Interaction of the Cytoskeletal Framework with Acetylcholine Receptor on the Surface of Embryonic Muscle Cells in Culture

J. PRIVES, A. B. FULTON, S. PENMAN, M. P. DANIELS, and C. N. CHRISTIAN
Department of Anatomical Sciences, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794, Laboratory of Developmental Neurobiology, National Institutes of Health, Bethesda, Maryland 20205, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02140, and Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT   To monitor the interaction of cell surface acetylcholine (AcCho) receptors with the cytoskeleton, cultured muscle cells were labeled with radioactive or fluorescent α-bungarotoxin and extracted with Triton X-100, using conditions that preserve internal structure. A significant population of the AcCho receptors is retained on the skeletal framework remaining after detergent extraction. The proportion of nonextracted AcCho receptors increases during myotube development. Both photographic images and quantitative fluorescence measurements indicate that AcCho receptors in patched or aggregated areas are retained on the cytoskeleton while the diffuse receptors are partially extracted by detergent. The skeleton organization responsible for restricting AcCho receptors to a patched region may also result in their retention after detergent extraction.

Despite an increasing appreciation of the physiological relevance of the spatial arrangement of many integral membrane proteins, the structural basis of the regulation of cell surface topography is poorly understood (for review, see reference 1). The acetylcholine (AcCho) receptor of skeletal muscle cells is a well characterized integral surface membrane protein that undergoes pronounced changes in distribution on the cell surface during differentiation and innervation of these cells (for review, see references 2–5). Initially, AcCho receptors on the surface of pre-innervated embryonic muscle cells are distributed randomly or homogeneously (3, 4). After innervation, the entire AcCho receptor population becomes concentrated into a small patch of membrane adjacent to the nerve ending (3, 6–8). Studies of muscle innervation in vitro suggest that preexistent surface AcCho receptors are redistributed to the patch at the innervation site (9, 10). Even in the absence of innervation, AcCho receptors on cultured embryonic muscle cells spontaneously form clusters (11–14) which have an elevated packing density and diminished lateral mobility (15) similar to those of the junctional AcCho receptors of innervated muscle.

Recent findings in other cell types suggest that most plasma membrane proteins are not extracted when lipids are removed with nonionic detergent (16–18). Rather, the surface proteins form a sheet or lamina that covers the internal skeletal framework remaining after detergent extraction (18). However, there are a few plasma membrane proteins which are extensively extracted from the surface lamina (16–18). We report here that in the early development of cultured muscle cells most of the

AcCho receptors are extracted by detergent. With maturation of these cells, a proportion of the receptors patch and become completely bound to the cytoskeletal framework. The association with cytoskeleton of AcCho receptors that are not recognizably clustered also appears to increase. A preliminary report of a portion of these results has appeared (19).

MATERIALS AND METHODS

Cell Cultures

Primary cultures of muscle cells were obtained from rat and chick embryos. Myogenic cells were isolated from breast muscle tissue of 12-d chick embryos and cultured as described (20). Rat muscle cultures were prepared as described (21) and grown in medium containing 0.5 μg/ml tetrodotoxin.

Labeling of Acetylcholine Receptor

AcCho receptor on the surface of intact cultured muscle cells was monitored by the binding of α-bungarotoxin (α-Bgt) labeled with ^{125}I (^{125}I-α-Bgt) or conjugated with tetramethylrhodamine (TMR-α-Bgt) (22). The cells were labeled with α-Bgt as described (14, 20, 23). Cultures were washed once in Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (1 mg/ml) and incubated with ^{125}I-α-Bgt or TMR-Bgt (10^{-8} M) for 1 h at 37°C. At the end of this period, cultures were washed four times with 3-ml volumes of DMEM to remove unbound toxin. Specificity of binding was established by the inhibition of α-Bgt labeling in the presence of the competitive inhibitor decamethonium (20 μM) as described (23).

Preparation of Triton X-100 Cytoskeletons

Muscle cells were extracted with Triton X-100 under conditions shown to result in the rapid removal of plasma membrane lipids and soluble cytoplasmic

proteins and retention of intact cytoskeletal frameworks on the substratum (18). The cultures were washed once with a 3-ml volume of 0.3 sucrose, 50 mM NaCl, 1 mM MgCl₂ and 10 mM HEPES, pH 7.4 (Buffer A), then extracted by incubation for various times at 22°C with a 1 ml volume of Buffer A supplemented with 0.5% Triton X-100 and, where stated, 1 mM EGTA. These extraction conditions do not alter the dissociation rate of α-Bgt from the AcCho receptor (5).

To measure the extraction of AcCho receptor from the muscle cell surface by detergent, cultures labeled with ^{125}I-α-Bgt were washed with Buffer A, and 1 ml of Buffer A containing 0.5% Triton X-100 was added to the culture dishes. After incubation at room temperature for the specified interval, the detergent buffer was removed from the monolayer and transferred to a vial for gamma counting. The material remaining on the culture plate was dissolved by a 2-h incubation with 1 N NaOH and counted. The radioactivity remaining in each plate after removal of the extraction solution was expressed as a percentage of the total amount of ^{125}I-α-Bgt bound to the intact cultures. The time-course of these data, over a period of 10 min, was fit to the equation $R = Se^{-st} + (100 - S)e^{-ft}$, where s and f are the extraction rate constants for the slow and fast components, respectively, and S is the percentage of receptors in the slowly extracting fraction. The curve-fitting routine was a least squares analysis carried out by computer.

Fluorescence Microscopy

The effect of the detergent treatment on the distribution of AcCho receptors was visualized by labeling cultured muscle cells with TMR-α-Bgt and inspecting the cultures with a Zeiss photomicroscope II, equipped with epi-illumination.

Cultures were transferred to Buffer A, and the same field was photographed before and after the addition of 0.5% Triton X-100.

For measurement of extraction rates of fluorescent-labeled AcCho receptors, glass cover slips with rat muscle cultures at 7 d or 12 d after plating were labeled with TMR-α-Bgt, mounted in a chamber containing 0.4 ml of Buffer A, and perfused with this buffer at a rate of 1 ml/min. The microscope objective was focused on an area of myotube surface containing either diffusely distributed AcCho receptors or a large aggregate of the receptors. Fluorescent light from a 37-μm diameter field was measured by a photomultiplier tube (R-928, Hamamatsu) attached to the microscope and powered by a spectrofluorometer (Farrand MK II). The output was sampled at 3 s intervals before and after addition of 0.5% Triton X-100 in Buffer A to the chamber.

Scanning Electron Microscopy

For scanning electron microscopy (SEM), previously extracted muscle cultures on glass cover slips were fixed in 1% (chick) or 2.5% (rat) glutaraldehyde in Buffer A plus 0.5% Triton X-100 for 0.5-1 h. Unextracted cultures were treated similarly but without detergent. The cultures were postfixed for 5 min in 1% OsO₄, dehydrated in ethanol, critical point-dried, coated with gold-palladium, and viewed in the scanning electron microscope. For transmission electron microscopy, previously extracted cultures in 35-mm plates were fixed (2.5% glutaraldehyde) and postfixed as for SEM, then stained en bloc with uranyl acetate, dehydrated, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate.
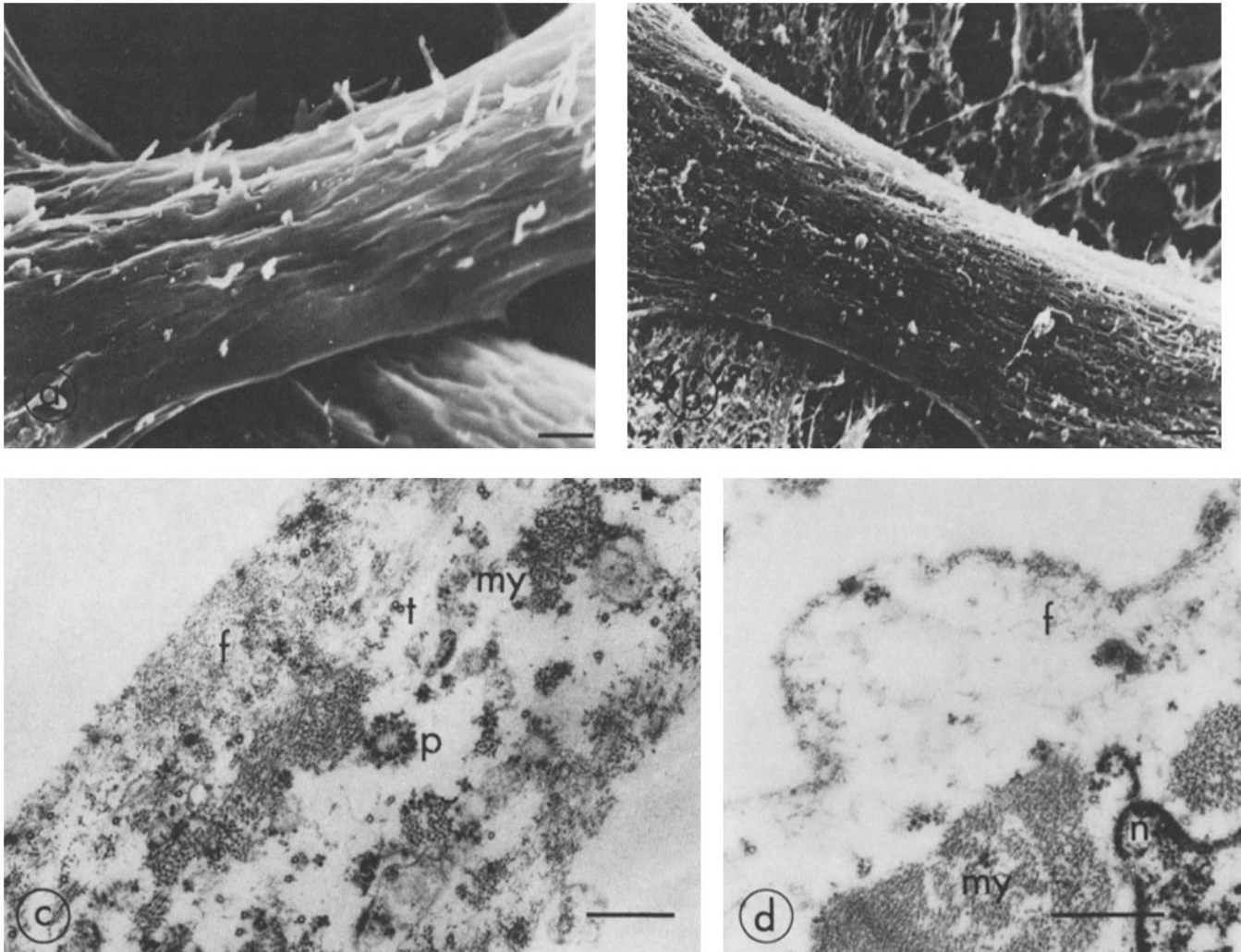

FIGURE 1 (a and b) Scanning electon micrographs of embryonic chick myotubes. (a) Intact day 4 myotube. (b) Detergent-extracted day 4 myotube showing the plasma lamina of the cytoskeleton. Bars, 1 μm. (c and d) Transmission electron micrographs of detergent-extracted day 6 rat (c) and day 10 chick (d) myotubes. n, nucleus. p, polyribosomes. my, myofibrils. t, microtubules. f, filaments. Bars, 0.5 μm. a, × 5,650. b, × 5,250. c, × 25,300. d, × 25,300.

Materials

RESULTS

Structure of Detergent-extracted Muscle Cells

Fig. 1 a and b are scanning electron micrographs of intact (a) and extracted (b) chick myotubes from 4-d-old cultures. At this stage of myotube development, the surface lamina covers most of the skeletal framework although numerous small openings remain (Fig. 1 b). The surface morphology resembles that of the intact cell (Fig. 1 a), suggesting that considerable structural integrity remains in the framework elements supporting the surface lamina. Similar results are obtained with SEM of extracted rat myotubes.

Fig. 1 c and d are transmission electron micrographs of transverse sections of an embryonic rat myotube after 6 d in culture (c) and an embryonic chick myotube after 10 d in culture (d). The plasma membrane and other membranous organelles are extracted, and large electron-transparent areas are seen within the cytoplasm. However, the cytoskeleton including microfilaments, intermediate filaments, microtubules, and myofibrils appears intact. The accumulation of filaments directly below the surface lamina is greater in rat than in chick myotube cytoskeletons.

Cytoskeletal Association of AcCho Receptors during Muscle Development

Figs. 2 and 3 show the results of representative experiments in which ^{125}I-labeled α-Bgt, a specific and highly irreversible ligand of AcCho receptors (24), is bound to embryonic chick or rat muscle cultures before extraction with Triton X-100. Fig. 2 shows the time-course of extraction of ^{125}I-α-Bgt-AcCho
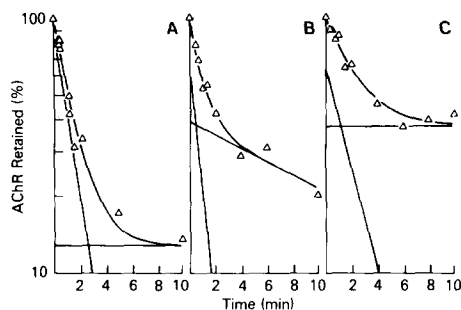

FIGURE 2 Extraction of AcCho receptors by detergent-treatment of embryonic chick muscle cells. The time-course of extraction of ^{125}I-α Bgt-AcCho receptor complexes from muscle at 4 d (A), 5 d (B), and 10 d (C) after plating are shown. In each panel, the percentage of total AcCho receptor that was retained on myotubes is plotted against time of exposure to detergent. Each point is the mean of duplicate plates. Total labeling, expressed as fmoles ^{125}I-α-Bgt bound per plate: 240 (A), 254 (B), and 187 (C). Nonspecific labeling, detected in the presence of decamethonium (20 μm) is <5% of the total. Given the rate of internalization of AcCho receptor α-Bgt complexes from the myotube surface (3), <3% of labeled receptors are internalized at the time of extraction. Also shown is the least sum of squares fit of these data to a decay curve with two exponential terms. The intercept of the slower exponential curve with the ordinate indicates the portion of AcCho receptors resistant to rapid extraction.
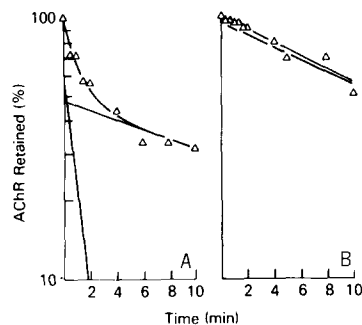

FIGURE 3 Extraction of AcCho receptors by detergent treatment of cultured embryonic rat muscle cells. Cultures were extracted at 4 d (A) or 10 d (B) after plating. Results are plotted as in Fig. 2. Each point is the mean of duplicate plates. Total labeling: 478 (A) and 577 (B) fmol ^{125}I-α-Bgt/culture dish. Nonspecific labeling is <5% of the total. The data in panel B are adequately fit by a single exponent.

receptor complexes from cultures of embryonic chick muscle at 4, 6, and 10 d after plating. In each panel of Fig. 2, the percentage of total AcCho receptor that is retained is plotted against time of exposure to detergent. Also shown is the least sum of squares fit of these data to a decay curve with two exponential terms. The intercept of the slower exponential curve with the ordinate indicates the portion of AcCho receptors resistant to rapid extraction. This portion is seen to increase substantially with time in culture. Thus, in 4-d-old chick muscle cell cultures, the major proportion of AcCho receptor is rapidly extracted with a half time of <1 min, while between 10% and 15% of total receptor is resistant to detergent extraction. In 6- and 10-d-old cultures, the proportion of total AcCho receptor that remains associated with the detergent-extracted cytoskeleton increases to ~40% of the total.

Qualitatively similar results are obtained with cultured rat muscle cells. The kinetic data in Fig. 3 show a rapidly extracted and a retained component in the 4-d-old cultures. The fraction of total AcCho receptor that is retained on the detergent-resistant cytoskeletal framework increases with time in culture, until at 10 d no rapidly extracted component is detected.

These results show that when AcCho receptors first appear on the surface of embryonic chick or rat muscle cells differentiating in culture, they are extensively released from the skeletal framework by the mild detergent extraction. However, even at early culture times, a proportion of these receptors is retained and this portion increases markedly as the culture matures.

Relationship of Cytoskeletal Association to Distribution of AcCho Receptors

Fig. 4 a is a fluorescence photomicrograph showing AcCho receptor distribution on an intact rat myotube stained with TMR-α-Bgt. There are two prominent regions of aggregated or patched AcCho receptors in this cell while the remaining receptors are more diffusely distributed. Fig. 4 b shows the same field 5 min after extraction with detergent. The aggregated AcCho receptor regions are still prominent while the diffuse receptor fluorescence has diminished below the threshold of visibility. Similarly, labeling 5-d-old embryonic chick muscle cells with TMR-α-Bgt reveals AcCho receptor aggregates together with regions of diffuse receptor staining (Fig. 4 c). The AcCho receptor aggregates on the surface of chick muscle cells are significantly smaller than on the rat myotubes (Fig. 4 a). As with the rat muscle cell (Fig. 4 a and b), extraction of the chick myotubes with detergent appears to preferentially
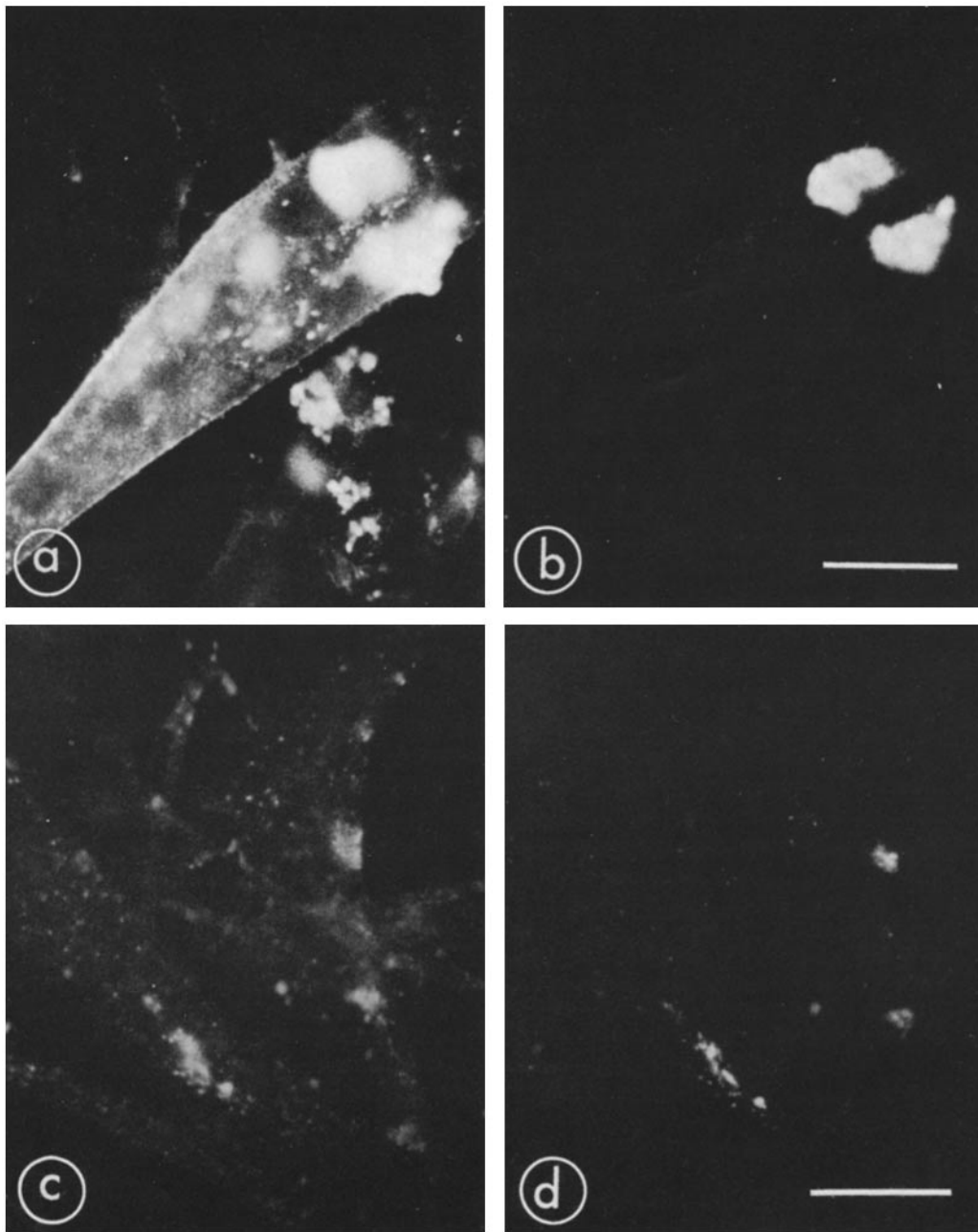
FIGURE 4 The effect of detergent extraction on distribution pattern of AcCho receptors on cultured myotubes. (a) A myotube in a 12-d-old rat muscle cell culture, prepared as described in Materials and Methods, except that dissociated cells were plated at a density of 0.7 × 10⁵ cells/16-mm culture dish containing a 12-mm collagen-coated glass cover slip. This procedure resulted in myotube cultures which developed more slowly than those prepared at higher plating concentrations on plastic culture dishes (Fig. 3). The cover slip was labeled with TMR-α-Bgt, mounted in Buffer A and examined, with a × 40 oil immersion objective NA 1.0. (b) The same field 5 min after addition of 0.5% Triton X-100 in Buffer A. (c) Intact myotubes in a 7-d-old chick muscle cell culture. The cultures were prepared in plastic culture dishes, labeled, and examined in Buffer A with a × 40 water immersion objective NA 0.75. (d) The same field 3 min after adding 0.5% Triton X-100 in Buffer A. Bars, 30 μm. a and b, × 625. c and d, × 635.

remove diffuse AcCho receptors and leave a large proportion of the aggregated receptors attached to the extracted myotube cytoskeleton (Fig. 4c and d). Receptor patches located on the upper surface and sides of myotubes as well as those situated on the basal surface were similarly resistant to detergent extraction, in chick (Fig. 4c and d) and in rat cultures (not shown). This rules out the possibility that these observations might reflect insufficient extraction of membrane regions "protected" by overlying structures or the collagen substrate. These qualitative results suggest that the diffuse receptors are pref-

erentially extractable while the patched receptors are stably bound to the cytoskeleton.

Quantitative measurements of the fluorescence in small areas of the TMR-α-Bgt-labeled rat muscle cell surface before and after detergent extraction are made with a photomultiplier detector. Representative results obtained in membrane regions containing diffuse or aggregated AcCho receptors are shown in Fig. 5. There is a continuous decay of fluorescence because of photobleaching of the TMR-α-Bgt probe, which is identical in areas of both diffuse and aggregated AcCho receptors. After

the addition of detergent (indicated by the arrow), there is no additional loss of fluorescence by clustered AcCho receptors above the photobleaching rate. In contrast, in areas of diffuse distribution of AcCho receptors, a rapid decrease of fluorescence intensity follows the addition of detergent (Fig. 5). After 1 min, the rapid partial extraction appears complete and the slower decay of fluorescence by photobleaching reappears.

Table I shows the rates of extraction in detergent of patched and diffuse AcCho receptors. These rates are calculated from fluorescence measurements of extraction such as those shown in Fig. 5. The values are estimated from the initial Triton X-100 extraction curves after correction for photobleaching. Comparison of extraction rates observed during the initial minute of detergent treatment shows that a portion of the diffusely distributed AcCho receptor is rapidly extracted. In contrast, AcCho receptors clustered on the myotube surface are resistant to extraction.

DISCUSSION

We have used a mild detergent extraction to detect the association of surface AcCho receptors with the cytoskeletal framework in cultured muscle cells. We find that this procedure distinguishes between two subpopulations of this integral membrane protein. The experimental results indicate the following: that (a) aggregated AcCho receptors are tightly bound to the myotube skeletal framework; that (b) a portion of the diffusely distributed AcCho receptors are not tightly bound and are extracted with detergent; and that (c) the proportion of the tightly bound AcCho receptors increases with muscle cell development.

Most of the surface proteins of cultured cells remain attached to the cytoskeletal framework when lipids and soluble proteins are removed by detergent extraction (18). These form a surface
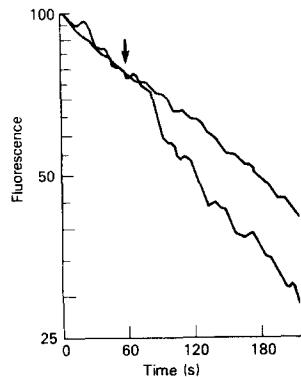

FIGURE 5 Quantitative fluorescence microscopy of TMR-α-Bgt-labeled AcCho receptor on day 7 cultured rat myotubes: comparison of extraction rates of aggregated (upper trace) and diffuse (lower trace) AcCho receptors in detergent. Arrow indicates addition of 0.5% Triton X-100 in Buffer A to the chamber.

TABLE I

Calculated Rates of AcCho Receptor Extraction in Triton X-100

	Diffuse AChoR	Aggregated AChoR
t½ (s)	185.8	>2,000
Rate constant, (τ) × 10⁻⁴	37.3 ± 9.28 (6)	1.28 ± 1.16 (5)

Rates of extraction in detergent of patched and diffuse AcCho receptors on cultured rat myotubes, calculated from the results of quantitative fluorescence measurements like those shown in Fig. 5. Rates of extraction of aggregated and diffuse AcCho receptors (AcChoR) during perfusion with 0.5% Triton X-100 in Buffer A were measured as described in Fig. 5 and Materials and Methods. t½ is the time at which 50% of AcCho receptors are extracted. Figures in parentheses are the number of experiments.

lamina which can be seen by SEM. In a study to be reported elsewhere (25), surface components of muscle cells were iodinated before detergent extraction. It was shown by direct measurements that most of the surface membrane proteins of prefusion myoblasts and postfusion myotubes remain with the cytoskeletal framework while three-quarters of total cellular proteins are removed. Thus, a fraction of AcCho receptors that are extracted belong to a minor class of surface proteins released from the cytoskeleton by the detergent. The developmental regulation of the cytoskeletal attachment of AcCho receptors may affect their distribution and function.

Tight binding of the AcCho receptor to the skeletal framework appears to accompany the formation of AcCho receptor patches. Quantitative measurements on rat myotubes indicate that there is no detectable loss of fluorescence-labeled AcCho receptors from the patched area when detergent is added. The fluorescence measurements further indicate that a fraction of the diffusely distributed receptors is also retained with the extracted skeletal framework. This is also evident in mature cultured rat myotubes where the AcCho receptors become completely bound to the framework although not completely patched. We cannot tell by the optical methods used here whether the diffuse receptors are actually aggregated on a submicroscopic scale.

Our findings, when related to recent measurements of AcCho receptor lateral mobility, indicate that cytoskeletal attachment limits the lateral movement of AcCho receptors. Axelrod et al. (15) have demonstrated that all AcCho receptors in patches are laterally immobile in the cell membrane. Diffusely distributed AcCho receptors can be divided into mobile and immobile fractions (15), and the immobile fraction increases with age in culture (D. Axelrod, personal communication). The close parallels between lateral immobility and resistance to detergent extraction lead us to propose that cytoskeletal attachment of AcCho receptors accounts for both phenomena.

An ultrastructural cytochemical study has shown the presence of an electron-dense fibrillar structure under areas of muscle cell surface containing high densities of AcCho receptors (26-28). Furthermore, a recent study (29) has found that aggregated AcCho receptors may be located near areas of concentration of vinculin, a component of the peripheral cytoskeleton associated with regions of cell attachment. Moreover, it is possible that components of the extracellular matrix which are retained on the framework after detergent-extraction may participate in the attachment of AcCho receptor aggregates. Recent observations suggesting the association of extracellular matrix structures (26-28) and antigens (30, 31) with AcCho clusters are consistent with this possibility.

The physiological relevance of our findings indicating the interaction of cytoskeletal elements with AcCho receptors is suggested by the similarities between the aggregation of these receptors on the surface of cultured muscle cells and the specialized subsynaptic regions of innervated muscle. These include high packing density of the receptors (3, 4, 6-8, 11-14) and reduced lateral mobility (15). The induction of aggregated AcCho receptors on cultured myotubes can result from innervation (9, 10) or from the addition of soluble neuronal factors (21, 32, 33). Moreover, the stimulation of AcCho receptor aggregation observed upon exposure of cultured muscle cells to neuron-conditioned medium (21) is accompanied by increased retention of these receptors on detergent-insoluble cytoskeleton (manuscript in preparation), as well as the reduction in lateral mobility of AcCho receptors (34).

We suggest that the capacity of aggregated cell surface

AcCho receptors to interact with cytoskeleton may contribute to the maintenance of high density regions of AcCho receptors characteristic of subsynaptic surface of innervated muscle. In more general terms, this study supports an interpretation of plasma membrane structural organization that stresses the role of the peripheral cytoskeleton in the expression of cell surface properties associated with the interactions between cells.

REFERENCES

1. Nicolson, G. L. 1976. Transmembrane controls of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys Acta.* 457: 57-108.
2. Karlin, A. 1980. Molecular properties of nicotinic acetylcholine receptors. *In* The Cell Surface and Neuronal Function. C. W. Cotman, G. Poste, and G. L. Nicolson, editors. Elsevier-North Holland Biomedical Press, New York. 191-260.
3. Fambrough, D. 1979. Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* 59:165-227.
4. Patrick, J., S. Heinemann, and D. Schubert. 1978. Biology of cultured nerve and muscle. *Annu. Rev. Neurosci.* 1:417-443.
5. Heidmann, T., and J. P. Changeux. 1978. Structural and functional properties of the acetylcholine receptor protein in its purified and membrane-bound states. *Annu. Rev. Biochem.* 47:317-357.
6. Barnard E., A. Wieckowski, and T. Chiu. 1971. Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. *Nature (Lond.).* 234:207-209.
7. Hartzell, H., and Fambrough, D. 1972. Acetylcholine receptors distribution and extra-junctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. *J. Gen. Physiol.* 60:248-262.
8. Fertuck, H., and M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-α-bungarotoxin binding at mouse neuromuscular junctions. *J. Cell Biol.* 69:144-158.
9. Anderson, M. J., M. W. Cohen, and E. Zorchyta. 1977. Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.).* 268: 731-756.
10. Frank, E., and G. D. Fischbach. 1977. ACh receptors accumulate at newly formed nerve-muscle synapses *in vitro. In* Cell and Tissue Interactions. J. Lash and M. Burger, editors. Raven Press, New York. 285-292.
11. Sytkowski, A., Z. Vogel, and M. Nirenberg. 1973. Development of acetylcholine receptor clusters on cultured muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 70:270-274.
12. Fischbach, G., and S. Cohen. 1973. The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* 31:147-162.
13. Hartzell, H. C., and D. Fambrough. 1973. Acetylcholine receptor production and incorporation into membranes of developing muscle fibers. *Dev. Biol.* 30:153-165.
14. Prives, J., I. Silman, and A. Amsterdam. 1976. Appearance and disappearance of acetylcholine receptor during differentiation of chick skeletal muscle in vitro. *Cell.* 7:543-550.
15. Axelrod, D., P. Ravdin, D. Koppel, J. Schlessinger, W. Webb, E. Elson, and T. Podelski. 1976. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle fibers. *Proc. Natl. Acad. Sci. U. S. A.* 73:4594-4598.
16. Yu, J., D. Fischman, and T. Steck. 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J. Supramol. Struct.* 1:233-248.
17. Sheetz, M. 1979. Integral membrane protein interaction with triton cytoskeletons of erythrocytes. *Biochim. Biophys. Acta.* 557:122-134.
18. Ben Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. *Cell.* 17:859-865.
19. Prives, J., C. Christian, S. Penman, and K. Olden. 1981. In Tissue Culture in Neurobiology. E. Giacobini, A. Verdanakis, and A. Shahar, editors. Raven Press, New York. 35-52.
20. Prives, J., and B. Paterson. 1974. Differentiation of cell membranes in cultures of embryonic chick breast muscle. *Proc. Natl. Acad. Sci. U. S. A.* 71:3208-3211.
21. Christian, C., M. Daniels, H. Sugiyama, Z. Vogel, L. Jacques, and P. Nelson. 1978. A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:4011-4015.
22. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80:585-592. *Erratum.* 83:336.
23. Paterson, B., and J. Prives. 1973. Appearance of acetylcholine receptor in differentiating cultures of embryonic chick breast muscle. *J. Cell Biol.* 59:241-245.
24. Lee, C. Y. 1972. Chemistry and pharmacology of polypeptide toxins in snake venoms. *Annu. Rev. Pharmacol.* 12:265-286.
25. Fulton, A., J. Prives, S. Farmer, and S. Penman. 1981. Development reorganization of the skeletal framework and its surface lamina in fusing muscle cells. *J. Cell Biol.* 91:103-112.
26. Weldon, P., and M. Cohen. 1979. Development of synaptic ultrastructure at neuromuscular contacts in an amphibian cell culture system. *J. Neurocytol.* 8:239-259.
27. Jacob, M., and T. Lentz. 1979. Localization of acetylcholine receptors by means of horseradish peroxidase-α-bungarotoxin during formation and development of the neuromuscular junction in the chick embryo. *J. Cell Biol.* 82:195-211.
28. Burrage, T., and T. Lentz. 1981. Ultrastructural characterization of surface specializations containing high-density acetylcholine receptors in embryonic myotubes in vivo and in vitro. *Dev. Biol.* 85:267-286.
29. Bloch, R., and B. Geiger. 1980. The localization of acetylcholine receptor clusters in areas of cell-substrate contact in cultures of rat myotubes. *Cell.* 21:25-35.
30. Sanes, J., and Z. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.* 83:357-370.
31. Fambrough, D., E. Bayne, J. Gardner, M. J. Anderson, E. Warshull, and R. Rotundo. 1981. *In* Neuroimmunology. J. Brockes, editor. Plenum Press, New York. In press.
32. Podelski, T., D. Axelrod, P. Ravdin, I. Greenberg, M. Johnson, and M. Saltpeter. 1978. Nerve extract induces increased and redistribution of acetylcholine receptors on cloned muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2035-2037.
33. Jessel, T., R. Siegel, and G. Fischbach. 1979. Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. *Proc. Natl. Acad. Sci. U. S. A.* 76:5397-5401.
34. Axelrod, D., H. Bauer, M. Stya, and C. Christian. 1981. A factor from neurons induces partial immobilization of nonclustered acetylcholine receptors on cultured muscle cells. *J. Cell Biol.* 88:459-462.