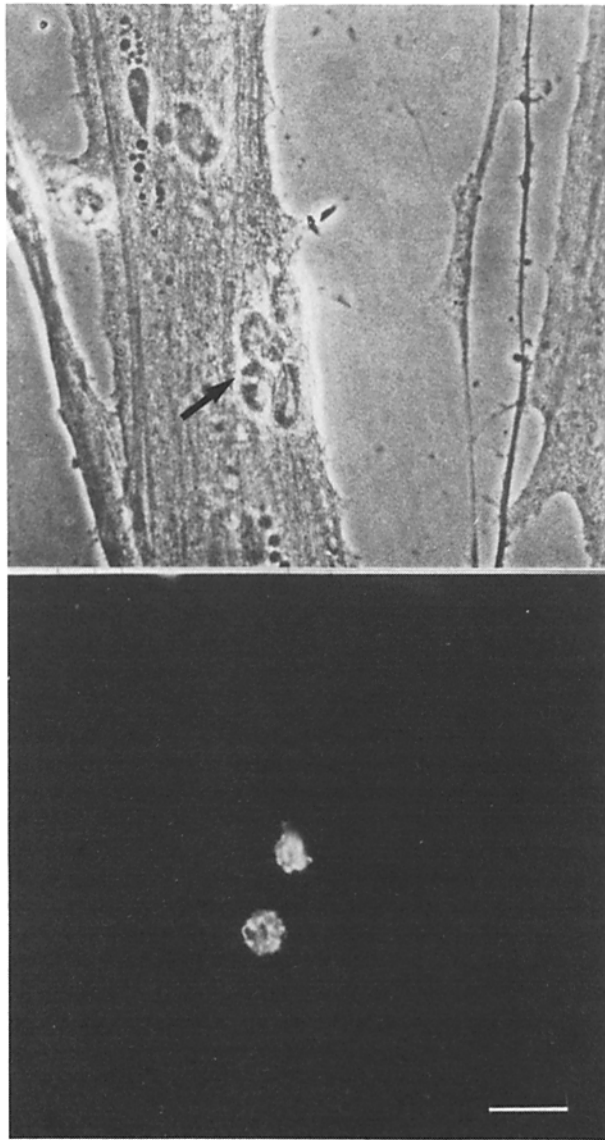


Figure 2. Relationship between AChR clusters and myonuclei. Rat muscle cells were treated for 24 h with clustering factor and then labeled with rhodamine alpha-bungarotoxin. Phase (*upper*) and fluorescence (*lower*) micrographs show two clusters situated near a group of nuclei (*arrow* in phase). Bar, 10 μm .

Nuclear Movement

Continuous Observation. The strong correlation between the positions of clusters and nuclei described above was made from populations of cells. Previous evidence (23) had suggested that clusters tend to maintain a constant position in the cell, while nuclei normally translocate throughout the length of the myotubes. This prompted us to investigate in detail the movement of nuclei with respect to clusters to see how moving organelles and stationary membrane components could become associated.

For this purpose, untreated chick muscle cells were examined continuously for several hours by time-lapse cinematography. We found that $\sim 85\%$ of the nuclei in these cells did move at a rate greater than $3\text{--}4\text{ }\mu\text{m}$ (a nuclear radius) per hour. Their average total rate of movement was $13.5\text{ }\mu\text{m/h}$.

While moving, they were able to pass around one another, thereby causing the sides of the myotube to distend. In addition, nuclei were able to tumble, i.e., to rotate very rapidly.

We also used time-lapse cinematography to examine nuclear movement in cells treated with the *Torpedo* clustering factor. In these cells, only $\sim 35\%$ of nuclei moved. However, mobile nuclei had an average rate of movement of $10.8\text{ }\mu\text{m/h}$, not much different from the average rate of nuclear movement in untreated cells.

Sequential Studies. The major known difference between control and factor-treated cells is that the latter have many more AChR clusters. One hypothesis that would account for the difference in the percent of moving nuclei in control versus treated cells is that nuclei beneath clusters are generally immobile, while those away from clusters are generally mobile. We investigated the behavior of individual nuclei near and away from identified clusters by labeling rat and chick muscle cells with rhodamine alpha-bungarotoxin, recording the positions of nuclei and clusters, and following them over time. Control experiments on unlabeled cells, followed by labeling with rhodamine alpha-bungarotoxin and identification of clusters, demonstrated that neither the labeling nor the brief exposure to UV light had any effect on movement of nuclei.

Nuclei in the chick cells used in these experiments behaved similarly to those described above. In all, 86.7% of nuclei in untreated cells, but only 42% of those in factor-treated cells, moved $>3\text{ }\mu\text{m/h}$. In both control and treated cells, nuclei within one nuclear diameter ($\sim 8\text{ }\mu\text{m}$) of a cluster were essentially immobile (Table II). Their average rate of movement was $\sim 1\text{ }\mu\text{m/h}$ and was never observed to exceed $3\text{ }\mu\text{m/h}$. In contrast, nuclei away from clusters moved an average of $\sim 6\text{ }\mu\text{m/h}$, with rates of up to $18\text{ }\mu\text{m/h}$. In many cases, nuclei beneath clusters simply shifted from one side of the cluster to the other. In addition, rates of moving nuclei

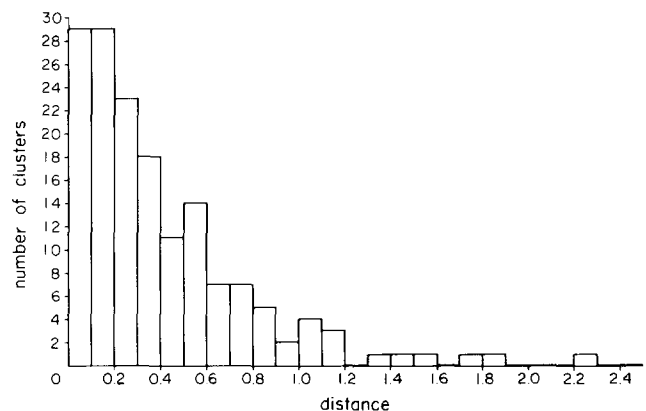


Figure 3. Histogram of distances from clusters to nearest nuclei. Chick muscle cells were treated for 36 h with clustering factor, incubated with rhodamine alpha-bungarotoxin, fixed, and stained with 4',6-diamidino-2-phenylindol-2HCl. Measurements were made from photomicrographs on a digitizing tablet, as described in Materials and Methods. The x-axis values in this figure represent the ratio between the actual cluster-nuclear distance and the expected distance if clusters were distributed randomly. These values are therefore relative, but strongly indicate that clusters are distributed much closer to nuclei than would be expected if they were placed randomly. 16 cells with a total of 165 clusters were examined.

Table II. Rates of Nuclear Movement in Chick Muscle Cells

Treatment	Cluster-associated	Unassociated
	$\mu\text{m/h}$	$\mu\text{m/h}$
Control ($N = 62$; $n = 5$)	1.4 (0–2.4)	5.4 (0–10)
Clustering factor ($N = 102$; $n = 7$)	1.2 (0–3)	5.9 (0–18)

Chick muscle cells treated as described in Materials and Methods were labeled with rhodamine alpha-bungarotoxin and visualized under fluorescence and phase optics to identify nuclei near and away from clusters. Rates of movement of nuclei (N) in separate cells (n) were determined and are given as the average rate (with range of rates in parentheses). In both types of cells, rates of movement of cluster-associated nuclei were significantly less than that of unassociated nuclei (two-tailed t test, $P < 0.001$).

were determined from periodic, rather than continuous, observation and were expected to be smaller than those in the previous section since individual nuclei can move in either direction at different times. Thus, it seems likely that the actual difference in total movement away from their starting location between nuclei associated with clusters and those unassociated was even greater than indicated here.

Observation of nuclei in rat cells provided similar results (Table III). Nuclei away from clusters in control and factor-treated cells moved at 8–9 $\mu\text{m/h}$ (Fig. 4) while those near clusters moved <2 $\mu\text{m/h}$ (Fig. 5).

Nuclei initially located near microaggregates were able to move rapidly, as long as clusters did not form in these regions. In total, the movement of 23 nuclei in six rat cells located near microaggregates was measured. 21 (91%) of the nuclei did move, the average rate being 8.3 $\mu\text{m/h}$. Thus, there was no statistically significant difference in their rate of movement compared with that of nuclei in areas of diffuse AChR distribution.

Cluster Formation and Dispersal. Particularly striking was our observation that even those clusters that initially formed away from any nuclei soon became associated with immobile nuclei (Fig. 6). Generally, the nearest available nucleus moved to the clustered region and stopped moving. These cluster–nucleus associations were as stable as those observed previously, so that they could be maintained for an entire 48-h observation period (Fig. 6, *d* and *e*).

Approximately 20–25% of the rhodamine alpha-bungarotoxin-labeled clusters dispersed during a 24–48-h observation period. The average rate of movement of 12 nuclei located near eight of these recently dispersed clusters increased to 8.4 $\mu\text{m/h}$ (Fig. 7). Thus, upon cluster dispersal,

Table III. Rates of Nuclear Movement in Rat Muscle Cells under Different Experimental Conditions

Treatment	Cluster-associated	Unassociated
	$\mu\text{m/h}$	$\mu\text{m/h}$
Control ($N = 111$; $n = 7$)	1.7 (1.4–1.8)	8.2 (7–10.4)
Clustering factor ($N = 170$; $n = 10$)	1.8 (0.3–3.1)	9.4 (4.8–16.8)
Cytochalasin D ($N = 81$; $n = 6$)	2.2 (0–4)	7.0 (0–15.2)
Cytochalasin D plus clustering factor ($N = 115$; $n = 7$)	1.8 (0–3.6)	8.5 (0–14.3)
Colchicine ($N = 5$; $n = 40$)	0.5 (0–1.6)	0.7 (0–1.0)
Colchicine plus clustering factor ($N = 47$; $n = 5$)	0.2 (0–1.2)	0.7 (0–1.2)

Rat muscle cells were treated as described in Materials and Methods and processed as detailed in the legend to Table II. Two-tailed t tests demonstrated that rates of movement of nuclei in colchicine-treated cells were significantly different ($P < 0.001$) from those in control cells. Cytochalasin D had no significant effect on nuclear movement.

the once immobile subcluster nuclei resumed moving at normal rates.

Nuclear Movement in Cytochalasin and Colchicine-treated Cells

Nuclear movement in cytochalasin-treated cells was virtually identical to that in nontreated cells (Table III). Thus, nuclei near clusters were relatively immobile in such cells, while those away from clusters generally moved more rapidly. Furthermore, when clusters formed away from any nuclei before the addition of cytochalasin, they could still migrate to the clustered region and become stabilized there even in the presence of cytochalasin (Fig. 8).

Nuclei in cells treated with colchicine appeared unable to move, regardless of whether they were near to or away from clusters (Table III). This effect was observed ~ 2 –4 h after colchicine addition and preceded marked shape changes in muscle cells.

Discussion

We have demonstrated in this paper that AChR clusters in cultured chick and rat myotubes are localized near myonuclei. This is true in both control cells, which have few clusters, and also in clustering factor-treated cells, which have many clusters. In the case of the treated cells, the associ-

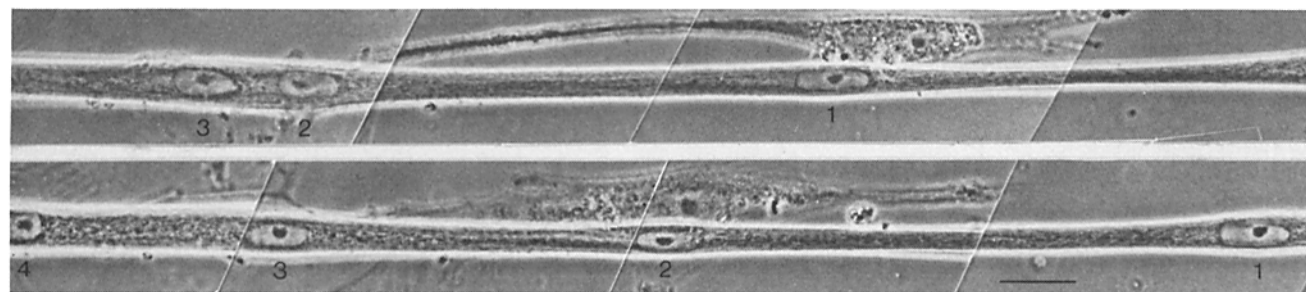


Figure 4. Nuclei not associated with clusters translocate along the myotube. Chick muscle cells were labeled with rhodamine alpha-bungarotoxin and examined using fluorescence optics (fluorescence micrograph not shown). Nuclei not in the vicinity of clusters were chosen for examination. Three of these nuclei moved rapidly between the start (*top*) and conclusion of this experiment 4 h later (*bottom*). Nuclei are numbered for ease of identification. Nucleus 4 was originally to the left, out of the field of view. Bar, 10 μm .

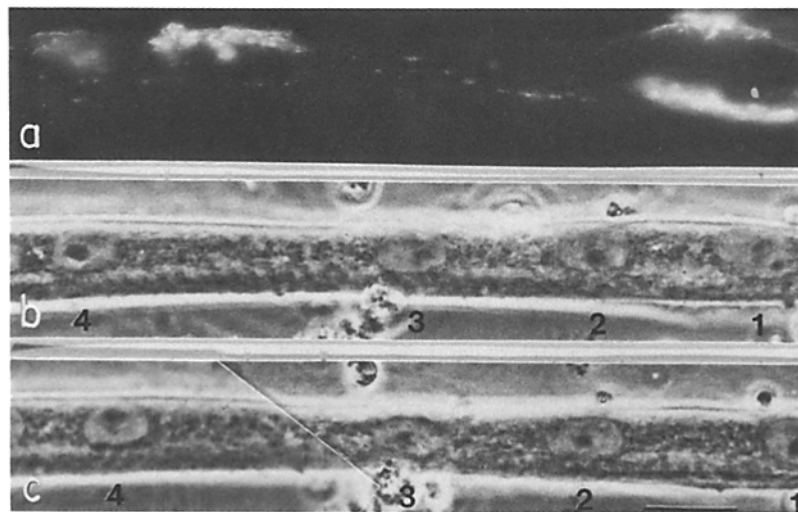


Figure 5. Nuclei beneath AChR clusters are immobile. Chick muscle cells were labeled and examined as in Fig. 4. (a) Fluorescence micrograph shows an area of a cell containing several clusters. Phase micrographs show positions of labeled nuclei at the start (b) and conclusion of this experiment 4 h later (c). These nuclei are obviously less mobile than those in Fig. 4. Bar, 10 μ m.

ation generally occurs within hours of cluster formation in spite of the fact that clusters can initially form away from nuclei by a process involving a redistribution of membrane AChRs. Experiments using thiamine pyrophosphatase staining for Golgi apparatus (36) suggest that the Golgi apparatus occupies a relatively diffuse, but generally circumnuclear, position and would be beneath clusters as well (data not

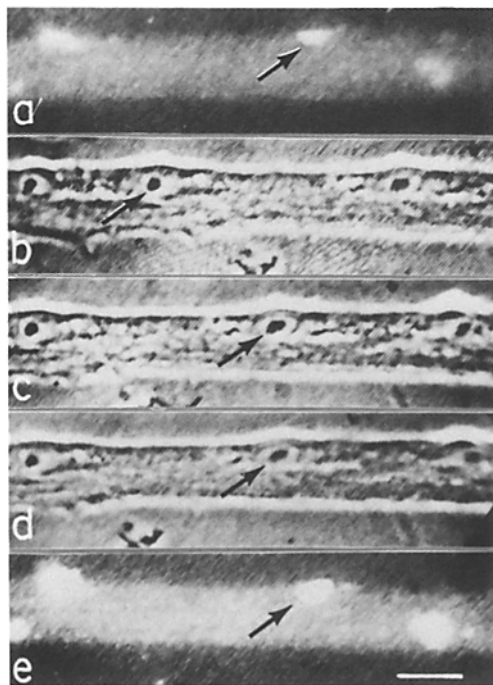


Figure 6. AChR clustering induces nuclear localization. Chick muscle cells were examined after clustering factor treatment and labeling with rhodamine alpha-bungarotoxin. Photos were taken from a video monitor. (a) Fluorescence micrograph taken at 0 h (2 h after factor treatment). Arrow indicates a cluster without an underlying nucleus. (b) Phase micrograph at 0 h showing a nucleus (arrow) that (c) moved underneath this cluster during the next 6 h. (d) After 18 h, this nucleus has remained beneath the cluster and the two other nuclei have also remained beneath clusters. (e) Fluorescence micrograph at 18 h demonstrating that the clusters themselves have been maintained. Bar, 10 μ m.

shown). These organelles could potentially provide a source of newly synthesized AChRs for insertion into the cluster and might be involved in cluster maintenance (although it is clear that clusters can form initially without their participation).

The association between clusters and organelles is explained by our further observation that nuclei localized beneath clusters in both types of cells were essentially immobile, while other nuclei in the same cells moved more rapidly along the myotube. The small amount of movement demonstrated by subcluster nuclei shifted them, in general, from one side of the cluster to the other, and might have been related to the displacement caused by the rather frequent muscle contraction seen in our cell cultures. Because nuclei beneath clusters in untreated cells comprise a relatively small proportion of these cells' nuclei, our findings are consistent with previous observations on nuclear mobility and AChR clusters (23). The role of nuclear movement and nuclear position (peripheral versus central) in muscle development and function has been of interest for quite some time (e.g., reference 22), and perhaps these results will contribute to an understanding of these aspects of muscle cell biology.

We also found that the smaller AChR microaggregates were found preferentially around nuclei as well, even though such nuclei continued to show normal movement. In part, this finding might be partially explained if the microaggregates themselves were not totally stationary. Also relevant is our observation that the Golgi apparatus, where AChRs are assembled into their final form and achieve the ability to bind alpha-bungarotoxin (19), seems to be localized around nuclei as well. Newly synthesized AChRs could well be inserted into plasma membrane regions directly above the Golgi (see, for instance, reference 10). Thus, at all times, membrane AChRs would be present at relatively high concentrations in the vicinity of nuclei, thereby increasing the chances of their being gathered into small or larger patches.

Our evidence favors the possibility that events initiated by AChR clustering are involved with organelle localization. First, we observed numerous instances in which clusters formed away from nuclei, but nuclei soon migrated to these regions and then became immobile for our entire observation period (up to 48 h). Second, in addition to having a markedly decreased lateral movement, most immobile nuclei also did

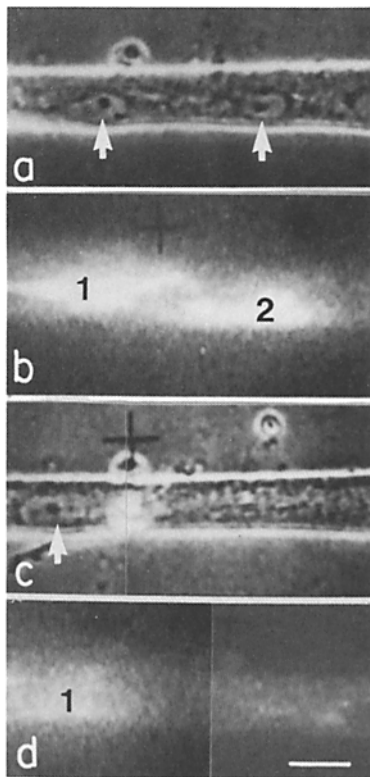


Figure 7. Nuclear movement resumes after cluster dispersal. Chick muscle cells were labeled with rhodamine alpha-bungarotoxin after treatment with clustering factor. Photos were taken from a video monitor. *a* and *c* are phase views at 0 and 6 h, respectively. *b* and *d* are corresponding fluorescence views. At the start of the experiment, this myotube had two large clusters (labeled 1 and 2) that both had associated nuclei (arrows; the resolution of these clusters is somewhat blurred by the video monitor images). During the next few hours, cluster 2 dispersed and its nucleus resumed moving (and is no longer in the picture frame) while the nucleus associated with cluster 1 (itself beginning to disperse) had not yet started to migrate. Bar, 10 μ m.

not rotate in the normal way, further suggesting that they were held in place. Finally, if clusters dispersed spontaneously, these same subcluster nuclei resumed normal migration. Such results suggest that AChR aggregation in the plasma membrane can have a profound effect on the organization of the muscle's cytoplasm. Almost certainly, AChR clustering initiates changes in the assembly of membrane-associated cytoskeletal elements that in turn restrict normal organelle movement. Not surprisingly, plasma membrane events have been thought for some time to have significant influences on processes inside the cell (e.g., reference 16).

In this paper we also present some data concerning the structural elements involved in associating nuclei and AChR clusters. Nuclei appear to move normally along microtubules, since movement is completely inhibited by colchicine treatment. On the other hand, colchicine treatment did not prevent cluster formation, nor did it disrupt pre-established cluster-nucleus complexes, so microtubules may not be involved directly in these processes. Cytochalasin D treatment did not alter nuclear movement, nor did it disrupt clusters or cause nuclei immobilized beneath clusters to resume mov-

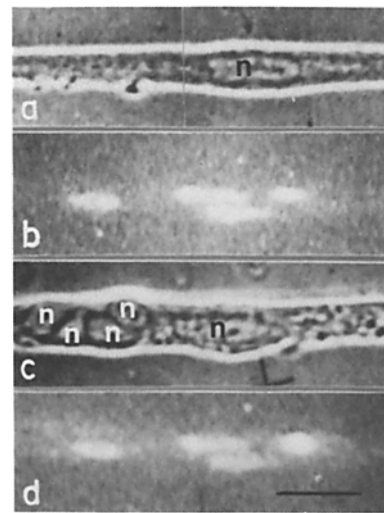


Figure 8. Clusters are maintained and can become associated with nuclei even in the presence of cytochalasin D. Photos were taken from a video monitor. Chick muscle cells were pretreated with clustering factor and cytochalasin D for 4 h and labeled with rhodamine alpha-bungarotoxin. *a* and *c* are phase images at the start of the experiment and 4 h later, respectively. *b* and *d* are corresponding fluorescence images. The three clusters on the right of the cell have an underlying nucleus (black *n*), while the left cluster has no associated nucleus. During the next 4 h, a group of nuclei (white *n*) migrated to the region of this cluster and then remained there for at least two additional hours. The clusters also remained intact. Bar, 10 μ m.

ing. However, this agent appeared to prevent clusters from forming.

Our results on the effects of colchicine on preformed clusters are in general agreement with those of Connolly (11), who studied chick muscle cells treated with a soluble clustering factor derived from chicken brain. They are different, however, from those of Bloch (5), who studied cluster dispersal in rat cells. Perhaps these differences were caused by variations in types of muscle cells used, culture conditions, and counting procedures. Conceivably, these differences could also reflect actual differences in the mechanism of cluster formation under the different experimental conditions.

Our results on the effects of cytochalasin are similar to those of Bloch (5), but apparently different from those of Connolly et al. (11, 12), who suggested that such treatment causes cluster dispersal in chick cells. We did not see this, however, even in an extensive examination of individual clusters labeled with rhodamine alpha-bungarotoxin and followed over time. We did find that cytochalasin blocks cluster formation and that a certain percentage of clusters disperse spontaneously. It is possible, then, that part of what Connolly et al. interpreted as cluster disruption in cytochalasin was actually a combination of normal dispersal without new cluster formation. Again, it is also possible that different mechanisms are used in different cell types or in response to different clustering factors.

Our findings suggest that actin-containing filaments may be involved in cluster formation itself and form part of a cytoskeletal network linking the membrane and the nuclei.

Certainly, previous work has suggested the presence of high concentrations of nonmyofibrillar actin in the vicinity of clusters (12, 32). There is also some evidence that the 43-kD AChR-associated protein binds actin (48). Therefore, this molecule may serve as an intermediate between the AChR and the rest of the cytoskeleton (8). Peng and Froehner (38) have seen high concentrations of this molecule beneath AChR clusters on *Xenopus* myocytes, and we have obtained similar results in cultures of rat muscle (44). Our results suggest that once the actin monomers have polymerized into a subcluster network, they are not as easily disrupted by treatment with cytochalasin. Thus these filaments must have become stabilized in some way. Recently, we have also found that a nonmyofibrillar, muscle-specific, 37-kD molecular mass tropomyosin is absent from cells transformed with Rous sarcoma virus that are completely unable to cluster AChRs (3, 4; Anthony, D. T., and L. L. Rubin, manuscript in preparation). So, this tropomyosin may be part of the network as well, possibly serving to stabilize the actin filaments. We are currently investigating the participation of other cytoskeletal elements in the complex.

We would envision that this network functions in some way in the initial redistribution of AChRs to form a large cluster. Since clusters can form in the presence of colchicine, microtubules are not necessary for this initial process. In the presence of colchicine, nuclei will not subsequently migrate beneath the clusters, so the prediction would be that these clusters could not be maintained as readily by insertion of newly synthesized AChRs. Since the cells eventually undergo radical shape changes in the presence of colchicine, we have been unable to determine if this is the case.

Based on our results and what is known about neuromuscular junction formation in tissue culture and in embryos, we would like to suggest the following sequence of events. It seems likely that motor nerve processes contact muscle fibers randomly and then secrete a factor that localizes AChRs to sites beneath the nerve endings. The first clustered AChRs include some that have moved from other regions of the muscle cell. These changes in the plasma membrane cause a random subset of migrating nuclei to assume a junctional position. Subsequently, these junctional nuclei and their surrounding Golgi apparatus may be responsible for providing a source of newly synthesized AChRs for insertion directly into the cluster to replace degraded AChRs. Thus, it may be that initial cluster formation and cluster maintenance occur via different mechanisms, although it might also be that local insertion and redistribution both function to some degree at all times.

Finally, it is possible that, in adult muscle, junctional nuclei transcribe a different set of genes than those transcribed by extrajunctional ones (34). Since our results suggest that a random set of myonuclei achieve a subcluster localization, differences among nuclei probably occur subsequent to their positioning. Also, these differences seem to be reversible, being determined, at least in the case of AChR synthesis, by levels of muscle contractile activity (30, 31). In other experiments we have shown that increases in muscle cell Ca^{2+} are associated with decreases in AChR synthesis (43). Perhaps, then, in normally contracting muscle, regional differences in gene transcription are caused by regional differences in cytoplasmic Ca^{2+} . This hypothesis is currently being tested by using Ca^{2+} -sensitive fluorescent dyes to measure Ca^{2+}

levels in different regions of adult muscle during contractile activity.

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References

1. Anderson, M. J., and M. W. Cohen. 1977. Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.)* 260:757-773.
2. Anderson, M. J., M. W. Cohen, and E. Zorychta. 1977. Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.)* 268:731-756.
3. Anthony, D. T., and L. L. Rubin. 1985. Molecular effects of Rous sarcoma virus transformation on cultured chicken myotubes. *Soc. Neurosci. Abstr.* 11:304.
4. Anthony, D. T., S. M. Schuetze, and L. L. Rubin. 1984. Transformation by Rous sarcoma virus prevents clustering of acetylcholine receptors on cultured chicken muscle fibers. *Proc. Natl. Acad. Sci. USA* 81:2265-2269.
5. Bloch, R. J. 1979. Dispersal and reformation of acetylcholine receptor clusters of cultured rat myotubes treated with inhibitors of energy metabolism. *J. Cell Biol.* 82:626-643.
6. Bloch, R. J., and Z. W. Hall. 1983. Cytoskeletal components of the vertebrate neuromuscular junction: Vinculin, α -actinin and filamin. *J. Cell Biol.* 97:217-223.
7. Burden, S. 1982. Identification of an intracellular postsynaptic antigen at the frog neuromuscular junction. *J. Cell Biol.* 94:521-530.
8. Burden, S. J., R. L. DePalma, and G. S. Gottesman. 1983. Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the β -subunit and the 43 kd subsynaptic protein. *Cell* 35:687-692.
9. Burden, S. J., P. B. Sargent, and U. J. McMahan. 1979. Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve. *J. Cell Biol.* 82:412-425.
10. Bursztajn, S., and G. D. Fischbach. 1984. Evidence that coated vesicles transport acetylcholine receptors to the surface membrane of chick myotubes. *J. Cell Biol.* 98:498-506.
11. Connolly, J. A. 1984. Role of the cytoskeleton in the formation, stabilization and removal of acetylcholine receptor clusters in cultured muscle cells. *J. Cell Biol.* 99:148-154.
12. Connolly, J. A., and A. J. Graham. 1985. Actin filaments and acetylcholine receptor clusters in embryonic chick myotubes. *Eur. J. Cell Biol.* 37:191-195.
13. Dennis, M. J. 1981. Development of the neuromuscular junction: inductive interactions between cells. *Annu. Rev. Neurosci.* 4:43-68.
14. Devreotes, P. N., and D. M. Fambrough. 1975. Acetylcholine receptor turnover in membranes of developing muscle fibers. *J. Cell Biol.* 65:335-358.
15. Durand, D. 1971. Stable Chaos: An Introduction to Statistical Control. General Learning Press, Morristown, NJ.
16. Edelman, G. M. 1976. Surface modulation in cell recognition and cell growth. *Science. (Wash. DC)* 192:218-226.
17. Englander, L. L., and L. L. Rubin. 1982. Co-localization of acetylcholine receptor clusters and nuclei in cultured myotubes. *Soc. Neurosci. Abstr.* 8:697.
18. Englander, L. L., and L. L. Rubin. 1985. Acetylcholine receptor clustering and nuclear movement in muscle fibers in culture. *J. Cell Biol.* 101:30a.
19. Fambrough, D. M., and P. N. Devreotes. 1978. Newly synthesized acetylcholine receptors are located in the Golgi apparatus. *J. Cell Biol.* 76:237-244.
20. Fischbach, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* 28:407-429.
21. Fischbach, G. D., and S. A. Cohen. 1973. The distribution of acetylcholine sensitivity over uninervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* 31:147-162.
22. Fischman, D. A. 1970. The synthesis and assembly of myofibrils in embryonic muscle. *Curr. Top. Dev. Biol.* 5:235-280.
23. Frank, E., and G. D. Fischbach. 1979. Early events in neuromuscular junction formation in vitro. Induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. *J. Cell*

24. Frank, E., K. Gautvik, and H. Sommerschild. 1975. Cholinergic receptors at denervated mammalian motor endplates. *Acta Physiol. Scand.* 95:66-76.
25. Froehner, S. C. 1986. The role of the postsynaptic cytoskeleton in AChR organization. *TINS.* 9:37-41.
26. Hall, Z. W., B. W. Lubit, and J. H. Schwartz. 1981. Cytoplasmic actin in postsynaptic structures at the neuromuscular junction. *J. Cell Biol.* 90:789-792.
27. Hirokawa, N., and J. E. Heuser. 1982. Internal and external differentiations of the postsynaptic membrane at the neuromuscular junction. *J. Neurocytol.* 11:487-510.
28. Kelly, A. M., and S. I. Zacks. 1969. The fine structure of motor endplate morphogenesis. *J. Cell Biol.* 42:154-169.
29. Kuromi, H., and Y. Kidokoro. 1984. Nerve disperses preexisting acetylcholine receptor clusters prior to induction of receptor accumulation in *Xenopus* muscle cultures. *Dev. Biol.* 103:53-61.
30. Lomo, T., and Rosenthal, J. 1972. Control of ACh sensitivity by muscle activity in the rat. *J. Physiol. (Lond.)*. 221:493-513.
31. Lomo, T., and R. H. Westgaard. 1975. Further studies on the control of ACh sensitivity by muscle activity in the rat. *J. Physiol. (Lond.)*. 252:603-626.
32. Lubit, B. W. 1984. Association of β -cytoplasmic actin with high concentrations of acetylcholine receptor (AChR) in normal and anti-AChR-treated primary rat muscle cultures. *J. Histochem. Cytochem.* 32:973-981.
33. McMahan, U. J., J. R. Sanes, and L. M. Marshall. 1978. Cholinesterase is associated with the basal lamina at the neuromuscular junction. *Nature (Lond.)*. 271:172-174.
34. Merlie, J. P., and J. R. Sanes. 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. *Nature (Lond.)*. 317:66-68.
35. Nakai, J. 1968. The development of neuromuscular junctions in cultures of chick embryo tissues. *J. Exp. Zool.* 170:85-106.
36. Novikoff, A. B., and S. Goldfischer. 1961. Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness for cytological studies. *Proc. Natl. Acad. Sci. USA.* 47:802-810.
37. Peng, H. B. 1983. Cytoskeletal organization of the presynaptic nerve terminal and the acetylcholine receptor cluster in cell cultures. *J. Cell Biol.* 97:489-498.
38. Peng, H. B., and Froehner, S. C. 1985. Association of the postsynaptic 43K protein with newly formed acetylcholine receptor clusters in cultured muscle cells. *J. Cell Biol.* 100:1698-1705.
39. Peng, H. B., and Phelan, K. A. 1984. Early cytoplasmic specialization at the presumptive acetylcholine receptor cluster: a meshwork of thin filaments. *J. Cell Biol.* 99:344-349.
40. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α -bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80:585-592.
41. Rogers, A. W. 1973. Techniques of Autoradiography. Elsevier, Amsterdam.
42. Role, L. W., V. R. Matossian, R. J. O'Brien, and G. D. Fischbach. 1985. On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. *J. Neurosci.* 5:2197-2204.
43. Rubin, L. L. 1985. An increase in intracellular calcium mediates changes in acetylcholine receptors and acetylcholinesterase caused by muscle contraction. *Proc. Natl. Acad. Sci. USA.* 82:7121-7125.
44. Rubin, L. L., D. T. Anthony, L. L. Englander, R. I. Lappin, and I. M. Lieberburg. 1986. Molecular modifications during nerve-muscle synapse formation. *Prog. Brain Res.* In press.
45. Rubin, L. L., and K. F. Barald. 1983. Neuromuscular development in tissue culture. In *Somatic and Autonomic Nerve-Muscle Interactions*. G. Burnstock, R. O'Brien, and G. Vrbova, editors. Elsevier, Amsterdam. 109-151.
46. Rubin, L. L., and U. J. McMahan. 1982. Regeneration of the neuromuscular junction: steps toward defining the molecular basis of the interaction between nerve and muscle. In *Disorders of the Motor Unit*. D. L. Schotland, editor. Wiley, New York. 187-196.
47. Sanes, J. R., and Z. W. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.* 83:357-370.
48. Walker, J. H., C. M. Boustead, and V. Witzemann. 1984. The 43-K protein, ν_1 , associated with acetylcholine receptor containing membrane fragments is an actin-binding protein. *EMBO J.* 3:2287-2290.
49. Ziskind-Conhaim, L., I. Geffen, and Z. W. Hall. 1984. Redistribution of acetylcholine receptors on developing rat myotubes. *J. Neurosci.* 4:2346-2349.