# Actin at Receptor-rich Domains of Isolated Acetylcholine Receptor Clusters

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*Abstract.* Acetylcholine receptor (AChR) clusters of cultured rat myotubes, isolated by extraction with saponin (Bloch, R. J., 1984, *J. Cell Biol.* 99:984–993), contain a polypeptide that co-electrophoreses with purified muscle actins. A monoclonal antibody against actin reacts in immunoblots with this polypeptide and with purified actins. In indirect immunofluorescence, the antibody stains isolated AChR clusters only at AChR domains, strips of membrane within clusters that are rich in receptor. It also stains the postsynaptic region of the neuromuscular junction of adult rat skeletal muscle. Semiquantitative immunofluorescence analyses show that labeling by antiactin of isolated AChR clusters is specific and saturable and that it varies linearly with the amount of AChR in the cluster. Filaments of purified gizzard myosin also bind preferentially at AChR-rich regions, and this binding is inhibited by MgATP. These experiments suggest that actin is associated with AChR-rich regions of receptor clusters.

Depletion of actin by extraction of isolated clusters at low ionic strength selectively releases the actin-like polypeptide from the preparation. Simultaneously, AChRs redistribute within the plane of the membrane of the isolated clusters. Similarly, brief digestion with chymotrypsin reduces immunofluorescence staining and causes AChR redistribution. Treatments that deplete AChR from clusters in intact cells also reduce immunofluorescent staining for actin in isolated muscle membrane fragments. Upon reversal of these treatments, cluster reformation occurs in regions of the membrane that also stain for actin.

I conclude that actin is associated with AChR domains and that changes in this association are accompanied by changes in the organization of isolated AChR clusters.

THE nicotinic acetylcholine receptor (AChR)[1] of vertebrate skeletal muscle is an integral membrane protein that binds the neurotransmitter acetylcholine and converts the energy of binding into a permeability change in the postsynaptic membrane (19). In adult muscle, most (>70%) of the AChRs are located in the postsynaptic membrane of the neuromuscular junction, which constitutes only ~0.1% of the total surface area of the muscle fiber (20, 21). The factors that help to maintain this high concentration of postsynaptic receptors have been under intense investigation in several laboratories.

Specializations on both the intracellular and extracellular faces of the muscle membrane appear to be involved in maintaining high junctional receptor concentrations. Extracellularly, the synapse-specific macromolecules include acetylcholinesterase (4, 40, 47, 49), a heparan sulfate proteoglycan (1), and several components that have been recognized immunologically but that have not yet been identified (27, 49). These components are assembled together with common components of the muscle basement membrane, such as collagen type IV, fibronectin, and laminin (48). Intracellularly, the postsynaptic region contains high concentrations of actin (30), vinculin, α-actinin, filamin, and talin (10, 52), a 43-kD polypeptide distinct from actin (24, 42, 45, 50, 51, 53), and a 51-kD polypeptide related to tonofilament protein (12). The 43-kD component has been shown morphologically and biochemically to be associated closely with postsynaptic AChRs (13, 42, 53). Neither the organization of the other macromolecules within the synaptic region nor their mode of interaction with AChR-rich membrane is yet understood.

To elucidate some of the molecular interactions involved in synaptic organization, it would be useful to study isolated membrane fragments that could be manipulated without many of the difficulties inherent in studies of intact muscle fibers. Although this approach has afforded much useful information on the postsynaptic membrane of the electric organs of fish, the purification of intact postsynaptic structures from muscle has not yet been achieved. I have developed a method for partially purifying AChR-rich membrane fragments from cultured rat muscle cells (7). The AChR aggregates, or clusters, in these fragments resemble those found at embryonic neuromuscular junctions at the earliest stages of synapse formation (58). Many are arranged rectilinearly, with strips of membrane rich in AChR, termed AChR domains,

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; FGAM, fluoresceinated goat anti-mouse immunoglobulin; R-BT, monotetramethylrhodamine conjugate of α-bungarotoxin.

interdigitating with receptor-poor strips, termed contact domains, located closer to the tissue culture substrate (9).

One of the major bands seen in SDS PAGE of isolated AChR cluster preparations has a molecular weight of ~43,000 (7). I report here that this band, termed p43, resembles actin and is localized at the AChR domains of isolated clusters. Like the postsynaptic region of the neuromuscular junction (30), isolated AChR clusters are therefore associated with actin. Unlike the junction, however, isolated receptor clusters can be experimentally manipulated to determine if there is a correlation between the presence of actin and the distinctive domain organization of AChR. I report here that treatments that remove or modify actin also cause the redistribution of AChR in the plane of the membrane.

# Materials and Methods

## Preparation of Isolated AChR Clusters

Rat myotubes were cultured from the hind limb muscles of newborn Sprague-Dawley rats, as described previously (5, 9). Cells were usually labeled with a tetramethylrhodamine conjugate of $\alpha$-bungarotoxin (R-BT; reference 46), and then subjected to extraction with saponin to isolate the AChR clusters, as described (7). In brief, cultures were washed with buffered saline (10 mM NaP, 145 mM NaCl, pH 7.2) and then with buffered saline containing 10 mg/ml bovine serum albumin, 10 mM $MgCl_2$, and 1 mM EGTA. Cultures were incubated with 1.5 ml of the same buffered saline solution supplemented with 0.2% saponin. Samples were shaken on a Tektator V rotary shaker (American Scientific Products, McGaw Park, IL) or a Luckham 802 suspension mixer (Tekmar Co., Cincinatti, OH) for 4–20 min. After this time, most of the cellular material was lost, but AChR clusters remained attached to the coverslips (7). Samples were usually collected as soon as possible after the bulk of the cellular material was shed (usually 5–8 min after the addition of saponin), but longer incubations did not significantly affect the results reported below. The isolated AChR clusters on the coverslip were then fixed in fresh paraformaldehyde (2% wt/vol, prepared in buffered saline) or manipulated further and then fixed before staining with antibodies or with smooth muscle myosin and antimyosin. Samples were routinely incubated in 0.1 M glycine in buffered saline before staining in order to inactivate any remaining free aldehyde.

When isolated AChR clusters were treated before fixation, the following procedures were used. For treatment in isotonic solutions, coverslips containing isolated clusters were removed from the saponin solution and dipped twice into beakers containing buffered saline. They were then blotted to remove excess buffer and placed into a clean petri dish containing buffered saline supplemented appropriately. Incubation was continued, usually for 5 min, after which the solution was aspirated and replaced with fresh paraformaldehyde (see above). When the effects of low ionic strength were investigated, coverslips were dipped from two to four times in 2 mM Tris-HCl, 0.2 mM ATP, pH. 8.0 (buffer A: reference 56) and incubated in the same solution, usually for 5 min, before fixation.

## Staining with Antiactin Antibodies

Antiactin antibodies were obtained from the following sources. A rabbit antibody against *Aplysia* body wall actin (37) was kindly donated by Dr. B. Lubit (Department of Neuroscience, New Jersey Medical School, Newark, NJ). Other antiactins were monoclonal IgM antibodies. One, HP249, generously donated by Dr. R. Anthony (Department of Pathology, University of Maryland School of Medicine, Baltimore, MD), was generated after immunization of mice with chicken skeletal muscle actin. The ascites fluid obtained from this clone and a control IgM antibody (HP285), were the gift of Litton Bionetics (Rockville, MD). Other antiactins, 1C1, 1C7, and 4D3, generated in mice injected with retinal proteins from *Drosophila*, were generously provided by Dr. F. Wong (Marine Biological Institute, Galveston, TX). Fluoresceinated goat anti-mouse immunoglobulin (FGAM; Litton Bionetics) was used to visualize bound antibody.

## Decoration with Myosin Filaments

Myosin was purified from chicken gizzard following the method of Groeschel-Stewart et al. (29) and stored at 1.1 mg/ml in 1 part glycerol/1 part 25 mM histidine, 1 M KCl, pH 6.8 (44). Storage was at −20°C until the myosin was ready for use, then at 4°C. Immediately before use, the myosin was diluted 20-fold into buffered saline at room temperature. Glass coverslips containing isolated receptor clusters were inverted over a droplet (50–80 $\mu$l) of the diluted myosin and incubated at room temperature for 5–30 min, then washed several times with buffered saline and fixed. Coverslips were then reacted with affinity-purified antibody to smooth muscle myosin (55) at 20–40 $\mu$g/ml, followed by fluoresceinated goat anti-rabbit immunoglobulin (Cappel Laboratories, West Chester, PA), used at a dilution of 1:100. Under these conditions, antimyosin did not not label isolated clusters unless they were preincubated with myosin filaments.

## Fluorescence Techniques

All fluorescence observations were made with a Zeiss IM35 microscope equipped for epifluorescence, and a Zeiss 63× Plan-Neofluar objective. For semiquantitative measurements, the Zeiss attachments (catalogue numbers 474230, 474280, and 476005) with a model 928A photomultiplier tube were used at a voltage of −850 V. The signal from the photomultiplier was processed by an I-V converter designed by Dr. W. G. Wier, with slight modifications by Mr. J. Michael (Departments of Physiology and Biophysics, University of Maryland School of Medicine). A small area (19.3 $\mu m^2$, created using the Zeiss 0.25-mm pinhole stop) in a total illuminated field of ~8,000 $\mu m^2$ (created by stopping down the diaphragm between the fluorescence light source and the sample) was exposed first to green light to excite the R-BT. This signal was stable for many seconds. Without refocusing, the illuminating wavelength was changed to excite the fluorescein. As the fluorescein signal decreased with an half-time of ~20 s, the initial value (<2 s) was read. All measurements are corrected for background, which was obtained by taking similar readings in regions of the coverslip that contained no cellular material. Background values were between 5 and 20% of the total signal obtained for brightly stained structures.

In many experiments, routine fluorescence photomicrography was performed. Exposures were usually 15–30 s. Ilford HP-5 film (Ilford Ltd., Basildon, Essex, U.K.) was processed to an ASA of 1200 with the Ilford developer Microphen.

## Gel Electrophoresis

Isolated AChR clusters were prepared as described above but without fixation. This material and material extracted from isolated clusters in buffer A were analyzed by SDS PAGE following the method of Laemmli (34). In the case of radiolabeled materials, ~75,000 cpm were applied to each lane. After electrophoresis, gels were fixed, soaked in EN$^3$HANCE (New England Nuclear, Boston, MA), washed briefly in water, and dried. The dried gels were exposed to Kodak X-OMat X-ray film in the presence of an enhancing screen (Cronex II, E.I. Du Pont de Nemours & Co., Wilmington, DE) for 1–3 d. For immunoblotting, ~10 $\mu$g of purified actins and 40 $\mu$g of protein of isolated cluster preparations were applied to SDS polyacrylamide gels and electrophoresed. The large amount of protein of isolated cluster preparations was necessary to obtain enough colored reaction product in immunoblots to photograph (see below). When appropriate, gels were stained with silver (43). The method of Burnette (14) was followed for Western blotting onto nitrocellulose paper (Schleicher & Schuell, Keene, NH). After blotting, the nitrocellulose paper was saturated with a mixture of albumin, gelatin, and hemoglobin. Antibody staining with HP 249 antiactin was then performed using slight modifications of published procedures. Antibody bound to the paper was visualized with anti-mouse immunoglobulin coupled to alkaline phosphatase (Cappel Laboratories) using naphthol AS-MX phosphate and fast red TR salt as substrates. After enzymatic staining, blots were photographed with Kodak Technical Pan film (Eastman Kodak Co., Rochester, NY). Development was with Kodak D19, following the manufacturer's specifications.

## Materials

Actins were purified as reported (56, 60). Other cytoskeletal proteins were purified as described (10). Prestained protein standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD). Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

# Results

The AChR clusters of cultured rat myotubes are frequently in close apposition to the tissue culture substrate. Upon exposure to saponin, most (~99%) of the cellular material in cultures of rat myotubes is shed from the coverslip within the first 5 min of incubation, but AChR clusters remain attached

to the substrate (7). Thereafter, ~0.5% of additional cellular material is shed gradually over the next 15 min. After exposure to saponin for 20–25 min, many AChR clusters are recovered essentially intact, with clearly delineated AChR and contact domains (7). Clusters recovered earlier, however, tend to retain more of their rectilinear organization than do clusters left for longer periods in saponin. For this reason, I performed most of the experiments described here on AChR clusters obtained by extracting cultures for only 5–10 min. Under these conditions, nearly all of the AChR clusters, but only ~1% of the cellular material in the original culture, remain attached to the coverslip (7).

One of the major bands of cellular origin seen in SDS PAGE of isolated AChR cluster preparations has a molecular weight of ~43,000 (Fig. 1 *B*, lane *1*; see also Figure 7 of reference 7). This polypeptide is referred to here as p43. In the results that follow, p43 is treated as a single polypeptide, although it probably contains more than one component. This is considered further in the Discussion.

### *Antiactin Labeling*

To learn if p43 shared antigenic determinants with actin, I used a monoclonal antiactin antibody, HP249. In immunoblots prepared after SDS PAGE, this antibody reacted with actins purified from rabbit skeletal muscle and chicken gizzard (Fig. 1 *A*, lanes *1* and *2*) but not with other standard proteins, present in equal amounts (Fig. 1 *A*, lane *3*). In templates prepared from isolated AChR clusters, HP249 reacted with p43 but did not react to any significant extent with other components (Fig. 1 *C*, lane *1*). This suggests that p43 resembles actin and that HP249 reacts preferentially with this polypeptide. HP285, a monoclonal IgM derived from the same parent cell line as HP249, failed to react in immunoblots with purified actin or with any of the polypeptides in isolated cluster preparations (not shown), further suggesting that the interaction of HP249 with actin and p43 is specific.

I performed immunofluorescence experiments to localize p43 in isolated cluster preparations. Cultures were labeled with R-BT to mark AChR and were extracted with saponin. They were then fixed and labeled with HP249 followed by FGAM. As shown in Fig. 2, *A* and *B*, antiactin labeled the AChR domains but not the interdigitating contact domains of the isolated membrane fragments. Labeling was weak and punctate in regions of the myotube membrane that did not contain clustered AChR, and at fragments of fibroblast membrane also present in these preparations (7). HP285, a nonspecific IgM, did not stain isolated AChR clusters or other membrane fragments. These results suggested that p43 was enriched at the AChR domains of isolated AChR clusters.

The intensity of the indirect immunofluorescence afforded by antiactin and FGAM seemed to vary in parallel with the fluorescence intensity resulting from R-BT bound to the isolated cluster. I used a photomultiplier attached to the fluorescence microscope (see Materials and Methods) to test this observation more quantitatively. Clusters were selected that showed a wide range of intensity of fluorescence emission due to bound R-BT. The fluorescein emission due to HP249 and FGAM bound in the same regions was then measured. The data in Fig. 3 show that immunofluorescence did indeed vary linearly with the intensity of R-BT fluorescence. The linear relationship between fluorescein and tetramethylrhodamine fluorescence in a particular culture was used to generate a ratio, $F/R$. This ratio varied from one set of cultures to another but was relatively constant among similar samples from a single set of cultures. This permitted semiquantitative comparisons of the HP249 labeling of isolated AChR clusters treated in different ways.

To optimize the conditions for doing such comparisons, I characterized the dependence of indirect immunofluorescence staining on the concentration of antiactin. The results (Fig. 4) show that staining was saturating, with apparent half-

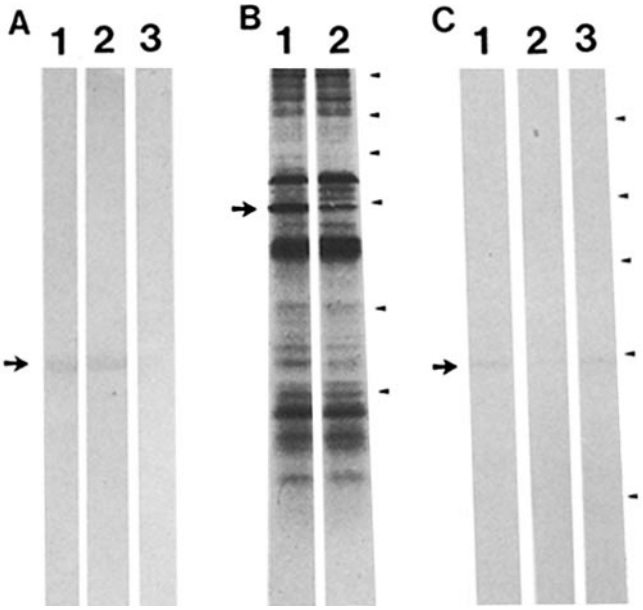



*Figure 1.* SDS PAGE of isolated AChR clusters and immunoblotting with antiactin HP249. (*A*) Actins purified from rabbit skeletal muscle (lane *1*, 10 μg) and chicken gizzard (lane *2*, 10 μg), and a mixture of standard proteins (lane *3*, 10 μg each), were subjected to SDS PAGE on 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. The blots were reacted with HP249 (10 μl ascites fluid/ml). Bound antibody was visualized using a chromogenic reaction. The results show that HP249 reacted with purified skeletal and gizzard actins (lanes *1* and *2*) but not with any of the standard proteins (lane *3*). The standard proteins were β-galactosidase, bovine serum albumin, ovalbumin, and lysozyme. (*B*) Rat myotube cultures were metabolically radiolabeled with [$^{35}$S]methionine, then extracted with saponin in the presence of protease inhibitors and serum albumin (see reference 7). Isolated clusters were then incubated in buffered saline, or in buffer A, as described in the text. Material remaining associated with the substrate was dissolved in SDS, electrophoresed on 12% polyacrylamide gels, and autoradiographed. The results show that p43 (arrow), present in high amounts in controls (lane *1*), was selectively depleted upon extraction with buffer A (lane *2*). (*C*) Unlabeled preparations were prepared as in *B*, above, but after SDS PAGE on 10% polyacrylamide gels they were transferred to nitrocellulose paper and incubated with HP249, as described above. HP249 reacted with p43 in controls (lane *1*), and this reaction was reduced in samples from which p43 was extracted with buffer A (lane *2*). Note also that HP249 did not label any of the other polypeptide chains present in preparations of isolated receptor clusters to any significant extent. HP249 was also reacted with a template prepared from materials extracted from isolated cluster preparations with buffer A. Here, too, HP249 labeled only p43 (lane *3*), the major component of this material. The arrowheads indicate the position of standard proteins. From high to low molecular weights, these are myosin heavy chain (200,000), phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (44,000), α-chymotrypsinogen (25,700), and β-lactoglobulin (18,400).

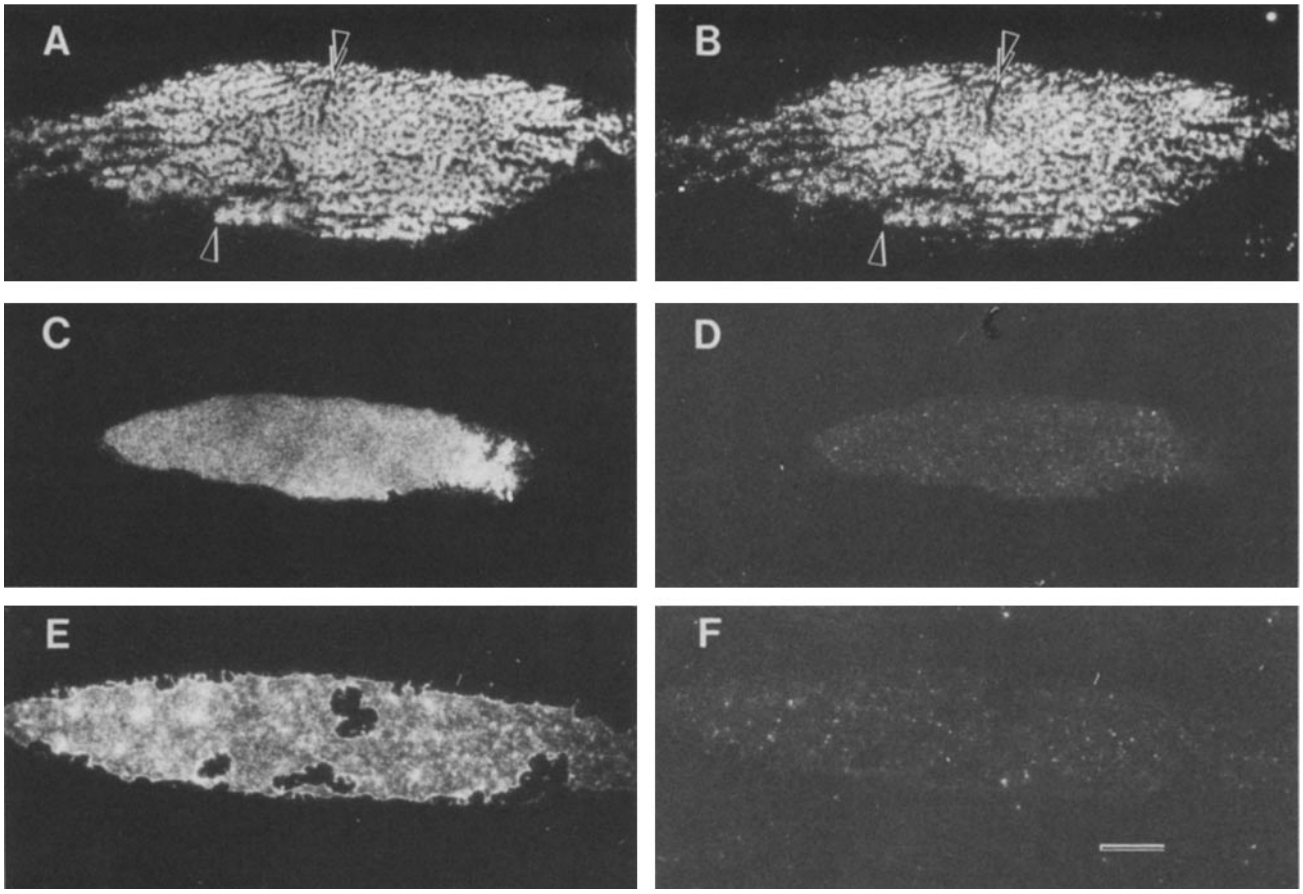



*Figure 2.* HP249 labels the AChR domains of isolated AChR clusters in controls but does not label clusters in which the organization of AChR has been disrupted. Rat myotube cultures were reacted with R-BT, extracted with saponin, and incubated for 5 min at 22°C in buffered saline (*A* and *B*), in buffer A (*C* and *D*), or in buffered saline containing 10 μg/ml chymotrypsin (*E* and *F*). After fixation, samples were labeled with HP249 (50 μl ascites fluid/ml). Bound antibody was visualized by incubating with FGAM. *A, C,* and *E* were photographed under rhodamine illumination; *B, D,* and *F* were photographed under fluorescein illumination. (*A* and *B*) Controls show strips of membrane, termed AChR domains (*A*, arrowhead), brightly labeled with R-BT, and poorly labeled strips, termed contact domains, (*A*, double arrowhead). In *B*, note that the AChR domains are labeled with antibodies (arrowhead) but that the contact domains are not (double arrowhead). This suggests that the antigenic binding site for HP249 is coincident with AChR domains. (*C* and *D*) A cluster extracted for 5 min with buffer A, showing a more uniform distribution of AChR (*C*) and much reduced staining by HP249 (*D*). (*E* and *F*) A cluster treated for 5 min with chymotrypsin (10 μg/ml in buffered saline) showing small aggregates of AChR (*E*) and much reduced staining by HP249 (*F*). Bar, 4 μm.

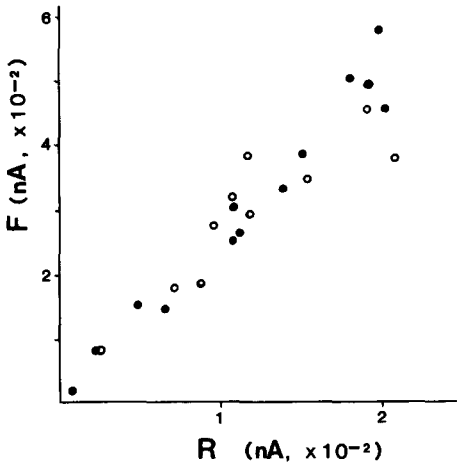



*Figure 3.* The fluorescence intensity due to labeling by HP249 and FGAM varies linearly with bound R-BT. Myotubes were labeled with R-BT, extracted with saponin, fixed, and stained with HP249 and FGAM, as described in Materials and Methods and in the legend to Fig. 2. Samples were examined on a fluorescence microscope equipped with a photomultiplier. Isolated AChR clusters were exposed to illuminate bound tetramethylrhodamine and fluorescein, and the displacement registered by the photomultiplier was measured, as described in Materials and Methods. After correction for background readings, the fluorescein signal, *F*, was plotted against the tetramethylrhodamine signal, *R*. The results suggest that the amount of antiactin and FGAM bound varied linearly with the number of R-BT-AChR complexes present. Open and closed circles represent data obtained from duplicate samples.

maximal saturation at ~10 μl ascites fluid/ml. For most of the experiments reported below, HP249 was used at 25 μl ascites fluid/ml, a concentration near saturating.

Staining of isolated clusters by HP249 could be blocked by purified skeletal muscle actin, with half-maximal inhibition found at 60 μg/ml, or at ~1.4 μM (Fig. 5). The maximum inhibition of staining varied from 60 to 90%, depending on the experimental conditions. Preliminary experiments suggested that actins in either the G or F form blocked staining by HP249 in the same concentration range (not shown). The low apparent affinity of HP249 for actin, demonstrated in

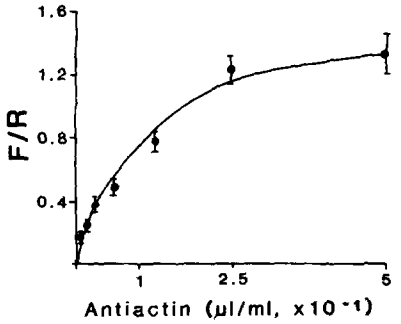



*Figure 4.* Staining by HP249 is saturating. Isolated AChR clusters were labeled and the bound label was quantitated as in the legend to Fig. 3, but the concentration of HP249 was varied. After correction for background readings, the ratio of the photomultiplier readings for fluorescein and tetramethylrhodamine ($F/R$) was plotted as a function of the concentration of HP249 used to label the clusters. Values are means ± SE; $n$ was 8 or 9 for all points. The results show that labeling by HP249 and FGAM became saturated at ~25 μl ascites fluid/ml, with half-saturation achieved at ~10 μl ascites fluid/ml.

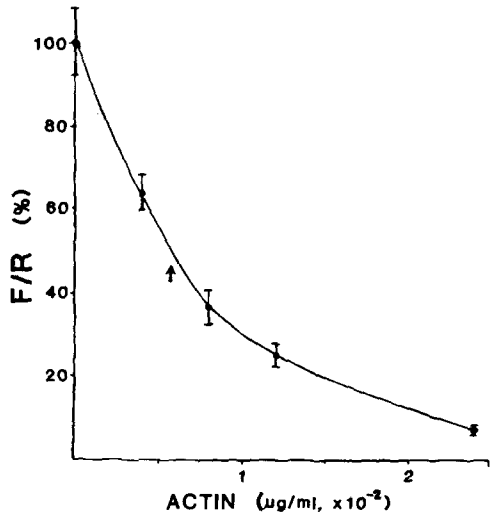



*Figure 5.* Purified skeletal muscle actin blocks labeling by HP249. Isolated AChR clusters were labeled and the bound label was quantitated as in the legend to Fig. 3, but HP249 was incubated with increasing amounts of G-actin before it was used. Incubation was for 30 min in three parts buffer A, one part buffered saline, supplemented with bovine serum albumin to keep the amount of protein added to all antibody samples constant at ~1 mg/ml. After incubation, samples were centrifuged briefly in a bench-top centrifuge. The supernatants were used to label fixed preparations of isolated AChR clusters. In this experiment, nearly complete inhibition of labeling by HP249 was achieved at 240 μg actin/ml, with half-maximal inhibition occurring at ~60 μg/ml, or ~1.4 μM. Values given are means ± SE; $n$ was between 10 and 14 for all points.

this experiment, probably explains why large amounts of protein from isolated cluster preparations must be used to detect p43 in immunoblotting (see Materials and Methods).

I tested other antiactin antibodies to learn if they, too, could interact with isolated AChR clusters. Rabbit antibodies specific for *Aplysia* body wall actin (37) label the postsynaptic region of the neuromuscular junction of the rat (30). For reasons that are still unclear, these antibodies stained the isolated AChR clusters only faintly, making further comparison with HP249 difficult. Monoclonal mouse antibodies against actin from *Drosophila* retina, 1C7, 1C1, and 4D3, did

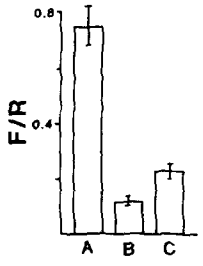



*Figure 6.* Semiquantitative analysis of the effects of chymotrypsin and buffer A extraction on HP249 staining of isolated AChR clusters. Samples were prepared and analyzed as described in the legends to Figs. 2 and 3. (*A*) Control clusters, incubated for 5 min in buffered saline. (*B*) Clusters incubated for 5 min in buffered saline with 10 μg/ml chymotrypsin. (*C*) Clusters incubated for 5 min in buffer A. Values are means ± SE, with $n$ = 10 for all three samples. The results show that exposure of isolated clusters to chymotrypsin reduced subsequent labeling by HP249 about fivefold, and that extraction with buffer A reduced subsequent labeling about threefold. These results are consistent with the photomicrographs of Fig. 2.

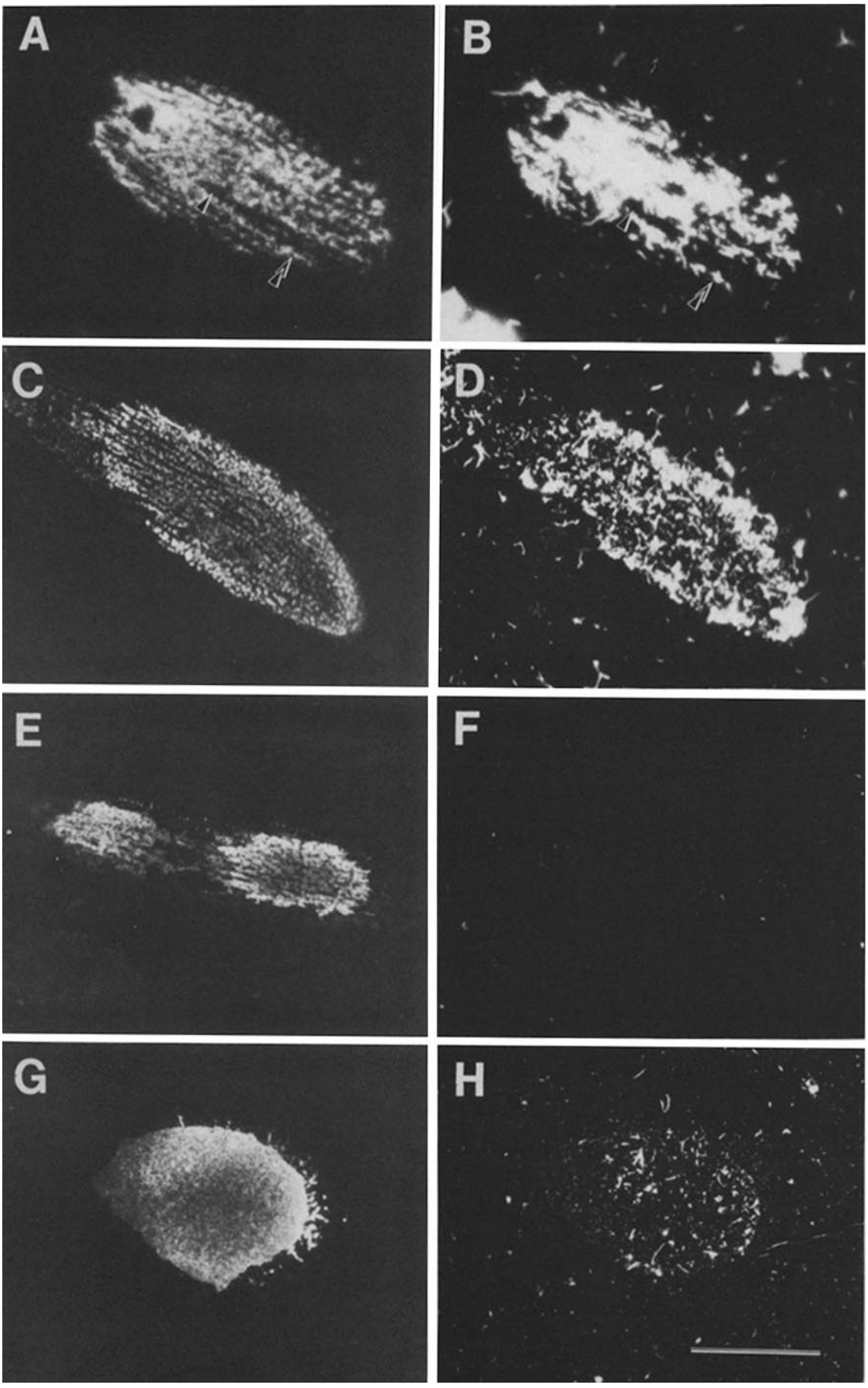
stain the isolated clusters, however. Antibody 1C7 was similar to HP249: it stained the AChR domains of clusters preferentially and reacted more weakly with other membrane fragments in the preparation. Antibodies 1C1 and 4D3, which gave weaker staining, reacted both with AChR clusters and with membrane devoid of clusters, either from muscle cells or from the mononucleate cells present in the original culture. As other antiactins also labeled the AChR domains of isolated clusters, HP249 probably did not react with an antigen that resembled but was distinct from actin.

### *Actin and Cluster Organization*

It could be argued that labeling by antiactin of the AChR domains of isolated AChR clusters was fortuitous. Muscle cells are rich in actin, and any subcellular fraction, including the AChR domains of isolated clusters, may be contaminated with this protein. I treated isolated AChR clusters in several ways before fixation and staining with antiactin to test this possibility. In preparations of isolated clusters extracted briefly with solutions of very low ionic strength, such as buffer A (56), subsequent labeling with antiactin antibody was greatly diminished (Figs. 2*D* and 6). Using diiodo-α-bungarotoxin to measure the total number of AChRs present in cluster preparations extracted under control conditions and at low ionic strength, I found that the total number of receptors remaining attached to the coverslips was diminished only slightly in samples extracted in buffer A (by 9 and 2%, in two experiments, well within the error of the assay). The organization of AChR in samples treated with buffer A was greatly changed, however. Instead of being limited to linear membrane domains, as in controls (e.g., Fig. 2*A*), AChR appeared to be more uniformly distributed within the plane of the membrane (Fig. 2*C*). Upon closer examination, AChR appeared to be organized into microaggregates that are close in size to the smallest structures resolvable by the light microscope (~0.25 μm).[2] The smallness and relatively uniform distribution of these spots probably account for the apparently uniform appearance of AChR in clusters extracted with buffer A. Similar results were obtained in cluster preparations extracted with buffer A solution and stained with the 1C7 antiactin (not shown).

Extraction with buffers of low ionic strength could have

[2] In some experiments, extraction with buffer A has yielded larger and more widely spaced spots of AChR labeled with R-BT. Most of these spots tend to label with HP249 and FGAM. I do not yet understand the cause of this variability.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removed many cytoplasmic and peripheral membrane proteins. I performed SDS PAGE on control and extracted cultures to learn which proteins were depleted from preparations of isolated AChR clusters after extraction with buffer A. Myotube cultures on glass coverslips were first metabolically radiolabeled with [$^{35}$S]methionine, then extracted with saponin. Samples were incubated for 5 min at 22°C in isotonic phosphate-buffered saline or in buffer A. The material that remained attached to the coverslip after these treatments was dissolved in SDS or Triton X-100, and, after concentration, was analyzed by SDS PAGE and autoradiography. This experiment (Fig. 1 *B*, lanes *1* and *2*) showed that extraction with buffer A selectively depleted p43, perhaps together with some minor bands. Two additional bands, barely visible in controls, appeared in buffer A–extracted samples just above the p43 band, with polypeptide chain molecular weights of ~48,000 and ~45,000. Higher molecular weight bands, not clearly visible in Fig. 1 *B*, were studied in other experiments by SDS PAGE using lower acrylamide concentrations. No change in the amounts or mobilities of these bands was observed in samples extracted with buffer A (not shown).

In immunoblotting, HP249 reacted to a much lesser extent with material from isolated clusters treated with buffer A (Fig. 1 *C*, lanes *1* and *2*), consistent with the depletion of the actin-like p43 polypeptide in these preparations. In several experiments the material extracted by buffer A from isolated cluster preparations was collected, concentrated, and subjected to SDS PAGE and immunoblotting. The major polypeptide present in this extract was p43, and it reacted with HP249 (Fig. 1 *C*, lane *3*). These results are all consistent with the idea that p43 is the actin-like protein of isolated AChR clusters and that extraction of p43 is associated with changes in the organization of AChR in clusters.

In a second set of experiments, isolated AChR clusters were treated briefly with chymotrypsin (10 μg/ml in buffered saline for 5 min) before fixation and staining with HP249. This treatment also caused most of the staining by HP249 to be reduced (Fig. 2, *E* and *F*) and AChR to redistribute in the muscle membrane. In this case, however, the distribution of AChR did not appear to be nearly uniform, as it was after extraction with buffer A. Instead, it appeared to be organized into large, bright patches of R-BT stain, separated by membrane in which R-BT stain was also apparent. This effect of chymotrypsin was not observed if the enzyme was first boiled or inactivated with phenylmethylsulfonyl fluoride. RNase and DNase, used at 10 μg/ml, did not affect cluster organization. The ability of HP249 to react with p43 after immunoblotting of chymotrypsin-treated material was also reduced (not shown). Mild proteolytic degradation therefore destroyed the ability of isolated AChR clusters to be labeled by antiactin and simultaneously destroyed the antigenicity of p43 on immunoblots. Although other macromolecules in preparations of AChR clusters may be destroyed by mild proteolysis (see Discussion), these results support the idea that changes in p43 are associated with changes in the organization of isolated AChR clusters.

### *Labeling with Myosin Filaments*

If actin is associated with the AChR domains of isolated AChR clusters, it should be possible to detect its presence using myosin. For this purpose, I prepared filaments of chicken gizzard myosin by diluting a stock solution of this protein into isotonic buffered saline and used the filaments that formed upon dilution to decorate isolated AChR clusters. AChR was visualized with R-BT, and the myosin filaments were visualized with rabbit antimyosin followed by fluorescein goat anti-rabbit immunoglobulin. In all cases, AChR clusters were intensely labeled (Fig. 7, *A–D*). In some cases the myosin filaments decorated the AChR domains preferentially (Fig. 7, *A* and *B*). This was seen clearly in only a few clusters, however, probably because the myosin filaments were often larger than the distance from an AChR domain to its nearby contact domains, and also because the filaments tended to aggregate, obscuring any underlying pattern.

Two additional observations suggested that the myosin filaments labeled AChR domains by virtue of their ability to interact with actin. First, filament decoration was blocked in the presence of 5 mM MgATP (Fig. 7, *E* and *F*), or of ATP alone (not shown), as observed for actin-myosin interactions in other systems. Treatment of isolated clusters with ATP before, but not during, incubation with myosin filaments did not reduce subsequent myosin-antimyosin labeling. Second, filament decoration of isolated clusters was greatly diminished when clusters were extracted with buffer A (Fig. 7, *G* and *H*) or digested briefly with chymotrypsin (not shown). These results demonstrate, without the use of antibodies, that actin is present at the AChR domains of isolated AChR clusters.

### *Cluster Disruption and Re-formation*

All the experiments described above were done on isolated AChR clusters. I wished to learn if the correlation between the presence of actin and the organization of AChR clusters could also be demonstrated in intact cells. Indirect immuno-

*Figure 7.* Decoration of isolated AChR clusters with myosin filaments. Rat myotubes were labeled with R-BT. AChR clusters were isolated by extraction with saponin and incubated in buffered saline (*A–F*) or in buffer A (*G* and *H*) for 15 min at 22°C. Samples were then incubated for 5 min with filaments of purified smooth muscle myosin, in buffered saline, and fixed with paraformaldehyde. Bound myosin was stained with affinity-purified anti-myosin, followed by fluorescein goat-anti rabbit immunoglobulin. *A*, *C*, *E*, and *G* were photographed under rhodamine illumination, *B*, *D*, *F*, and *H* under fluorescein illumination. (*A* and *B*) A control AChR cluster (*A*) with a clear linear pattern of AChR domains (e.g., double arrowheads) and contact domains (e.g., single arrowhead) and bound myosin filaments. In this example, the AChR domains were labeled with myosin, but the contact domains were not. (*C* and *D*) A control AChR cluster labeled with myosin filaments but showing a more typical result. The membrane domains, clearly visible in staining for AChR (*C*), were not apparent in the myosin pattern (*D*), but the cluster was heavily labeled. (*E* and *F*) A control AChR cluster incubated with myosin filaments in the presence of 5 mM MgATP. The cluster (*E*) did not bind any myosin (*F*). (*G* and *H*) An AChR cluster extracted with buffer A before incubation with myosin. Note that the AChR became more evenly distributed in the plane of the membrane (*G*), as also shown in Fig. 2, and that myosin labeling was much reduced (*H*). Bar, 12.5 μm for *A* and *B*, 30 μM for *C–H*.

fluorescence studies of intact cells could not be done, however, because HP249 binding to intracellular proteins obscured the labeling of the AChR clusters. I therefore took an indirect approach to this question by altering the stability of AChR clusters in intact cells then isolating clusters by extraction with saponin and labeling them for actin.

Nearly all of the AChR clusters of cultured rat myotubes can be disrupted by treating the cells with sodium azide, carbachol, or medium depleted of $Ca^{2+}$ (5, 6, 8). Although many of the clusters are completely dispersed by these treatments, some still display limited regions with bright R-BT staining. After extraction with saponin, the membrane fragments obtained from these partially disrupted receptor clusters are also retained on the coverslip. To learn if actin is lost from partially disrupted clusters associated with the AChR domains of clusters, I stained these preparations with HP249 and FGAM. I found that the samples obtained from cells incubated with azide (Fig. 8, *A* and *B*) or carbachol (not shown), or in the absence of $Ca^{2+}$ (Fig. 8, *C* and *D*) showed greatly reduced amounts of actin. Very few membrane fragments containing undisturbed AChR clusters were obtained after these treatments, consistent with earlier observations on intact cells (5, 6, 8). Fragments with large accumulations of actin were also rare. In contrast, fragments that labeled poorly for AChR and actin were commonly seen, consistent with the idea that they arose from clusters that had been partially dispersed in the treated cells. These results suggest that actin is lost (or its association with the isolated membrane fragments is destabilized) when AChR clusters are dispersed in intact rat myotubes.

The correlation between actin and AChR is further supported by the observation that much of the antiactin labeling still visible in these samples was associated with the spots at which R-BT-AChR complexes were present (e.g., Fig. 8, *A* and *B*, arrowheads). Little antiactin label was associated with regions of the isolated membrane fragments that were depleted of AChR. A similar correlation was observed in reforming AChR clusters. Upon removal of sodium azide from cultures of rat myotubes, small foci of aggregated AChR appear, around which further clustering occurs (5). After extracting and labeling cultures in which clusters were reforming, I found that all of these small foci were also associated with actin (Fig. 8, *E* and *F*, double arrowheads). These results support the idea that actin is associated with the AChR-

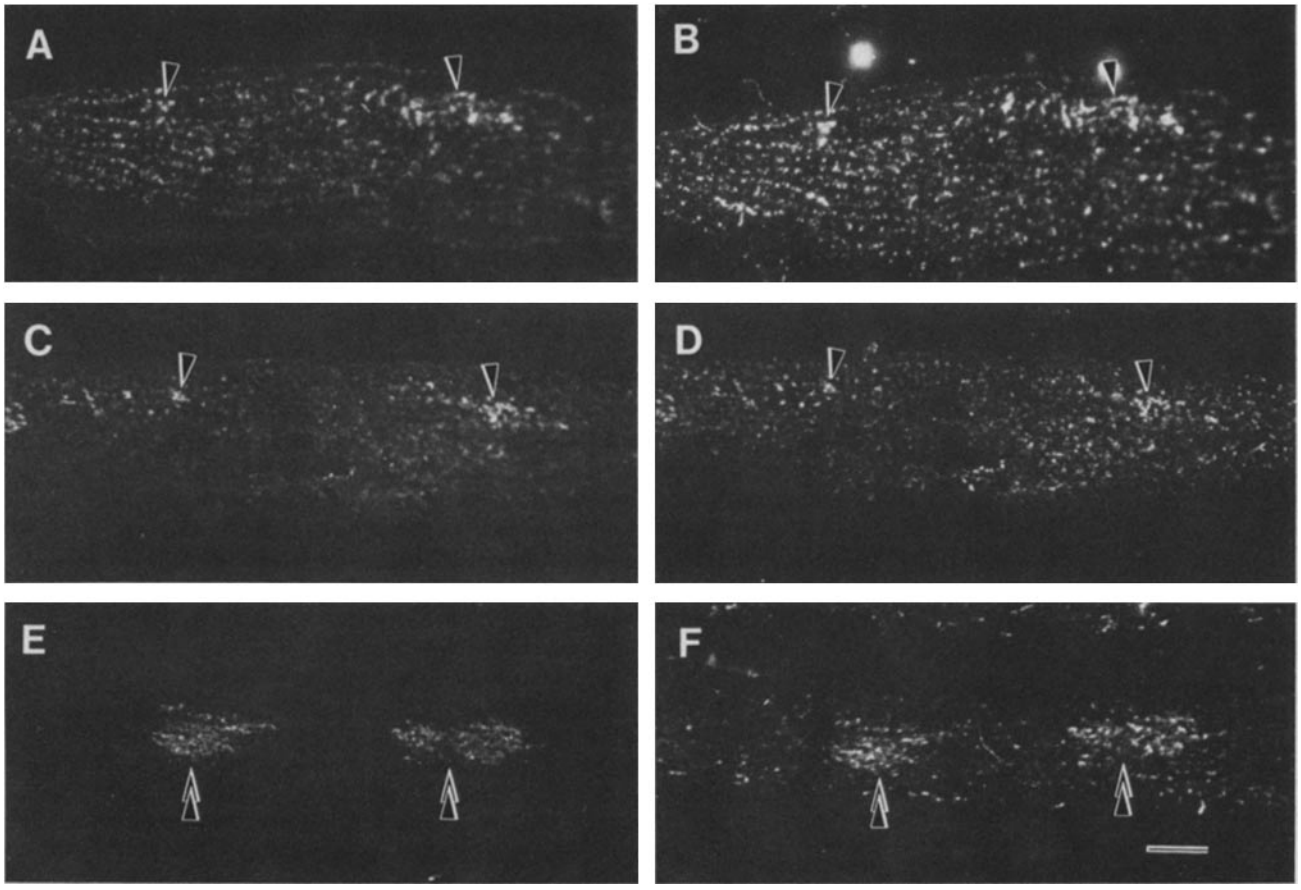


*Figure 8.* Cluster disruption and re-formation are accompanied by changes in actin distribution. Rat myotube cultures were treated for 6 h with 5 mM sodium azide or in medium depleted of $Ca^{2+}$, to disrupt AChR clusters (5, 6). Additional cultures were washed free of azide and allowed to recover for 4.5 h. After these treatments, cultures were labeled with R-BT, extracted with saponin, fixed, and stained with HP249 and FGAM. *A*, *C*, and *E* were photographed under rhodamine illumination, *B*, *D*, and *F* under fluorescein illumination. (*A* and *B*) A receptor-rich membrane fragment from an azide-treated culture (*A*), containing small "speckles" of R-BT labeling (e.g., arrowheads) which were also labeled with HP249 (*B*, arrowheads). (*C* and *D*) A similar example obtained from a culture incubated in the absence of $Ca^{2+}$. (*E* and *F*) A reforming cluster from a culture treated with azide and allowed to recover. The smaller AChR aggregates (*E*, double arrowheads), typical or reforming clusters (5), were associated with sites of antiactin labeling (*F*, double arrowheads). Bar, 4 $\mu$m.

rich regions of isolated clusters and further suggest that this association is regulated by the metabolic state or the intracellular ionic milieu of the intact muscle cell.

### *The Neuromuscular Junction*

Hall et al. (30) have reported that actin is present at the postsynaptic region of the neuromuscular junction of the rat. To learn if the actin-like antigen recognized by HP249 is present at the postsynaptic region of the neuromuscular junction, I prepared frozen sections through the junctional regions of hemidiaphragms from control rats and from rats that had been denervated for 5 wk. After this period of denervation, no structures derived from the nerve terminus or Schwann cell remain apposed to the postsynaptic element (41). Upon staining with HP249 and FGAM, intact junctions, visualized with R-BT, were labeled (Fig. 9). Labeling was not always

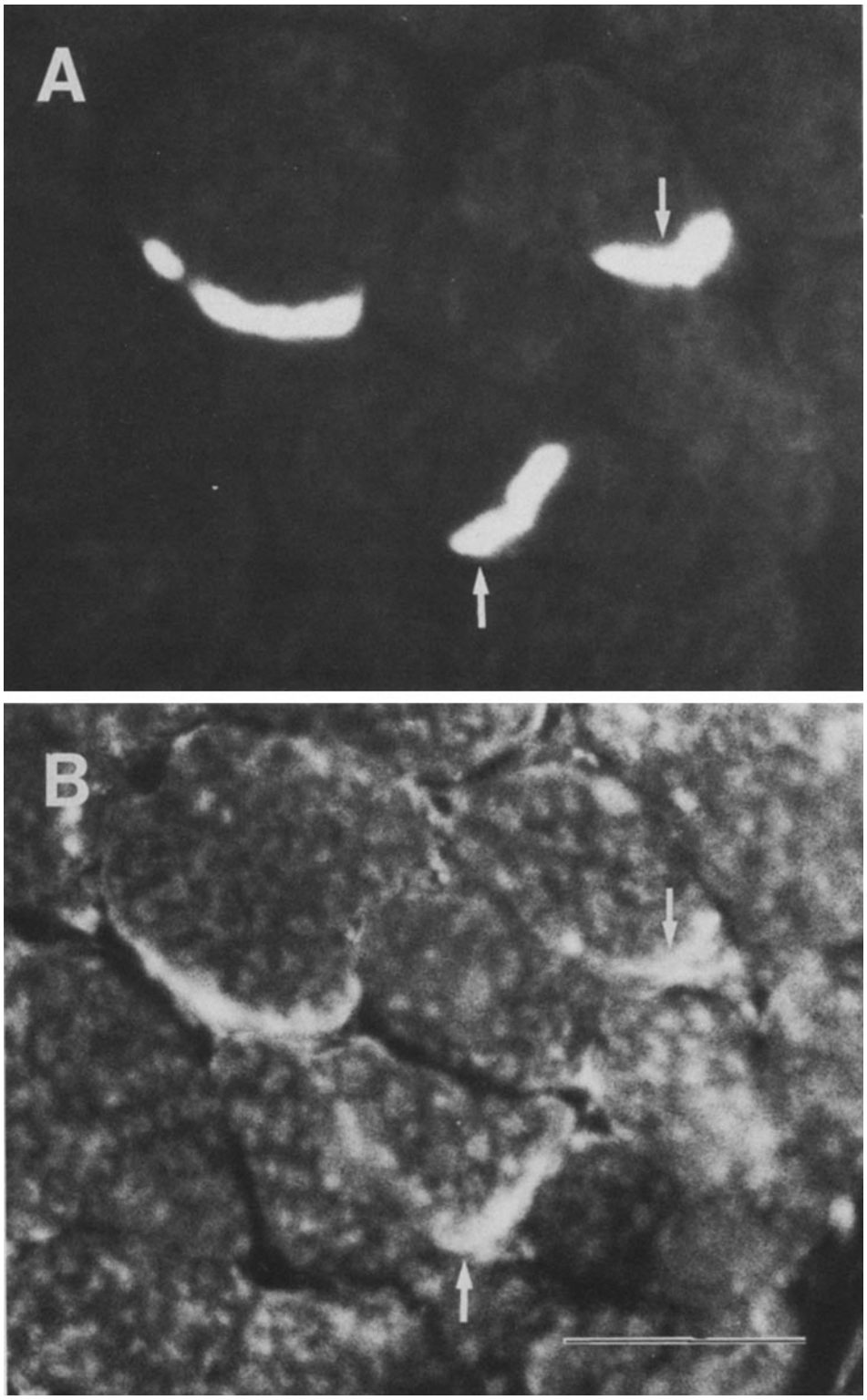


*Figure 9.* HP249 stains the neuromuscular junction of rat diaphragm. Frozen sections, 4 $\mu$m thick, were cut through the junctional region of intact rat diaphragm, as described (10). Sections were stained with HP249 (50 $\mu$l ascites fluid/ml), and counterstained with FGAM and R-BT. Neuromuscular junctions visualized with R-BT (*A*) were labeled with HP249 and FGAM (*B*). In addition, the contractile apparatus within the myofibers also stained, making it difficult to distinguish the junctional regions with the fluorescein label (e.g., arrows). Bar, 50 $\mu$m.

much brighter at endplates than over the contractile apparatus, however. This is consistent with the observation (e.g., Fig. 1), that HP249 reacts with contractile as well as non-contractile forms of actin. Similar results were obtained with 5-wk-denervated diaphragm (not shown). These experiments agree with those of Hall et al. (30) and suggest that actin is a constituent of the postsynaptic region of the neuromuscular junction of the rat. HP249 is not, however, specific enough for cytoplasmic forms of actin to be used to advantage in these experiments.

## Discussion

Actin and several proteins associated with actin have been reported to be components of the postsynaptic region of the vertebrate neuromuscular junction (10, 30, 52) and of AChR-rich regions in cultured muscle cells (9). The way these proteins interact with AChR in membranes remains obscure, however. In cultured muscle cells, vinculin, $\alpha$-actinin, and filamin appear to be associated preferentially with sites involved in cell-substrate contact, which contain few, if any, AChRs (9; my unpublished observation). As these proteins can be removed from clusters upon extraction with saponin without significantly changing the appearance of the cluster, they are almost certainly not required for the distinctive organization of clustered AChR (7). They may, however, be involved in stabilizing the attachments of muscle cells to the tissue culture substrate or to the elaborate extracellular matrix found at the neuromuscular junction (e.g., reference 54).

Actin itself is difficult to localize in muscle cells in structures other than the contractile apparatus. One approach to overcome this problem has been to develop antibodies to the non-contractile isoforms of actin, termed beta- and gamma-actins, and to use these antibodies to stain frozen sections of muscle. Craig and Pardo used antibodies specific for gamma-actin to visualize a cytoskeletal lattice adjacent to the sarcolemma in chicken skeletal muscle (18). Hall et al. (30) showed that antibodies prepared against actin from *Aplysia* body wall labeled the postsynaptic region of the neuromuscular junction of rat skeletal muscle. These antibodies, reported to be specific for a beta isoform of actin, label areas of the cultured muscle cells where AChR clustering usually occurs (36). They do not, however, appear to associate preferentially with AChR-rich membrane (36), which is consistent with my observation that they label isolated receptor clusters only very weakly.

An alternative way to localize actin near the plasma membrane is to isolate membrane fragments or complexes free of cytoplasm. The classic preparation for such studies is the human erythrocyte ghost (28, 39, 57), but other cell membranes have been examined using a similar approach (e.g., references 16, 22, 31, 33, 38). In membrane fragments from excitable tissue, actin has been identified in postsynaptic densities from mammalian brain (32) and in the AChR-rich postsynaptic membrane from *Torpedo* electric organ (45, 59; Wray, B. E., B. Paschal, and R. Sealock, manuscript submitted for publication).

The study of cytoskeletal proteins at the AChR clusters of cultured rat myotubes is facilitated because clusters form preferentially at sites of myotube-substrate attachment (e.g., references 2 and 9). This reduces the potential difficulties posed by cytoplasmic contamination. In intact cells, clusters can be visualized by total internal reflection fluorescence microscopy (2), which samples only a thin optical section through the substrate-associated membrane and adjacent cytoplasm. Fragments of myotubes containing AChR clusters can also be separated from cytoplasmic contaminants because they remain firmly attached to the substrate when the bulk of the myotube is removed either by physically shearing the cultures (9) or by extracting with saponin (7). The saponin extraction procedure, used in the present study, yields AChR clusters that are purified 100-fold or more (7) and have little cytoplasmic contamination, as determined by electron microscopy (Pumplin, D. W., and R. J. Bloch, manuscript in preparation). Upon immunofluorescence labeling of material prepared by saponin extraction, actin associated with AChR clusters can be visualized without interference from the cytoplasm or the contractile apparatus.

The results of these experiments show that actin is present at the AChR domains of isolated receptor clusters. Isolated cluster preparations contain a band of molecular weight 43,000 in SDS PAGE, p43, which reacts with HP249 antiactin in immunoblots. This antibody labels the AChR domains, but not the contact domains, of isolated clusters. In addition, myosin filaments label the AChR domains of isolated clusters, and this labeling is inhibited by MgATP. These studies indicate that actin is associated with regions of the muscle membrane rich in AChR, as also found by Hall et al. (30) in embryonic and adult rat muscle.

One advantage of using isolated receptor clusters, rather than intact cells or frozen sections, is that the preparations can be manipulated and the effects of the manipulations on the receptor distribution and the polypeptide composition can be compared. Two treatments described here, extraction with buffer A and digestion with chymotrypsin, reduce the amount of HP249-immunoreactive p43 in cluster preparations. These treatments also reduce or eliminate the labeling of isolated clusters by antiactin or by myosin filaments and simultaneously cause a significant redistribution of AChR in membrane fragments. Furthermore, dispersal of AChR clusters in intact cells reduces the subsequent labeling of isolated membrane fragments by antiactin. These studies indicate that the removal of actin from AChR domains is associated with changes in the distribution of receptors in the membrane. This correlation cannot be readily explained in terms of a nonspecific association of actin with the AChR domains of isolated clusters.

Another advantage of studying isolated AChR clusters is that the amount of a particular antigen relative to the amount of AChR can be quantitated. By sampling the fluorescence arising from small (5-$\mu$m diam) areas of receptor-rich membrane, the ratio of fluoresceinated antibody to R-BT can be determined. The variance of this ratio in a given culture is quite small. This permits reliable comparisons of the actin content of clusters that have been treated differently before fixation and labeling. Antiactin labeling of isolated clusters is reduced several-fold by buffer A or chymotrypsin. If appropriate calibrations can be developed, such measurements could eventually be used to establish the stoichiometry of membrane complexes containing AChR.

One aspect of the experiment using buffer A that I cannot yet explain is the appearance in buffer A–extracted samples of additional bands at ~48 and ~45 kD. They may be present at approximately equal levels in controls but appear more clearly in buffer A–treated fractions if minor bands that

obscure the region of the gel above actin are extracted together with p43. Alternatively, the additional bands may appear as a result of autolytic reactions which occur more readily in the presence of buffer A.

It is not yet clear how endogenous or exogenous proteases affect the organization of AChR. Proteases added to isolated cluster preparations can certainly alter the distribution of AChR within isolated clusters while destroying the antigenic binding sites for HP249. However, chymotrypsin causes AChR to form larger aggregates than are formed after extraction with buffer A, suggesting that the effects of exogenous proteases are distinct. The observation that the AChR distribution is different after digestion with chymotrypsin suggests that there may be proteins in AChR clusters other than p43 that affect AChR distribution and that are susceptible to proteolysis. The effects of proteolytic enzymes on the organization and polypeptide content of isolated cluster preparations are now being studied further.

Although the experiments reported here suggest that actin may play a role in receptor clustering, it is difficult to rule out the possibility that polypeptides present in only small amounts are altered or extracted without being readily detected on SDS PAGE or immunoblotting. These polypeptides might react with HP249 and the other antiactins I have used, but their reaction would be difficult to detect in blots because of the small amounts of the hypothetical antigen. One possibility that has concerned me is that HP249 might react with AChR or with the 43K protein, first identified in electric organ and shown to be distinct from actin both biochemically and immunologically (45). In preliminary experiments, Dr. S. Froehner and I have found that the 43K protein is indeed present at AChR clusters. However, immunofluorescent labeling with anti-43K antibody is not reduced in clusters extracted with buffer A or treated with chymotrypsin. Immunofluorescent labeling with anti-AChR antibodies is also not affected by these treatments (Bloch, R., and S. Froehner, unpublished results). These observations suggest that neither AChR nor the 43K protein is likely to provide the primary binding site for HP249. It is still possible, however, that another minor component of clusters binds HP249 and accounts for some of the results presented here.

The presence of the 43K protein at isolated AChR clusters suggests that the entity I have designated as p43 is composed of at least two distinct polypeptides. Indeed, p43 may be even more heterogeneous. In addition to the 43K protein, there are probably at least two isoforms of actin present in isolated cluster preparations. One isoform is concentrated at clusters and reacts preferentially with antiactin HP249. The other isoform reacts with antiactins 1C1 and 4D3 and is present at fragments of fibroblast membrane and of muscle membrane lacking clusters. Because p43 is heterogeneous, its presence on a gel cannot be strictly equated with the presence of actin in the preparation. It seems clear, nevertheless, that p43 contains an isoform of actin that binds HP249 and myosin. The isoforms of actin and the relative amounts of actin and 43K protein present at AChR clusters are now under investigation.

How is actin organized at receptor domains? The possibility that actin is arranged in bundles of microfilaments seems unlikely for at least two reasons. First, vinculin, $\alpha$-actinin, and other proteins that are present where bundles of actin filaments insert into the plasma membrane (15, 25, 26, 35; see also reference 11) are not detectable in isolated AChR clusters (7; my unpublished results). Second, ultrastructural studies of isolated clusters show no evidence of microfilament bundles (Pumplin, D. W., and R. J. Bloch, manuscript in preparation). Another possibility is that actin is present in a stoichiometric complex with AChR. A complex containing actin together with a membrane glycoprotein and a 58-kD polypeptide has been isolated from the filopodia of adenocarcinoma cells by detergent extraction (16). The binding of actin to the membrane glycoprotein appears to be direct and may be stabilized by the 58-kD component (16). If this model applies to receptor clusters, then a complex of actin with AChR should be present in detergent extracts of isolated AChR clusters. A strictly stoichiometric arrangement is unlikely, however, as there is considerably more actin present in isolated cluster preparations than there is AChR. A third possibility is that actin and AChR are organized into a structure resembling that found in the human erythrocyte membrane. Erythrocyte actin is organized into short double-helical oligomers which form the foci of a network of spectrin (23, 28). Spectrin binds on the intracellular face of the membrane to ankyrin, a specific peripheral membrane protein that itself is bound to an integral membrane protein involved in ion transport (3, 17; see references 28 and 39 for reviews). If this model applies to the cytoskeleton associated with AChR domains, a spectrin-like protein and a membrane binding site for spectrin should link actin to clustered receptors.

In summary, the experiments reported here demonstrate that actin is associated with the AChR domains of receptor clusters. They further suggest that actin is involved in maintaining the distinctive organization of AChR in clusters. Several other cytoskeletal proteins besides actin have been found at the postsynaptic region of the neuromuscular junction (10, 12, 24, 52), and some probably participate in the stabilization of the postsynaptic receptor aggregate. AChR clusters isolated from cultured rat muscle cells seem to be a good preparation in which to study these proteins and to learn how they interact with receptors in muscle membrane.

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