Cytoplasmic Actin in Postsynaptic Structures at the Neuromuscular Junction

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ABSTRACT We used an antibody prepared against *Aplysia* (mollusc) body-wall actin that specifically reacts with certain forms of cytoplasmic actin in mammalian cells to probe for the presence of actin at the neuromuscular junction. Immunocytochemical studies showed that actin or an actinlike molecule is concentrated at neuromuscular junctions of normal and denervated adult rat muscle fibers. Actin is present at the neuromuscular junctions of fibers of developing diaphragm muscles as early as embryonic day 18, well before postsynaptic folds are formed. These results suggest that cytoplasmic actin may play a role in the clustering or stabilization of acetylcholine receptors at the neuromuscular junction.

The postsynaptic membrane at the adult neuromuscular junctions is highly specialized with several characteristic features that distinguish it from the surrounding nonjunctional membrane. The membrane underneath the nerve terminal is elaborately folded and, along its entire contour, has a thin layer of electron-dense material attached to its cytoplasmic face. The underlying cytoplasm is rich in filaments whose identity has, thus far, not been established (10, 12, 18, 30). At the crests of the folds of the postsynaptic muscle membrane, acetylcholine receptors (AChRs) are densely packed (14): their density in the membrane is several orders of magnitude higher than that found elsewhere on the muscle surface (13, 22). The biochemical basis of these specializations at the neuromuscular junction is unknown. Because of the presence of filaments beneath the postsynaptic membrane, and because contractile proteins have been implicated in the clustering of integral membrane proteins in nonmuscle cells (7, 11), we have investigated whether actin is concentrated at or near the postsynaptic surface of the neuromuscular junction. For these experiments we have used a recently characterized antibody with an unusual specificity; it does not bind myofibrillar actin in vertebrate muscles but recognizes certain forms of cytoplasmic actin (26).

A preliminary report of these findings has been published (27).

MATERIALS AND METHODS

Antiactin antibody was elicited in rabbits by immunization with actin purified from *Aplysia* (mollusc) body-wall muscle. For immunocytochemistry the antiactin was purified from serum by adsorption to Sephacryl-200 to which highly purified *Aplysia* actin had been coupled by the cyanogen bromide method (26). The fraction of serum that did not bind to the column was used as a control. Bound antiactin was eluted with 3 M ammonium thiocyanate and was dialyzed against 0.15 M NaCl at 4°C.

Actin, used to block antibody binding to tissue sections, was prepared by differential extraction of *Aplysia* thin filaments followed by polyacrylamide gel electrophoresis in SDS (24). The protein band corresponding to actin was eluted from the gel with 10 mM Tris–HCl (pH 7.6) containing 50 μM ATP. The actin preparation contained a trace of tropomyosin which is not recognized by the antiserum (26).

Frozen sections (4 or 6 μm) of rat diaphragm muscle containing endplates were cut in a cryostat as described previously (34). They were incubated with antiactin (43 μg/ml) diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) in a moist chamber for 60 min at room temperature. After rinsing with PBS, the sections were incubated for 30–60 min at room temperature with fluorescein-conjugated goat anti-rabbit IgG (N.L. Cappel Laboratories, Inc., Cochranville, Pa.) diluted 1:100 in PBS-BSA that also contained 0.05–0.15 μM α-bungarotoxin conjugated to rhodamine (the generous gift of Drs. Randall von Wedel and Regis Kelly, Department of Biochemistry, University of California, San Francisco). The slides were then rinsed with PBS, mounted in Elvanol (DuPont and Co., Inc., Wilmington, Del.), and examined with a Zeiss photomicroscope equipped with filters allowing rhodamine (filters 48–77–15) and fluorescein (filters 48–77–09) to be viewed separately with little or no interference from one another (34). Photographs were made with Kodak Tri-X (ASA 400) black and white film.

To test the binding of antiactin to intact fibers, we prepared single fiber segments from endplate-rich regions of rat diaphragm by gentle homogenization in a VirTis blender (The VirTis Co., Gardiner, N. Y.) after light fixation (15 min in 1% formaldehyde) as described by Reiness and Weinberg (33). These were incubated for 60 min at room temperature with 43 μg/ml antiactin, followed by a horseradish peroxidase (HRP)-conjugated second antibody. Fibers were then fixed and stained as described by Sanes and Hall (34), mounted in 90% glycerol, and viewed in bright-field optics. Staining could be detected on 1 fiber in 200. In parallel experiments, as positive controls, single fiber segments were either stained for acetylcholinesterase by the method of Karnovsky (19) or were stained with a rabbit antiserum to rat muscle AChR (39) followed by HRP-labeled second antibody. In both cases, endplates could be identified in ~10% of the segments. Control experiments with muscle sections showed that reactivity with the antibody was not abolished by the fixation procedure.

RESULTS

To test for the presence of actin at endplates we incubated frozen sections of rat diaphragm muscle with an affinity-purified antibody to *Aplysia* body-wall actin. Binding of the antibody was detected with a fluorescein-labeled second antibody; endplates were identified in the same sections with rhodamine-α-bungarotoxin, which binds to the acetylcholine receptors at the endplates. Endplates stained intensely with the antiactin antibody and the stained areas coincided exactly with the area occupied by the bound toxin (Fig. 1 *a*, *b*; see also Fig. 3). Diffuse staining throughout the muscle was also seen, as described previously (26). Control experiments established that the immunofluorescence observed at endplates was specific for actin, or an actinlike molecule: the staining was abolished by preincubation of the antibody with purified *Aplysia* actin (Fig. 2 *c*, *d*) and was faint or absent when sections were incubated at the same protein concentration used for the affinity-purified antibody with the fraction of serum that was excluded from the actin affinity column (Fig. 2 *e*, *f*). Endplates in denervated muscle fibers, lacking nerve terminals and Schwann cells, also bind the antibody (Fig. 2 *g*, *h*). Therefore, the actin must be present postsynaptically; we cannot determine from these experiments whether or not it also occurs in presynaptic terminals. Although actin has been reported to be in most preparations of AChR-rich membranes isolated from *Torpedo* (36), no specific association with postsynaptic structures has previously been shown. To see whether actin is on the cell surface, we

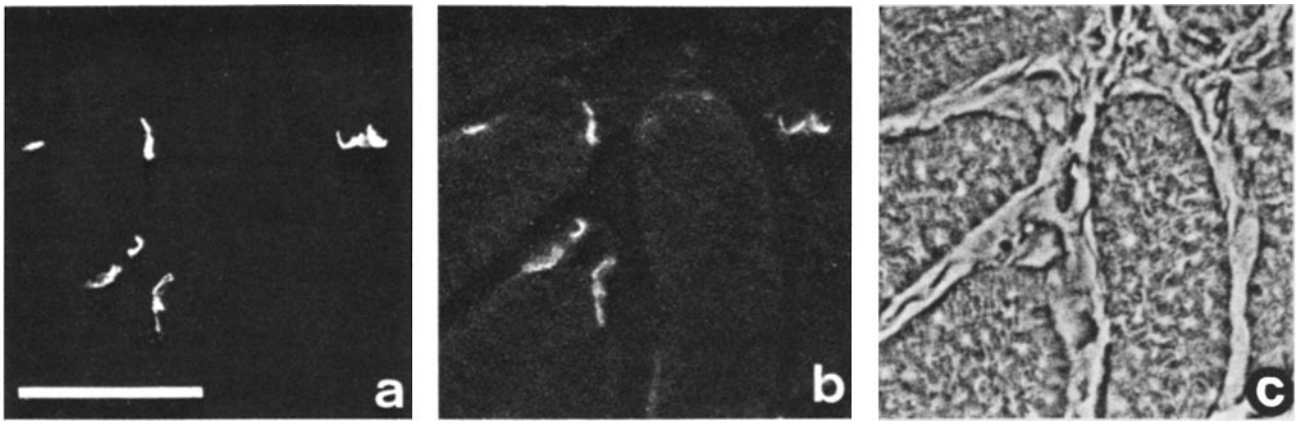

FIGURE 1 Antiactin binds at the neuromuscular junctions of adult rat muscle. A frozen section (4 μm) of rat diaphragm is viewed with (*a*) rhodamine, (*b*) fluorescein, and (*c*) phase optics after incubation with antiactin, followed by a fluorescein-labeled second antibody and rhodamine α-bungarotoxin. The sites of bungarotoxin binding and antibody binding are identical. Bar, 50 μm. × 470.

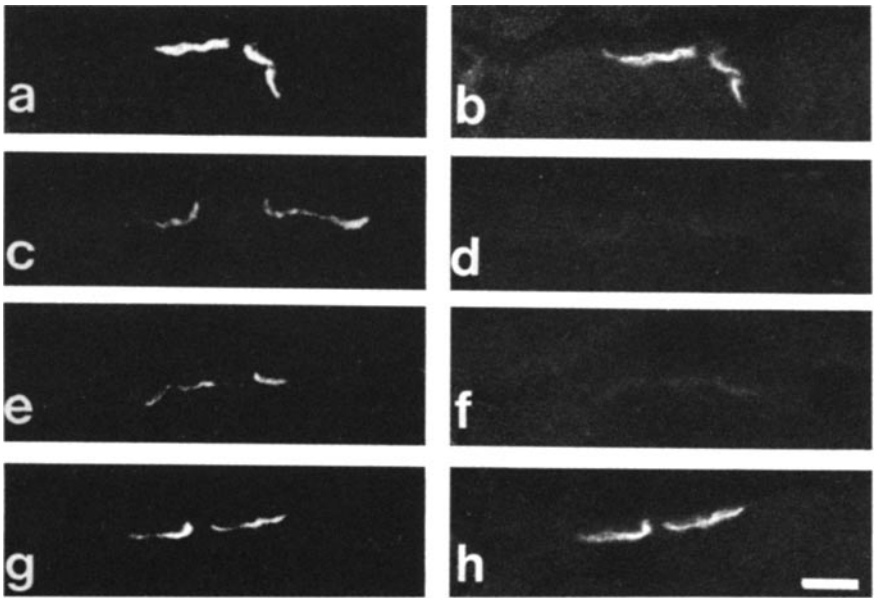

FIGURE 2 Antiactin binds to endplates from both normal and denervated muscle, and the binding is specific. Frozen sections (4 μm) of rat diaphragm muscle viewed with rhodamine (*a*, *c*, *e*, *g*) and fluorescein (*b*, *d*, *f*, *h*) optics. (*a*, *b*) Normal muscle incubated with antiactin (43 μg/ml). (*c*, *d*) Normal muscle incubated with antiactin that had been preincubated overnight at 4°C in a 10-fold molar excess of purified actin from *Aplysia*. In other experiments, immunofluorescence staining was abolished with a 2.5-fold excess of actin over IgG binding sites. (*e*, *f*) Normal muscle incubated with the fraction of serum that did not bind to the actin affinity column (43 μg/ml). (*g*, *h*) Denervated muscle (11 wk) incubated with antiactin. At this stage of denervation, nerve terminals are no longer present and Schwann cells have retracted from the endplate (reference 29). Exposures were the same for each set of rhodamine prints and for each set of fluorescein prints. Bar, 10 μm. × 750.

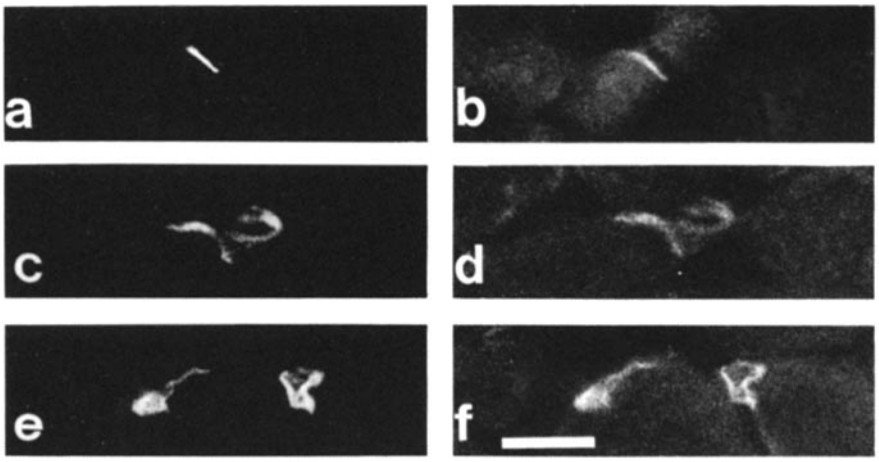

FIGURE 3 Antiactin binds to endplates in developing muscle. Frozen sections (6 μm) of rat diaphragm muscle viewed with rhodamine (*a, c, e*) and fluorescein (*b, d, f*) optics. (*a, b*) Muscle from an embryo at 18 d gestation. (*c, d*) Muscle from 6-d pup. (*e, f*) Muscle from 12-d pup. Exposures were essentially the same for each set of rhodamine prints and each set of fluorescein prints, with minor variations for clarity. The difference in configuration of AChRs at different ages was too consistent to be ascribed to a chance difference in the plane of section. Bar, 10 μm. × 750.

incubated intact fibers with antiactin, followed by an HRP-labeled second antibody, as described in Materials and Methods. In several experiments we were unable to detect staining at endplates, and we conclude that actin is intracellular.

During formation of endplates in rat muscle, receptor clusters first appear in the postsynaptic membrane during embryonic development (3, 9), whereas folding of the membrane does not occur until the 2nd wk of postnatal life (21, 23, 37). This same period marks the transition of the configuration of postsynaptic receptors from a simple plaquelike structure to the more complex collection of clusters seen in the adult (35). To see whether actin was associated with endplates in both early and late stages of development, we examined diaphragm muscle fibers at embryonic day 18 and at postnatal days 6 and 12. Between embryonic day 18 and postnatal day 6, we observed that the configuration of acetylcholine receptors clustered at endplates, when seen in cross-section, changed from a simple arc at the periphery of the fiber to much more elaborate forms (Fig. 3*b, d, f*). We found actin at all three stages, although it could not be detected with certainty at every endplate at embryonic day 18. The distribution of antiactin immunofluorescence at all stages coincided with that of the acetylcholine receptors (Fig. 3).

DISCUSSION

Our experiments show that actin is concentrated at the endplates of developing and adult rat muscles. Because it is present at endplates of denervated muscles that lack both nerve terminals and Schwann cells, it must be associated with structures at or under the postsynaptic membrane. Intact muscle fibers are not stained by the antibody and thus we assume that the actin is intracellular. Immunocytochemical experiments cannot establish conclusively whether the immunoreactive substance at the synapse is actin rather than a cross-reactive protein that shares immunological determinants with actin. The antibody that we have used appears to be highly specific, however. It was prepared by affinity chromatography from a serum raised against *Aplysia* body-wall actin; it cross-reacts with cytoplasmic actin prepared from bovine brain and, in mammalian cells, stains only structures known to contain cytoplasmic actin (26). The antiserum also removed actin from crude extracts of goldfish, cat, and human brain, as well as from *Aplysia* and octopus nervous tissue.[1]

There are several postsynaptic structures at the neuromuscular junction with which actin could be associated. A thin layer of electron-dense material lining the cytoplasmic surface of the postsynaptic membrane is distributed along the entire length of the folds (10, 30). This postsynaptic dense material appears early during development of the neuromuscular junction and can be seen at about the same time (day 17) that receptor clusters first appear (3, 21). Prominent postsynaptic densities are also associated with synapses in the central nervous system. Although synaptosomal fractions that are enriched for these structures contain actin (6, 20, 28), actin has not been shown directly to be a component of the postsynaptic density, and could be associated with contaminating components.

A high concentration of filaments is also a prominent postsynaptic morphological feature of muscle cells. These form dense bands running within the folds and also occur in sarcoplasm between the folds and the myofilaments (10, 18, 30). Filaments also appear beneath the postsynaptic membrane of *Torpedo* electroplax (17) and in the cytoplasm beneath receptor clusters in cultured muscle cells (31). Electron microscopic studies will be required to determine whether actin is associated with these filamentous structures and with the postsynaptic density.

What role might actin play at the adult endplate? We found it to be present as early as embryonic day 18. Because endplates were smaller and the binding of antiactin throughout the fiber (26) was increased in younger muscles, actin could not reliably be identified at every endplate in day-18 embryonic muscle, but in favorable sections was seen at most of them. Ultrastructural investigations show that, at this time in development, the postsynaptic membrane has a simplified structure, with only occasional slight invaginations; postsynaptic folds resembling those seen in the adult are first seen in rat muscle at about postnatal day 4, and continue to develop in complexity over the next few weeks (21, 23, 37). Although actin could be required to form and maintain the elaborate structure of the

[1] Lubit, B. W., and J. H. Schwartz. Manuscript in preparation.

postsynaptic membrane, its association with endplates as early as a week before folds appear makes it unlikely that this is its sole function.

Another possibility is that actin is required to achieve the high density of receptors that is characteristic of endplates. Actin is found in the caps formed after ligand-induced clustering of surface membrane receptors in fibroblasts and lymphocytes (8, 16, 38) and is thought to play an important part in the mechanism of cap formation (2, 7, 11, 15). Nerves and neural extracts are capable of inducing the redistribution of preexisting receptors on the surface of muscle cells (1, 32), and it is possible that the clustering of acetylcholine receptors during formation of synapses occurs by similar mechanisms. Vinculin, a protein associated with sites of attachment for actin-containing microfilaments at the cell surface, has recently been found to occur in acetylcholine receptor patches on myotubes in primary cell cultures (4).

There have been several suggestions, based on indirect evidence, that acetylcholine receptor clusters at developing endplates become stabilized to dispersal by association with the cytoskeleton at a time that is subsequent to clustering (5, 32). Such an association of receptors with the cytoskeleton could also explain the presence of cytoplasmic actin at the endplate. It should be noted that the concentration of actin is not correlated with the metabolic stability of receptors at endplates, because we found it at endplates as long as 11 wk after denervation, a time when endplate receptors are rapidly turning over (25).

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