Cytoskeletal Components of the Vertebrate Neuromuscular Junction: Vinculin, α-Actinin, and Filamin

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ABSTRACT We have used immunocytochemical methods to investigate the cytoskeletal constituents of the vertebrate neuromuscular junction. Specific, affinity-purified antibodies to three cytoskeletal proteins, vinculin, α-actinin, and filamin, bound to neuromuscular junctions in sections of normal rat, mouse, chick, and *Xenopus* muscles. All three antibodies bound to the synaptic regions of denervated rat muscle fibers, indicating that the proteins recognized by these antibodies are associated with postsynaptic structures. The three proteins are present at the neuromuscular junction in muscle fibers of embryonic and neonatal animals, and therefore, may play an important role in its differentiation.

The postsynaptic membrane of the adult vertebrate neuromuscular junction has a highly specialized structure that is distinct from the extrasynaptic membrane that surrounds it. Within its borders, the postsynaptic membrane is organized into distinct domains that are either rich or poor in acetylcholine receptors (AChR). The AChR-rich domains, which contain paracrystalline arrays of AChR (16, 27, 30), are closely apposed to the nerve terminals (10), whereas the AChR-poor domains are invaginated to form more or less elaborate folds according to the species and the muscle type (41).

Although the factors responsible for the differentiation of the postsynaptic membrane and its organization into AChR-rich and AChR-poor domains are not yet understood, the structures in close association with the membrane on each of its surfaces may play important roles. The postsynaptic membrane is bounded extracellularly by a basal lamina that has unique structural and functional properties (17, 31) and that, in adult muscle, can direct the differentiation of both presynaptic and postsynaptic membranes (1, 7, 25, 31, 32). The postsynaptic membrane is associated intracellularly with a layer of electron-dense material that is coextensive with the AChR-rich domain and with an extensive network of cytoskeletal filaments (16–18, 29, 30). The involvement in other cells of cytoskeletal elements in generating and maintaining membrane protein aggregates and specialized configurations of the membrane suggest that these may also be important in determining the structure of the postsynaptic membrane at the neuromuscular junction. Two proteins, one that resembles actin (15) and another that is immunologically related to the 43,000-dalton protein in *Torpedo* (12, 33, 34), have been associated with postsynaptic structures at the neuromuscular junction. Here we report that proteins related to vinculin, α-actinin, and filamin are also concentrated postsynaptically.

MATERIALS AND METHODS

Staining of Frozen Sections:: Frozen muscle sections were cut as described previously (15, 31). Briefly, muscles were dissected and frozen in liquid nitrogen or in hexane precooled in a dry ice-acetone bath. Muscles from embryonic and newborn rats were wrapped in a strip of adult rat diaphragm before freezing and sectioning. Pieces (~3 mm × 5 mm × 8 mm) were trimmed, mounted on a brass block, and sectioned with a cryostat. Sections of adult and neonatal muscles were 4 μm thick; sections of embryonic diaphragm were 6 μm thick. Sections were picked up onto glass slides, dried at room temperature, and stored with dessicant at −70°.

Antigens in the frozen sections were detected by indirect immunofluorescence. In most cases, the sections were first incubated with the antibody of interest, diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), followed by fluorescein-conjugated goat anti-rabbit IgG (fGAR; Cappel Laboratories Inc., Cochranville, PA) diluted 1:100 in PBS-BSA. Tetramethylrhodamine-labeled α-bungarotoxin (R-α-BuTx), prepared according to the method of Ravdin and Axelrod (28), was routinely added (0.5 μg/ml) to the fGAR solution to label AChR at the motor endplates. In some cases, we used a more sensitive immunofluorescence procedure in which the fGAR was replaced by biotinylated goat anti-rabbit IgG (20 μg/ml), prepared with biotin succinimide ester (Biosearch, San Rafael, CA). Sections were subsequently incubated in a mixture of fluorescein-conjugated avidin (40 μg/ml; Vector Laboratories Inc., Burlingame, CA) and R-α-BuTx. The fluorescein-avidin was adsorbed before use with a membrane fraction from rat muscle to reduce background staining. When the sensitivity of the fGAR and biotin-avidin procedures were compared on sections of rat muscle, using serial dilutions of a rabbit antiserum to rat muscle AChR as a test system, the biotin-avidin method was found to be approximately eightfold more sensitive.

After staining, the sections were rinsed with PBS, mounted in Elvanol or glycerol, and viewed in either a Leitz Diavert or a Zeiss IM 35 microscope.

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Photographs were made with Kodak Tri-X or Ilford HP-5 film; exposure were for 15–60 s. Tri-X film was developed with D-76 (Kodak) to an ASA of 400; HP-5 film was processed with Microphen (Ilford) to an ASA of 1,200. Further details are given elsewhere (4, 15).

Preparation of Proteins: Vinculin, α-actinin, and filamin were purified from chicken gizzard (Type II from Pel-Freeze Biological, Rogers, AR) according to the methods of Feramisco and Burridge (11). Tubulin from rat brain, and actin from rabbit muscle and from chicken gizzard were purified following published procedures (35, 37, 38). Tropomyosin from chicken gizzard was purified as described by Bailey (2) through the first isoelectric precipitation step. The precipitate was dissolved in 0.01 M Tris-HCl, 1 M KCl, pH 8.0, applied to a column (2.5 × 90 cm) of Sepharose 6B-CL (Sigma Chemical Co., St. Louis, MO), and chromatographed in the same buffer at 20 ml/hr. Fractions of 3 ml were collected and analyzed by SDS PAGE (21). Those fractions showing the tropomyosin A and B chains, and containing no higher molecular-weight contaminants, were pooled and dialyzed against 5 mM Tris-HCl and 50 mM $MgCl_2$, pH 8.0 (23).

Preparation of Antibodies: Virgin female rabbits were immunized with 0.2–0.5 mg of protein in buffered saline that had been emulsified in an equal volume of Freund's adjuvant. Half of the emulsion was injected into the hind footpads and the remainder was injected subcutaneously along the back. The rabbits were boosted 4 wk later, and at 1–2 wk intervals thereafter, by subcutaneous injection of 50 μg protein in incomplete Freund's adjuvant. Blood, collected regularly after the first boost, was allowed to clot at room temperature, then incubated at 4° overnight. After the clots were removed, the sera were stored frozen at −20 or −70°C in 10–35-ml aliquots.

For purification of specific antibodies, sera were thawed and subjected to precipitation by 50% ammonium sulfate. The partially purified IgG fraction was dialyzed against buffered saline, then repeatedly passed through a column (5–10 ml) consisting of Sepharose 4B (Pharmacia Fine Chemicals Div., Pharmacia, Inc., Piscataway, NJ) covalently linked to the appropriate protein (24). Unbound IgG was stored for later use as a control for specificity of immunofluorescent staining. The bound antibody was eluted with 0.1 M glycine, 0.5 NaCl, pH 2.7, and collected in 2-ml aliquots into tubes containing 0.1 ml of 2 M Tris HCl, pH 8.0. Purified antibodies were stored at 4° in PBS supplemented with 10 mM sodium azide.

We used an enzyme-linked immunosorbent assay, as described by Engvall (9), to test the specificities of the purified antibodies. Plates (Immulon II) were purchased from Dynatech Laboratories, Inc. (Alexandria, VA); goat anti-rabbit IgG coupled to alkaline phosphatase, bovine serum albumin (type V), and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Optical density was read in a Titertek Multiscan (Flow Laboratories Inc., Rockville, MD).

The results of the binding assays are presented in Fig. 1. It is clear that each antibody was highly specific for its appropriate antigen and shows little or no binding to any of the other seven purified antigens to which it was exposed. The seven nonreactive proteins include many of those which are most likely to contaminate the purified protein preparations. The staining pattern of each affinity-purified antibody on cultured rat fibroblasts and myotubes was also examined. In each case, a specific pattern characteristic for that antibody was obtained.

Animals: New Zealand white rabbits were obtained from Dutchland Lab Animals (Denver, PA). White leghorn chickens were hatched from fertilized eggs obtained from Spafas (Lancaster, PA) and raised in the laboratory for 29 d before sacrifice. Sprague-Dawley rats were purchased from Charles River Breeding Labs, Inc. (Wilmington, MA). *Xenopus laevis* adults, 6–7 cm long, were obtained from Nasco, Inc. (Fort Atkinson, WI).

RESULTS

Our goal in these experiments was to determine if vinculin, α-actinin, or filamin is enriched in the postsynaptic region of the vertebrate neuromuscular junction. We generated specific antibodies against the three proteins (Fig. 1), and used them to stain the neuromuscular junction in frozen rat muscle sections by indirect immunofluorescence. By co-staining with R-α-BuTx, which specifically labels AChR at the motor end plate, we were able to compare the sites of antibody labeling with that of the postsynaptic membrane. Given the limitations imposed by the light microscope and by the thickness of our sections, we believe that immunofluorescence can localize antigens to within ~0.5 μm of the postsynaptic membrane.

Our most extensive series of initial experiments were performed on sections of adult rat muscle. In each case the antibodies gave positive staining that was coincident with staining by R-α-BuTx (Fig. 2). Similar results were obtained with antivinculin and anti-α-actinin when sections were fixed with 2% paraformaldehyde before staining (not shown). In addition to the neuromuscular junction, other structures in the muscles were also stained. Antivinculin staining was occasionally apparent in some extrajunctional regions. Anti-α-actinin weakly stained the contractile apparatus and capillaries. Antifilamin showed moderate staining of capillaries and occasional weak staining of extrajunctional regions. Some examples of such extrajunctional staining are pointed out in the legends to Figs. 3–5.

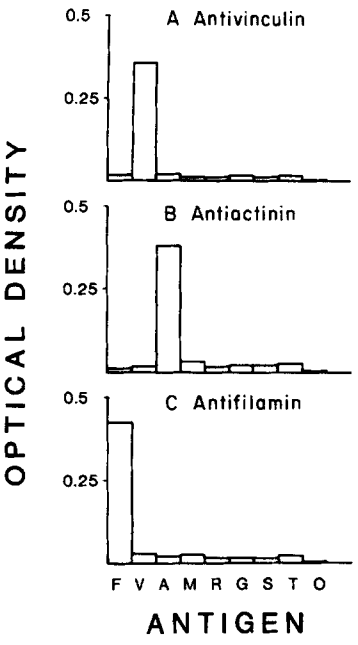

FIGURE 1. Specificity of the antibodies for their respective antigens. Multiwell plates were coated with 50 ng of the protein designated as antigen, in 50 μl of buffered saline. All remaining, nonspecific binding sites for protein were then saturated with BSA. Solutions in buffered saline of antivinculin (48 ng/ml), anti-α-actinin (144 ng/ml), antifilamin (208 ng/ml), and antimyosin (160 ng/ml) were introduced in 50-μl aliquots and incubated for 1.5 h at 37°. Unbound antibody was washed out, and goat-anti-rabbit IgG coupled to alkaline phosphatase (270 ng/ml) was introduced and incubated, as described. Bound antibody-linked alkaline phosphatase was assayed using *p*-nitrophenyl phosphate (0.6 mg/ml in diethanolamine buffer, pH 9.8). More details are given in reference 9. *F*, filamin; *V*, vinculin; *A*, α-actinin; *M*, myosin; *R*, tropomyosin; *G*, gizzard actin; *S*, skeletal muscle actin; *T*, tubulin; and *O*, blank. Similar results were obtained with sixfold higher antibody concentrations. The results show that each antibody is highly specific for its appropriate antigen.

We tested the specificity of staining at the junction in two ways. First, we incubated the sections with the IgG fractions that failed to bind to the affinity columns (see Materials and Methods). In each case, little or no junctional staining was observed. This is illustrated in Fig. 3, *a–d*, for vinculin; identical results were obtained with nonspecific IgG fractions from antifilamin and anti-α-actinin sera. Second, we adsorbed each of the specific antibody preparations with a 6–20-fold molar excess of the appropriate antigen. Preadsorption with vinculin greatly reduced the junctional staining by antivinculin, as illustrated in Fig. 3, *e–h*. Similar results were obtained for antifilamin and for anti-α-actinin (not shown). On the basis of these experiments we conclude that proteins resembling vinculin, α-actinin, and filamin are concentrated at the neuromuscular junction.

To determine if the specific junctional staining was localized to the postsynaptic region, we examined rat diaphragms that had been surgically denervated for three or more weeks, a time when both nerve terminals and Schwann cells have completely disappeared from the junction (26). In each case, the denervated end plates were stained. The results with

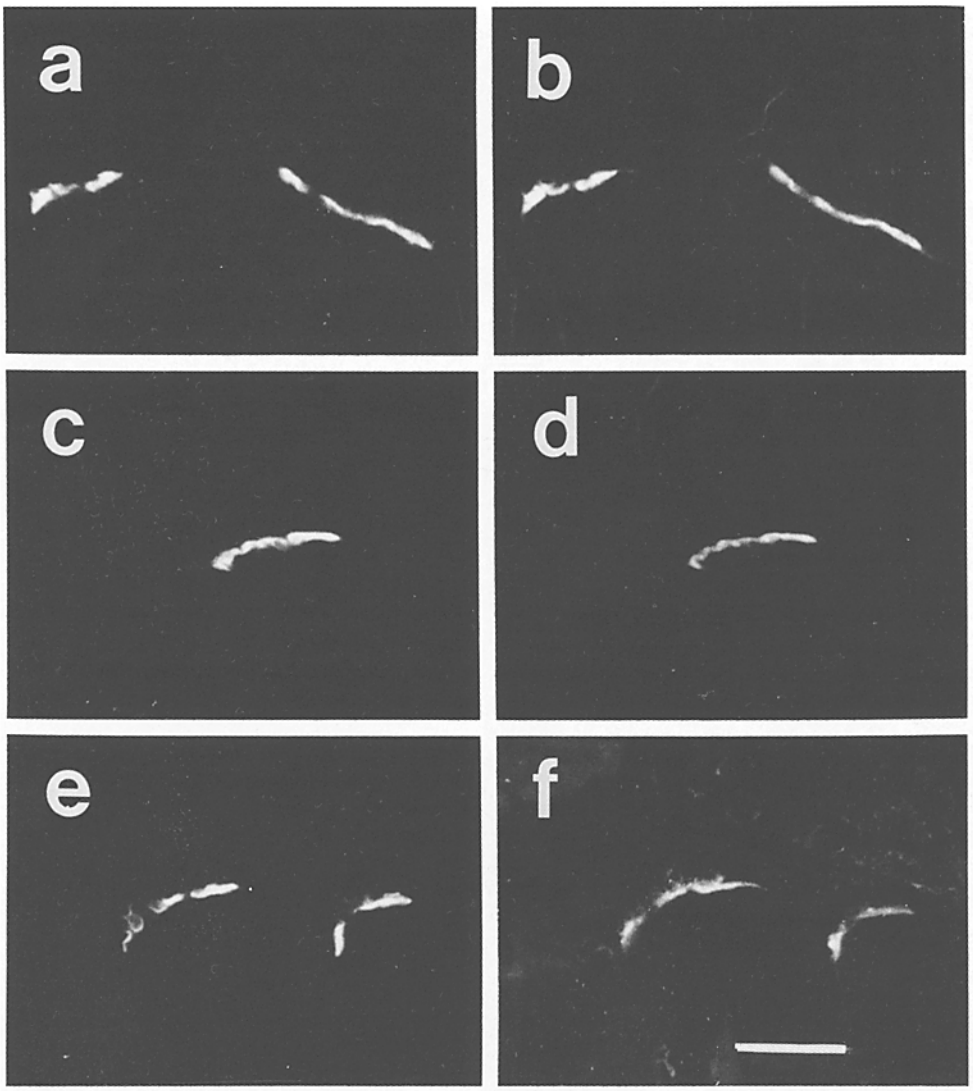

FIGURE 2. Antibodies against vinculin, α-actinin, and filamin stain the neuromuscular junction of rat diaphragm. Frozen transverse sections were cut through junctional regions of normal, adult rat diaphragm. Sections were reacted with (*a* and *b*), antivinculin (12 μg/ml); (*c* and *d*), anti-α-actinin (36 μg/ml); (e and *f*) antifilamin (34 μg/ml). Counterstaining was with R-α-BuTx and either fGAR in *a–d*, or with biotinylated GAR and fluoresceinated avidin, in *e–f*. After staining, samples were mounted in Elvanol and observed under epi-illumination to visualize fluorescein (*b*, *d*, *f*) and tetramethylrhodamine (a, c, e). The results show that the three antibodies react with junctional regions, labeled with R-α-BuTx. Bar in *f*, 25 μm.

antivinculin are shown in Fig. 3, *i* and *j*; similar results were obtained with anti-α-actinin and antifilamin (not shown). No staining of 3-wk denervated junctional regions was seen when nonspecific IgG fractions were used. These results suggest that proteins related to vinculin, α-actinin, and filamin are enriched in postsynaptic structures.

To determine if these proteins are present in developing muscles, we stained frozen sections of diaphragms from rats of the following ages: embryonic day 18, neonatal, and 6–d postnatal. Antivinculin, anti-α-actinin, and antifilamin stained most end plates in muscles at all three ages. Examples of end plates from neonatal animals are shown in Fig. 4. Staining of the junctional regions of these muscles by nonspecific IgG fractions was in all cases negligible. Thus proteins related to vinculin, α-actinin, and filamin are concentrated at end plates within several days after the onset of synaptogenesis.

We also tested the antibodies against muscle sections of mouse diaphragm, *Xenopus sartorius*, and chicken posterior latissium dorsi muscles. In all cases but one, specific staining of the junctions was clearly evident (Fig. 5). In *Xenopus* muscle, our preparation of anti-α-actinin showed extensive staining of the contractile apparatus, which made it impossible to determine if the end plates were also stained.

DISCUSSION

Using indirect immunofluorescence techniques, we have shown that affinity-purified antibodies to vinculin, α-actinin, and filamin stain the neuromuscular junctions of vertebrate skeletal muscles. We judged the staining to be specific by three criteria: (*a*) Enzyme-linked immunosorbent assays indicated that each of the antibodies is specific for the immunogen used to make it, and that cross-reaction with seven other cytoskeletal proteins is minimal. (*b*) IgG fractions from which the specific antibodies had been removed by affinity chromatography gave no significant junctional staining. (*c*) The intensity of staining was greatly decreased by preadsorption with the appropriate antigens. In addition to the results described here, we have, in preliminary experiments, been unable to observe antimyosin staining of denervated junctions (unpublished result). This further suggests that staining of neuromuscular junctions by antibodies to cytoskeletal proteins is specific.

The nature of the antigens at the junction is still unknown. Proteins such as α-actinin occur in different isozymal forms. Each of the antibodies we used were prepared using proteins purified from avian smooth muscle. The antibodies reacted only weakly with skeletal myoplasm, enabling us to detect

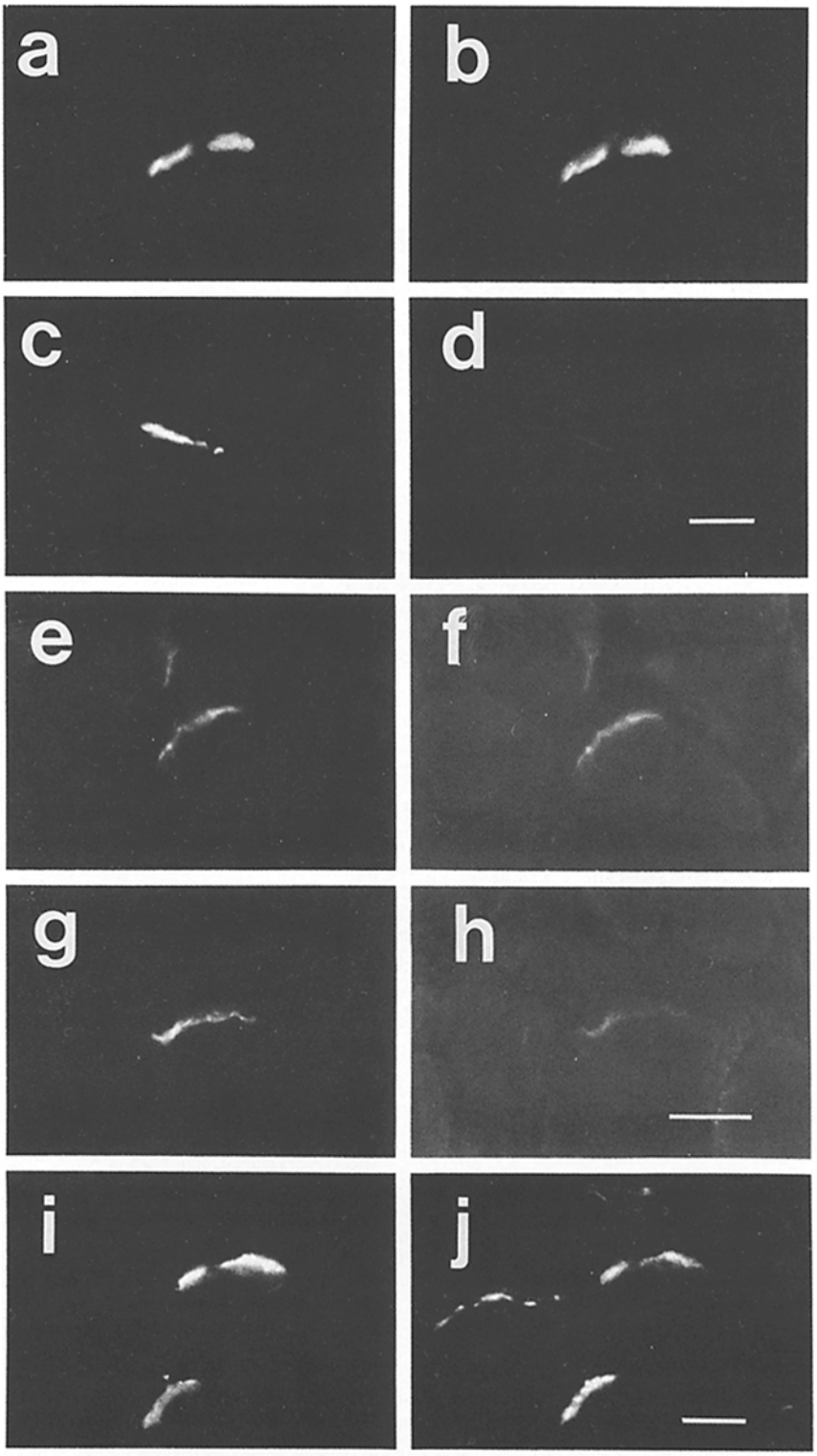

FIGURE 3. Specificity of antivinculin staining of the neuromuscular junction. The procedure given in the legend to Fig. 1 was followed, but only antivinculin was used. *b*, *d*, *f*, *h*, and *j* show fluorescein fluorescence obtained after staining with fGAR. *a*, *c*, e, *g*, and *i* show staining of the AChR in the same sections with R-α-BuTx. the antibodies used were (*a* and *b*) antivinculin (12 μg/ml); (c and *d*) nonspecific IgG from antivinculin antiserum (12 μg/ml), which shows essentially no staining; (e and *f*) antivinculin (0.38 μg/ml); (*g* and *h*) antivinculin (0.38 μg/ml) preadsorbed with purified vinculin (7.6 μg/ml) for 15 min at 22°, which shows significantly lower staining; (*i* and *j*) antivinculin (12 μg/ml) staining of a section through the junctional region of a rat diaphragm that had been denervated 3 wk before freezing and sectioning. The results show that antivinculin staining of the neuromuscular junction is specific and that it persists in long-term denervated muscle. Note the appearance of intense extrajunctional stain in denervated muscle (*j*). Bars, 20 μm; the bar in *d* applies to *a–d*; in *h*, to *e–h*; and in *j*, to *i* and *j*.

specific staining of the neuromuscular junction. The junctional antigens are therefore likely to resemble their smooth muscle counterparts. Had the antibodies cross-reacted extensively with the skeletal muscle isozymes, junctional staining probably would have been obscured. Such cross-reaction may account for our inability to detect specific junctional staining of α-actinin in *Xenopus* muscle.

Because a cytoplasmic form of actin has previously been localized to the postsynaptic region of the neuromuscular junction of rats (15), it is not surprising that other cytoskeletal proteins, often associated with actin, are also present. Nevertheless, the roles postulated for these proteins in other tissues suggest that the postsynaptic cytoskeleton may have some interesting features.

Both in cultured and in vivo cells, vinculin and α-actinin have been localized to sites at which microfilamentous structures are attached to the cytoplasmic face of the cell membrane (13, 14, 36). These are often sites of adherence of cells

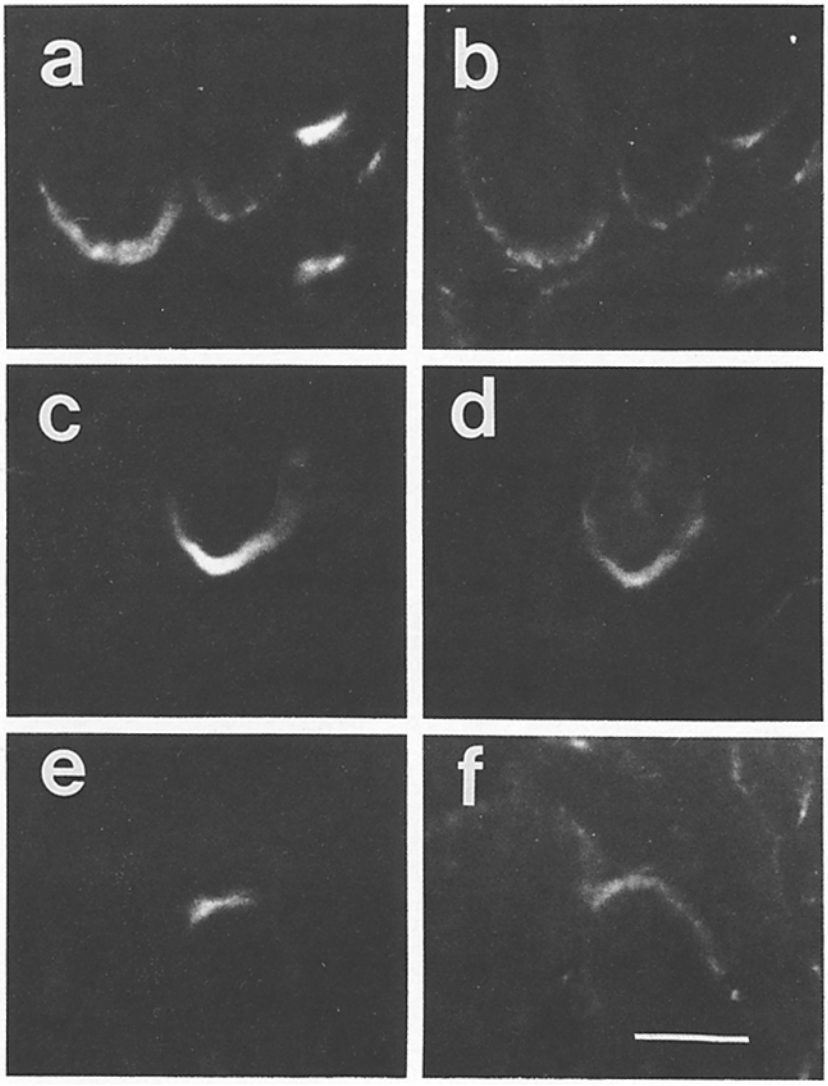

FIGURE 4 Antibody staining of junctional regions from newborn rats. Frozen sections (6 μm) through diaphragm obtained from newborn (<24-h-old) rats are shown. Conditions are as described in the legend to Fig. 1. The antibodies are (*b*), antivinculin; (*d*), anti-α-actinin; (*f*), antifilamin. *a*, *c*, and *e* show corresponding R-α-BuTx staining of the same sections. The results indicate that junctional regions of newborn diaphragm show antivinculin, anti-α-actinin, and antifilamin staining. Note, however, that extrajunctional staining is also apparent (e.g., *f*). Bar in *h*, 10 μm.

to substrate, to other cells, or to extracellular matrix (5, 8, 13, 14, 22, 36). At the adult neuromuscular junction, a highly differentiated extracellular matrix lies between the nerve and muscle, and appears to be connected to both nerve and muscle membrane by filamentous structures (17). Subjacent to AChR-rich membrane is a fine microfilamentous network (16–18, 29, 30). Vinculin and α-actinin may thus be involved in anchoring extracellular materials to intracellular actin filaments. Neither vinculin nor α-actinin seem to bind directly to AChR in the muscle membrane (5; R. J. Bloch, manuscript in preparation). Actin (15) or other postsynaptic proteins (12, 33, 34) may perform this function, however.

Filamin, also localized at postsynaptic structures, is likely to play a different role from that of α-actinin or vinculin. Filamin is a large polypeptide that is capable of cross-linking individual actin filaments into bundles or sheets (40). It is postulated that in regions of cell-cell contact, filamin cross-linking of microfilaments stabilizes contact (40). If it is localized immediately below the AChR-rich membrane of the motor end plates, filamin may be involved in cross linking the postsynaptic microfilamentous network.

Postsynaptic folds do not appear in rat diaphragm until 1 wk after birth (19, 20, 39), many days after proteins related to vinculin, α-actinin, and filamin can first be detected at the motor end plate. Therefore, membrane folding, with its associated increase in postsynaptic surface area, cannot account for the enrichment of these proteins in junctional regions of embryonic and neonatal rats. These proteins appear at AChR-rich membrane within 2 d after onset of AChR accumulation at the developing neuromuscular junction (3, 6). The temporal and spatial association of proteins related to vinculin, α-actinin, and filamin with the developing motor end plate suggest that they may play an important role in its morphogenesis.

We are grateful to many of our colleagues for their assistance and support: to Dr. D. W. Pumplin, for dissecting the sartorius muscle from *Xenopus*; to Dr. C. Shear, for dissecting the posterior latissimus dorsi; to Dr. C. Hall-Craggs, for the use of his freezing microtome; to Ms. W. Resneck, for her expert supervision of the immunizations and for assistance in purifying some of the antigens; to Ms. N. Robitaille-Giguere, for cutting the frozen sections of all the mammalian samples we examined; to Ms. A. Ford and V. Weisz, for collecting antisera; to Mr. J. Worley, III, for purifying tropomyosin and tubulin; to Dr. R. Anthony, for the use of the Titertek Multiscan. We also thank Dr. L. Silberstein for her useful suggestions and Dr. S. Heinemann for his encouragement and support at the earliest stages of antibody preparation. Z. Hall also thanks Dr. N. Hirokawa, who participated in an earlier series of experiments on α-actinin. Our research is supported by grants to R. J. Bloch and to Z. W. Hall from the National Institutes of Health and from the Muscular Dystrophy

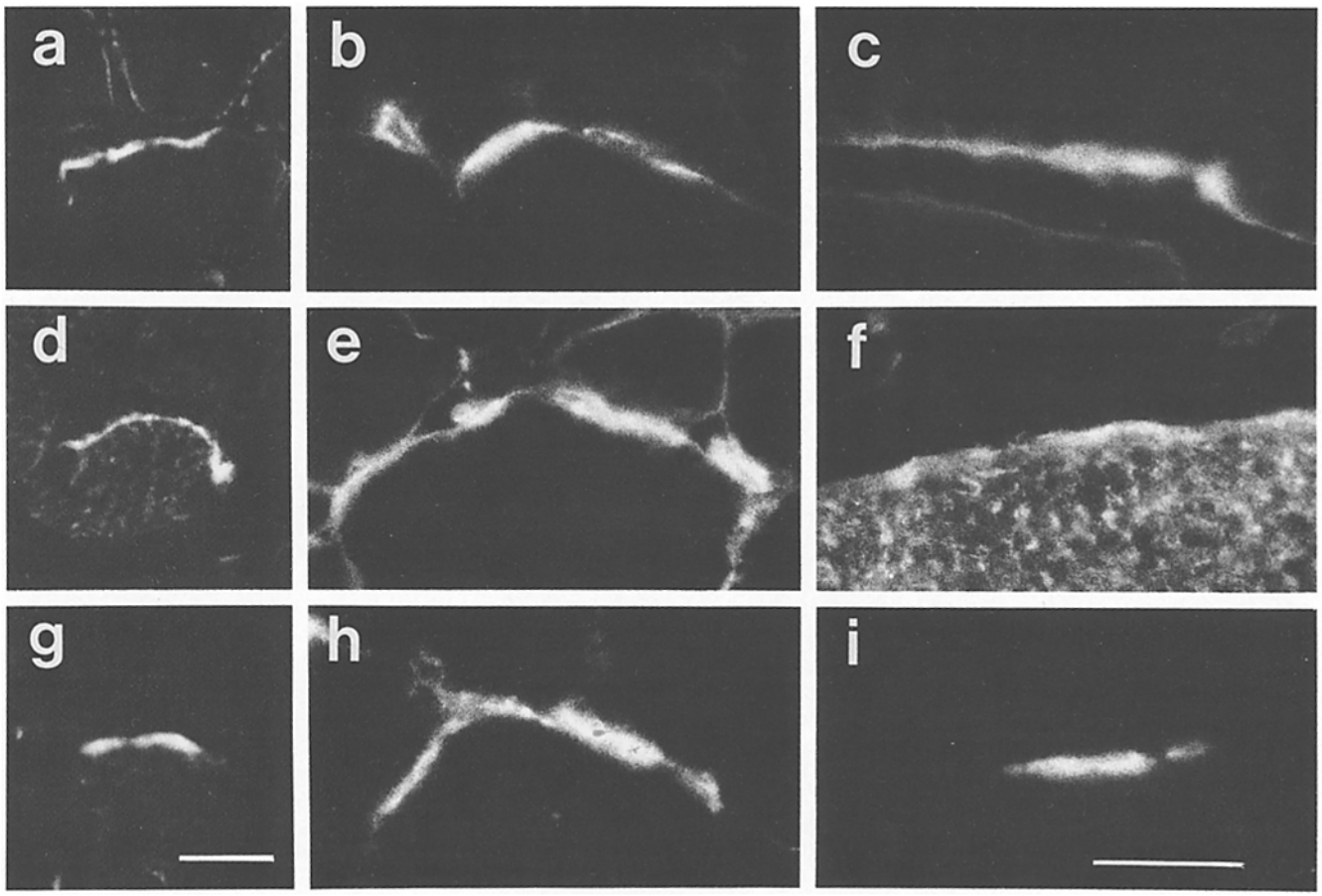

FIGURE 5 Staining of neuromuscular junctions of mouse, chicken, and *Xenopus*. Frozen sections through junctional regions of mouse diaphragm (*a*, *d*, and *g*), chicken posterior latissimus dorsi (*b*, *e*, and *h*), and *Xenopus* sartorius (*c*, *f*, and *i*) muscles were stained with antibodies. Methods were as described in Fig. 1, except that fluorescein-avidin was used only in antifilamin staining of mouse muscle and staining of chicken muscle used 10-fold lower concentrations of antivinculin and ninefold lower concentrations of fGAR. R-α-BuTx stained regions (not shown) in all cases coincided with the brightly staining, membrane-associated structures pictured here. The antibodies used were antivinculin, (a–c); anti-α-actinin, (*d–f*); antifilamin, (*g–i*). The results show that, except for anti-α-actinin reaction with *Xenopus* muscle (*f*), junctional regions of all three species are stained by the antibodies. Some weak extrajunctional staining is also apparent in *a* and *e*; staining of the contractile apparatus is evident in *f*, and, more faintly, in *d*. Bars, 20 μm. The bar in *g* also applies to *a* and *d*, and the bar in *i* applies to the rest of the panels.

Association. R. J. Bloch is the recipient of a McKnight Scholar's Award and a Research Career Development Award.

Received for publication 14 December 1982, and in revised form 10 March 1983.

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