Vinculin in Subsarcolemmal Densities in Chicken Skeletal Muscle: Localization and Relationship to Intracellular and Extracellular Structures

CHARLES R. SHEAR* and ROBERT J. BLOCH[‡]
Departments of *Anatomy and ‡Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT   Using immunocytochemical methods we have studied the distribution of vinculin in the anterior and posterior latissimus dorsi skeletal (ALD and PLD, respectively) muscles of the adult chicken. The ALD muscle is made up of both tonic (85%) and twitch (15%) myofibers, and the PLD muscle is made up entirely of twitch myofibers. In indirect immunofluorescence, antivinculin antibodies stained specific regions adjacent to the sarcolemma of the ALD and PLD muscles. In the central and myotendinous regions of the ALD, staining of the tonic fibers was intense all around the fiber periphery. Staining of the twitch fibers of both ALD and PLD muscles was intense only at neuromuscular junctions and myotendinous regions. Electron microscopy revealed subsarcolemmal, electron-dense plaques associated with the membrane only in those regions where vinculin was localized by immunofluorescence. Using antivinculin antibody and protein A conjugated to colloidal gold, we found that the electron-dense subsarcolemmal densities in the tonic fibers of the ALD contain vinculin; no other structures were labeled. The basal lamina overlying the densities appeared to be connected to the sarcolemma by fine, filamentous structures, more enriched at these sites than elsewhere along the muscle fiber. Increased amounts of endomysial connective tissue were often found just outside the basal lamina near the densities. In tonic ALD muscle fibers, the subsarcolemmal densities were present preferentially over the I-bands. In partially contracted ALD muscle, subsarcolemmal densities adjacent to the Z-disk appeared to be connected to that structure by short filaments. We propose that in the ALD muscle, through their association with the extracellular matrix, the densities stabilize the muscle membrane and perhaps assist in force transmission.

The latissimus dorsi muscle of the chick is composed of anterior and posterior slips. The anterior latissimus dorsi (ALD)[1] muscle of the adult is made up largely of tonic muscle fibers, and the posterior latissimus dorsi (PLD) muscle is made up entirely of twitch muscle fibers (19, 44). The intracellular and surface membranes of tonic and twitch muscle fibers have distinctive properties. In the twitch myofibers the sarcoplasmic reticulum is far more extensive and uniformly distributed than in the tonic myofibers (42). Also, the Ca^{2+} uptake system of twitch muscle fibers is better developed and more efficient than in the tonic myofibers. The transverse

tubular system (T-system) of the twitch muscle fibers is likewise more extensive and more uniformly distributed than in the tonic myofibers (42). In the twitch muscle fiber, elements of the T-system encircle each sarcomere of the myofibril at the A-I band junction whereas in the tonic muscle fiber the T-system is more randomly distributed, and is often present only at the Z-disk (17, 23, 24, 42, 50). Mitochondrial content and oxidative enzyme activity are considerably higher in the tonic myofibers than in twitch myofibers. The tonic ALD and twitch PLD myofibers also have strikingly different sarcolemmal properties. Muscle fibers of the PLD receive a single "en plaque," focal innervation, and the sensitivity of the myofibers to acetylcholine receptor agonists applied in the bath is low (12, 16). In contrast, the tonic ALD muscle fibers receive

---

[1] *Abbreviations used in this paper:* ALD and PLD, anterior and posterior latissimus dorsi, respectively.

multiple "en grappe," innervation at ~750-μm intervals along their length (16) and respond to receptor agonists applied in the bath with prolonged contracture (12, 16). The tonic ALD and twitch PLD myofibers also differ in their transverse membrane resistances and their membrane time constants by ~10-fold and in their length constants by more than twofold (12).

The factors that affect the distinctive physiological properties of the sarcolemma in chicken ALD and PLD myofibers are poorly understood. Although it is clear from a variety of studies (21, 24, 25) that neural influences on these properties may be strong, the way in which a muscle fiber alters its sarcolemma in response to stimulation and use is still unknown. We have begun a series of immunochemical and morphological studies of the sarcolemma in the chick ALD and PLD muscle fibers. Here we report some significant differences in the membrane-associated cytoskeletal protein, vinculin (14), and its relationship to the contractile apparatus and to extracellular materials closely apposed to the sarcolemma.

MATERIALS AND METHODS

In this study we used ALD and PLD muscles from 33 adult (120 to 365 d old) laboratory-reared female White Leghorn chickens (germ-free fertilized eggs purchased from SPAFAS, Inc., Lanchester, PA). Newly hatched chicks were transferred to battery brooders and given food and water ad lib. The birds were killed with an overdose of nembutal (Abbott Laboratories, North Chicago, IL), and both the ALD and PLD muscles were immediately removed.

Electron Microscopy: For electron microscopy, the muscles were exposed, dissected free of surrounding connective tissue, and fixed in situ for 5–15 min with cold (4°C) 2.5% glutaraldehyde fixative buffered by phosphate at pH 7.2 and containing 0.54% glucose. The muscles were then tied to an applicator stick, removed, and placed in the larger volume of the same fixative at 4°C for 2 h more. We were careful not to damage the myotendinous ends of the muscle. After fixation each muscle was divided into three parts (origin, belly, and insertion). Each part was minced, washed briefly in cold (4°C) 0.1 M phosphate buffer and postfixed for 2 h in 1% OsO₄ buffered to pH 7.2 with 0.1 M phosphate buffer and containing 0.54% glucose. The muscles were either dehydrated in ethanol and embedded in Araldite (CIBA, CY 212) or stained en bloc with 2% uranyl acetate in 50% ethanol and then dehydrated and embedded. We allowed some muscles to shorten by ~10–30% of their total absolute rest length in situ i.e., that length at which maximum isometric tension is obtained (43), pinned transversely as described by Pierobon-Bormioli (37), and then tied to an applicator stick. The shortened muscles were then fixed in 1% glutaraldehyde in the presence of 0.5% saponin and 2% tannic acid, as described by Maupin and Pollard (29), or fixed in a 1:1 mixture of phosphate-buffered 1% OsO₄ and zinc iodide (37). The saponin-tannic acid treated muscles were postfixed with buffered OsO₄ and rapidly rinsed in phosphate buffer, dehydrated, and embedded in Araldite. The osmium-zinc iodide–treated muscles were rinsed, dehydrated, and embedded in Araldite without postfixation. Semithin sections were cut and evaluated by light microscopy. Ultrathin sections that showed a gray interference color were taken from each part (origin, belly, and insertion), collected on naked grids, stained with uranyl acetate and lead citrate solutions (39), and examined with a Philips EM201 or EM410 electron microscope.

To determine whether the distribution of subsarcolemmal dense plaques could be correlated to the subjacent myofibrillar organization, we measured directly the absolute length of each plaque from electron micrographs of myofibers taken from the belly of the ALD. The absolute length of the sarcolemma that overlay 30 adjacent sarcomeres (not interrupted by grid bars) from 20 muscle fibers ($n = 600$) was measured directly from electron micrographs, and the length of sarcolemmal membrane associated with electron dense regions seen immediately over either the I-Z-I band, or the A-band was determined and expressed as a percentage of the total measured sarcolemmal length.

Ultrastructural Localization of Vinculin with Protein A-Gold: We used the protein A-gold technique described by Bendayan and Zollinger (3) to label the vinculin antigenic sites on osmium-fixed or postfixed myofibers. The staphylococcal protein A-gold complex was obtained directly from Polysciences Inc. (Warrington, PA; 20-nm gold particles) or prepared as

described by Bendayan et al. (4). The antibody to vinculin has been described elsewhere (7). Ultrathin sections showing dark gold interference were taken from tissue prepared and embedded as described above. The sections were mounted on uncoated nickel grids, treated with periodate as described by Bendayan and Zollinger (3), and then reacted with antivinculin. After a rapid wash in phosphate-buffered saline, they were incubated with the protein A-gold complex for 30 min at room temperature. They were then thoroughly washed in phosphate-buffered saline, rinsed in distilled water, and dried. Staining with uranyl acetate and lead citrate was performed before examination with a Philips EM201 electron microscope. Control experiments carried out to demonstrate the specificity of labeling included the use of nonimmune IgG and incubation with protein A-gold alone.

Frozen Sections: For immunochemistry, the muscles were dissected, placed on a small sheet of aluminum foil, and frozen in hexane precooled in a dry ice-acetone bath. Pieces (~3 × 5 × 8 mm) were trimmed, mounted on a brass block and sectioned on a SLEE cryostat (South London, U.K.). For preparation of longitudinal sections, muscles were pinned at their in situ resting length to an applicator stick, fixed in buffered 4% formaldehyde for 5 min, placed in 30% sucrose for 1–3 h, and then frozen (see above). 4-μm sections were picked up with a glass slide, allowed to dry at room temperature, then stored with dessicant at −70°C.

Immunofluorescence: Frozen sections on glass slides were thawed, stained with affinity-purified antibodies to chicken gizzard vinculin, washed, and counterstained with fluoresceinated goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA) diluted 1:900. Some sections were costained with tetramethylrhodamine-labeled α-bungarotoxin (38). All staining was performed in solutions consisting of 10 mM NaP, 145 mM NaCl, pH 7.2, and containing 1% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, MO). After they were stained samples were mounted in a solution consisting of nine parts glycerol to one part 1 M Tris-HCl, pH 8.0. Samples were viewed with epifluorescence, Nomarski, and phase-contrast optics on either a Zeiss IM35 or Zeiss M5 microscope. For fluorescence microscopy, photographic exposures ranged from 15 to 30 s. HP5 film (Ilford Ltd., Basildon, Essex, U.K) was processed to an ASA of 1200 using the Ilford developer, Microphen.

Gel Electrophoresis and Electroblotting: SDS PAGE was performed according to Laemmli (27). We obtained muscle samples by removing the ALD and PLD from adult birds, as above, and quickly freezing them in hexane cooled in a dry ice-acetone bath. The muscles were lyophilized. Dried pieces from the central regions were removed, weighed, and boiled in sample buffer (0.5 ml/mg dry wt of tissue) preparatory to electrophoresis (27). Aliquots containing the equivalent of 10–100-μg dry wt of muscle were applied to polyacrylamide gels. After electrophoresis, gels were either stained with silver (34) or electroblotted onto nitrocellulose (10) using a Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Nitrocellulose blots were reacted first with affinity-purified rabbit antivinculin (0.12 μg/ml) and then with ¹²⁵I-protein A at 2.5 μCi/ml (New England Nuclear, Boston, MA) using slight modifications of published methods. Autoradiography revealed bands reactive with the antibody and protein A. Standard proteins, including vinculin as a positive control, were always electrophoresed and blotted as controls.

RESULTS

Immunofluorescence

Our initial experiments used rabbit antivinculin and fluoresceinated goat anti-rabbit IgG to reveal sites of vinculin enrichment in chicken ALD and PLD muscles. As illustrated in Figs. 1–3, we found several regions of intense antivinculin staining. (a) In the belly of the ALD, the large diameter, tonic myofibers stained brightly around their peripheries but only slightly in their intracellular regions (Fig. 1 A, fiber L). (b) Myofibers at the myotendinous junction of the PLD showed intensive antivinculin staining (Fig. 1, B and C, arrows). (c) As also reported elsewhere (7), we found the neuromuscular junction of the PLD to be rich in vinculin (Fig. 2, A and B). The neuromuscular junction of the tonic fiber of the ALD was also rich in vinculin but was apparently no more enriched in this protein than the surrounding extrajunctional region[2]

---

[2] Sanes (40) has described antigens of the neuromuscular junction as being either junctional, extrajunctional, or shared, i.e., found in both junctional and extrajunctional regions. Our findings with chicken muscle suggests that this classification may depend on muscle fiber type.
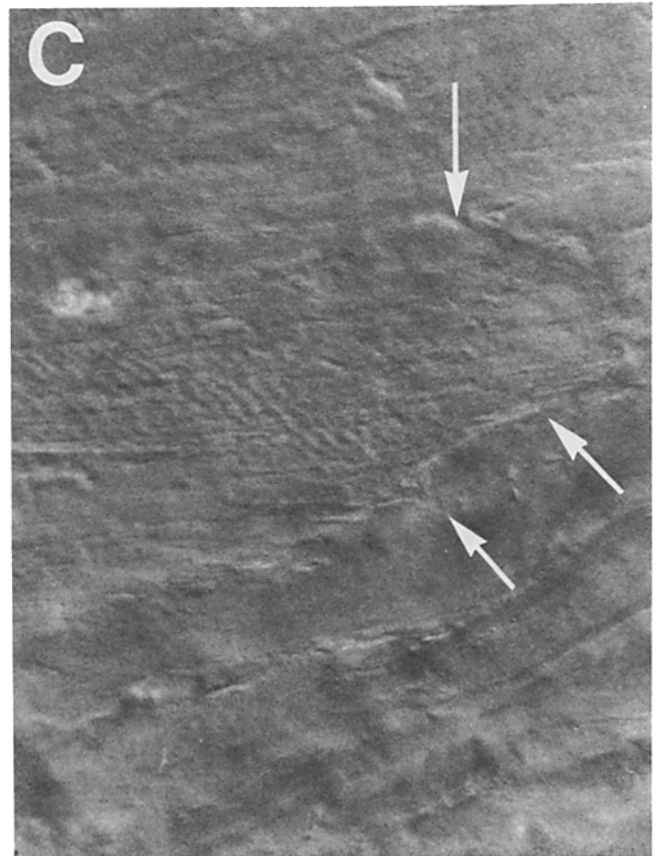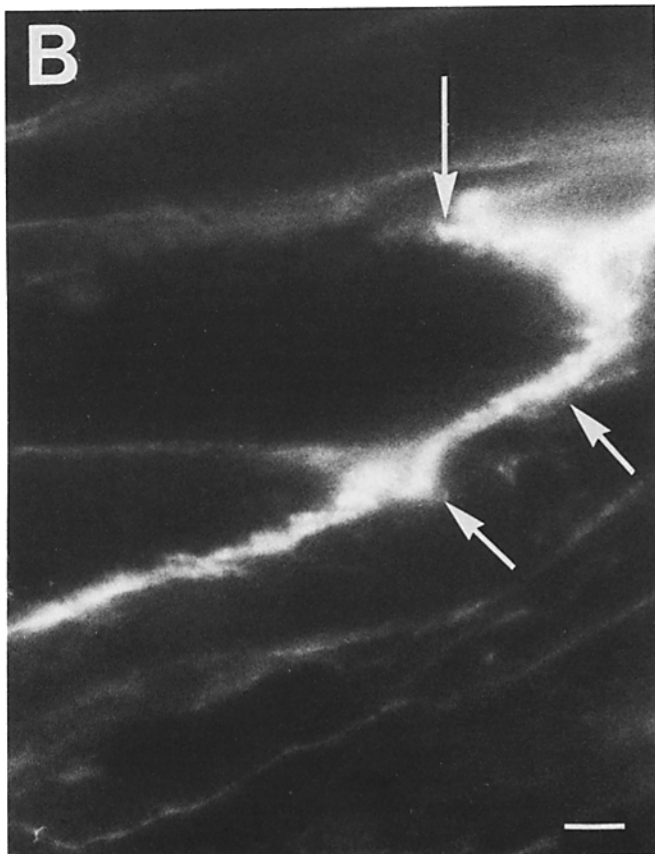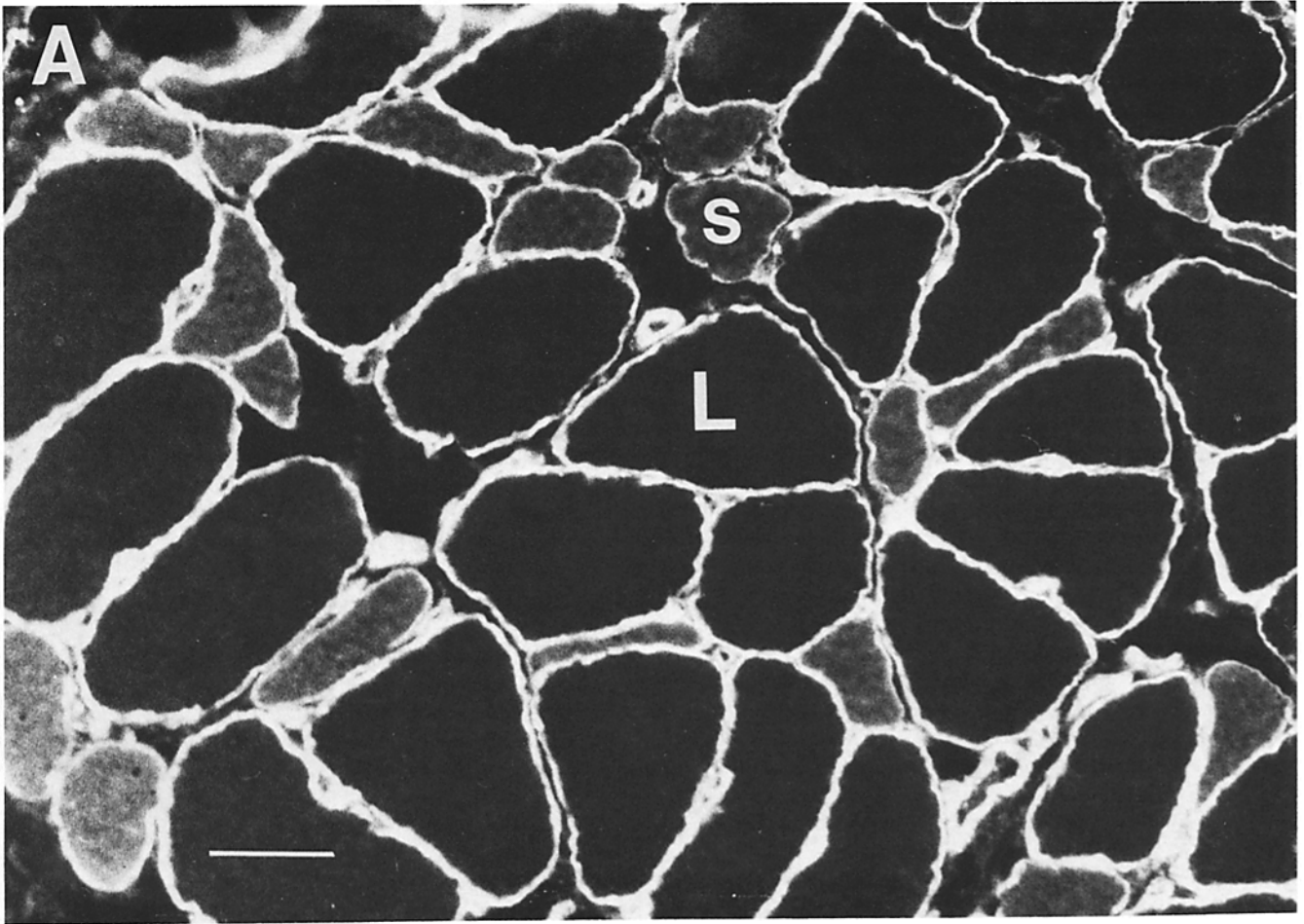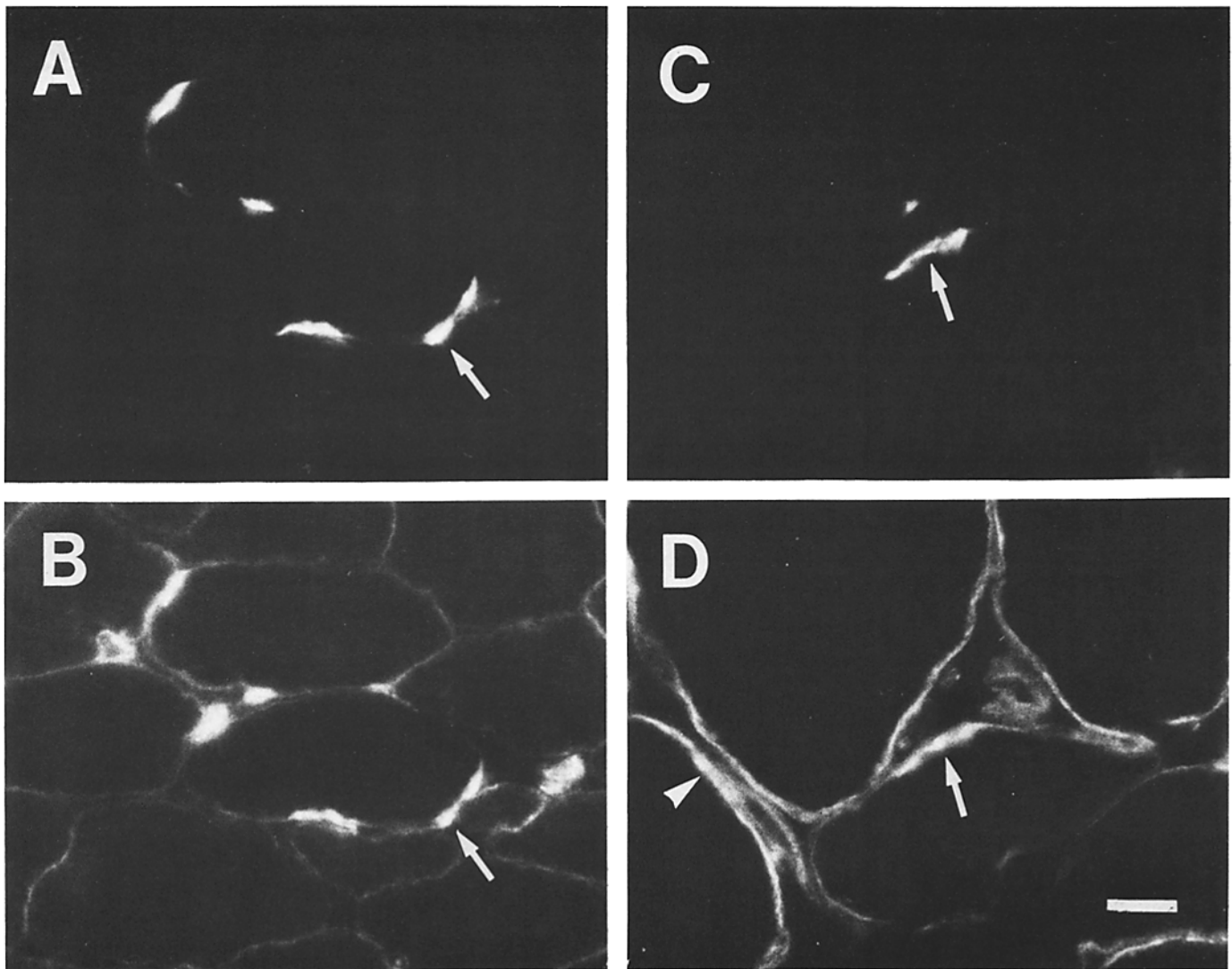
FIGURE 2 Vinculin is enriched at the neuromuscular junction. Frozen sections were cut and stained as in the legend to Fig. 1, except that tetramethylrhodamine-labeled α-bungarotoxin was added to visualize the postsynaptic regions of the neuromuscular junction (A and C). Immunofluorescent staining with antivinculin was done on the same sections (B and D). (A and B) Vinculin was enriched at the junctional regions of the PLD muscle (e.g., arrows). (C and D) Vinculin is enriched at the junctional regions of the small twitch fibers of the ALD (such as the one marked S in Fig. 1). In this case the antivinculin staining at the junction (arrow in D) is approximately as bright as the intense subsarcolemmal staining of the larger, tonic fibers of the ALD (arrowhead in D). Bar, 10 μm; × 1,100.

(not shown). The ALD muscle contains a small percentage (~15%) of small twitch myofibers (44). We found that the neuromuscular junctions of these twitch ALD myofibers were also rich in vinculin (Fig. 2, C and D). Furthermore, the intensity of antivinculin staining at the synapse was comparable to that observed anywhere around the periphery of the larger, tonic ALD myofibers. The staining observed in all of these structures was judged to be specific, as it was not seen if nonspecific IgG from antivinculin serum was used or if antivinculin antibody was first adsorbed with a 20-fold molar excess of purified gizzard vinculin (not shown). In contrast to these regions of intense antivinculin staining, the rest of the PLD stained only faintly with antivinculin. The small twitch fibers of the ALD (Fig. 1 A, fiber S) also stained less intensely

FIGURE 1 Immunofluorescent localization of vinculin in the ALD (A) and PLD (B and C) muscles of the adult chicken. ALD and PLD muscles were removed from adult chicken and immediately frozen in hexane cooled in a dry ice-acetone bath. 4-μm sections were cut on a cryomicrotome. Sections were stained first with affinity-purified rabbit antivinculin antibody (2 μg/ml), then with fluoresceinated goat anti–rabbit IgG. (A) Transverse section of the belly of the ALD muscle. Note the two fiber types, the large fibers (L), showing bright peripheral staining and little internal staining, and the small fibers (S), showing more internal and less peripheral stain. The sarcolemmal regions of the large fibers appear enriched in vinculin. (B and C) Longitudinal section of the origin of the PLD muscle, visualized by fluorescence (B) and Nomarski (C) optics of the same section. Note that the muscle fibers and the endomysial connective tissue stain poorly with antivinculin but that the interface, the myotendinous junction, stains brightly (arrows). Thus, the myotendinous junction also appears rich in vinculin. Bars: A, 40 μm; B, 10 μm. A, × 440. B, × 850.

FIGURE 3 A longitudinal section shows immunofluorescence localization of vinculin in an extended tonic myofiber from the belly of the ALD muscle. A demonstrates vinculin localized in discrete bands at the periphery of the myofiber (arrows). B is a phase-contrast micrograph of A. Arrows in both A and B point to the same regions, indicating that vinculin is enriched over the I-bands. Note that there is less intense fluorescent staining over the Z-disk at the center of the I-band. Bar, 5 μm; × 2,000.
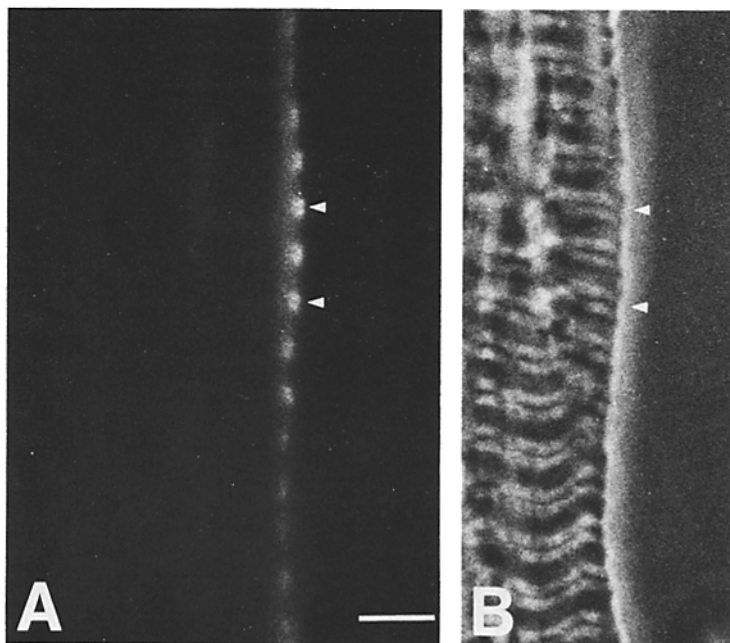
with the antibody. These results suggest that vinculin near the sarcolemma is enriched at distinct locations within chicken ALD and PLD muscles.

We examined longitudinal sections through the belly of the ALD to learn if vinculin is uniformly enriched along the sarcolemma. Our results (Fig. 3) show that the immunofluorescence is strongest over the I-bands of the tonic ALD myofibers and weak over the A-bands. We also observed a weaker signal from the myofiber surface overlying the Z-disk, but, because the Z-disks of the tonic ALD myofibrils are irregular, we saw this only occasionally (Fig. 3). These results suggest that along the sarcolemma, vinculin is enriched over the I-band, but may be depleted immediately over the Z-disk. These results agree with those reported by Pardo et al. (35, 36). As is the case with cross-sections, we found that the antivinculin staining of longitudinal sections of the twitch myofibers through both the ALD and PLD muscles was much fainter than that of the tonic ALD myofibers, except near the neuromuscular junctions and myotendinous ends of the myofibers.

Electron Microscopy

SUBSARCOLEMMAL DENSITIES: We used electron microscopic techniques to learn if particular ultrastructural features are associated with sites of intense antivinculin staining. We found that at all locations within the ALD and PLD muscles where vinculin appears to be enriched, distinctive electron-dense plaques were present on the cytoplasmic side immediately adjacent to the sarcolemma. We refer to these as subsarcolemmal densities. These structures are illustrated in the middle part of a tonic fiber from the belly of the ALD muscle (Fig. 4). We did not see subsarcolemmal densities in extrajunctional regions along most of the length of the twitch myofibers from either the PLD or the ALD muscles (Fig. 5). We did, however, observe subsarcolemmal densities near the myotendinous junctions of all muscle fibers examined (i.e., within 1–2 mm of the myotendinous junction of the twitch myofibers from ALD and PLD and in tonic myofibers from the ALD; Fig. 6). As illustrated in Fig. 7, similar subsarcolemmal densities were present at the postsynaptic membrane of

neuromuscular junctions of the twitch PLD myofibers. We did not see densities in either twitch or tonic myofibers along regions of the sarcolemma subjacent to satellite cells (Fig. 4), beneath the cell membrane in Schwann cells, or in presynaptic terminals, with the possible exception of the densities associated with active zones (Fig. 7).

The subsarcolemmal densities have a close but mutually exclusive spatial relationship with caveolae, both in the central regions of the tonic myofibers from the ALD and near the myotendinous junctions of tonic and twitch fibers of both the ALD and PLD muscles. This is illustrated in Figs. 4, 6 b, 8, and 11a. The caveolae appeared to be situated between subsarcolemmal densities. Densities were never associated with membranes of caveolae. We also observed caveolae at the neuromuscular junctions of the ALD and PLD, where they were present much less frequently and were similarly excluded from regions displaying subsarcolemmal densities.

To determine more precisely if the subsarcolemmal densities contain vinculin, we performed ultrastructural immunocytochemical localization experiments. We etched both surfaces of ultrathin plastic-embedded sections prepared through the belly of the ALD muscle with a saturated solution of periodate (3) to reveal vinculin antigenic sites. The etched grids were then reacted with antivinculin antibody and protein A conjugated to colloidal gold. Although some sections reacted well with the immunocytochemical probes, other sections did not react at all. We cannot explain this variability. In all the regions and sections that reacted, however, the only structures that labeled with antivinculin and protein A-gold were the subsarcolemmal densities (Fig. 9). We saw no significant labeling of the extracellular matrix or of the contractile apparatus. We never saw labeling of the subsarcolemmal densities in periodate-etched sections if a nonspecific IgG, as first antibody, or protein A-gold alone was used. Although the variability in protein A–gold staining remains problematic, these results suggest that the antivinculin staining we observed at the subsarcolemmal densities was specific. Based on both the distribution of the subsarcolemmal densities and the immunocytochemical labeling, we conclude that the densities are the principle sites of enriched vinculin localization

FIGURE 4 A longitudinal section shows two tonic ALD muscle fibers. Note the subsarcolemmal densities (arrowheads). Fine filamentous structures are seen in the lamina rara adjacent to the subsarcolemmal densities. For a higher magnification of a similar region, see Fig. 8. The sarcolemma underlying the satellite cell (SC) has no subsarcolemmal densities and no intervening basal lamina. × 32,700.

in chicken tonic ALD muscle fibers.

CONTRACTILE APPARATUS: We made morphometric studies of the ALD to learn if the distribution of the subsar-

colemmal densities was related to the underlying contractile structures. We found that the subsarcolemmal densities more frequently overlay the I-bands than the A-bands of the sub-
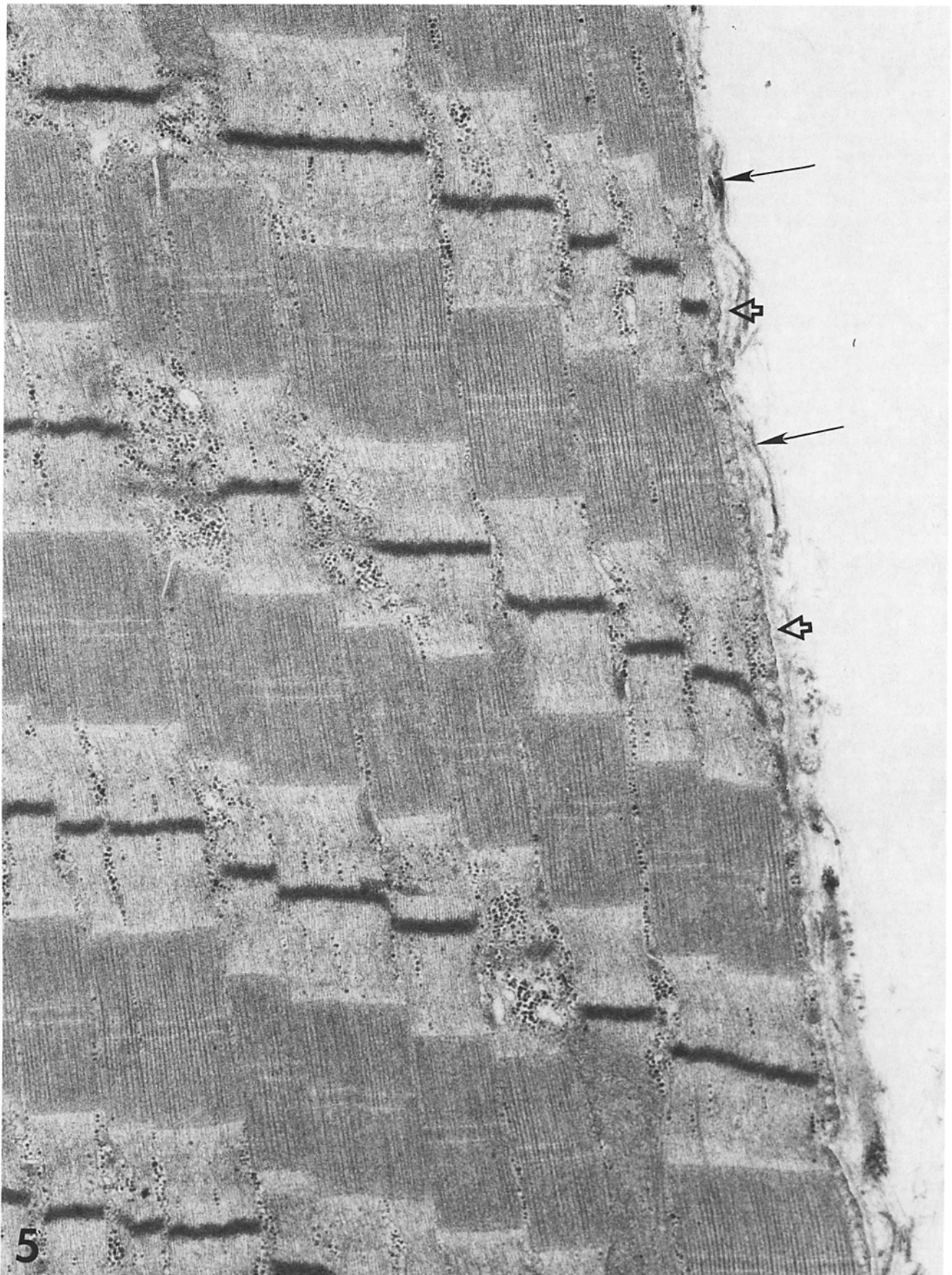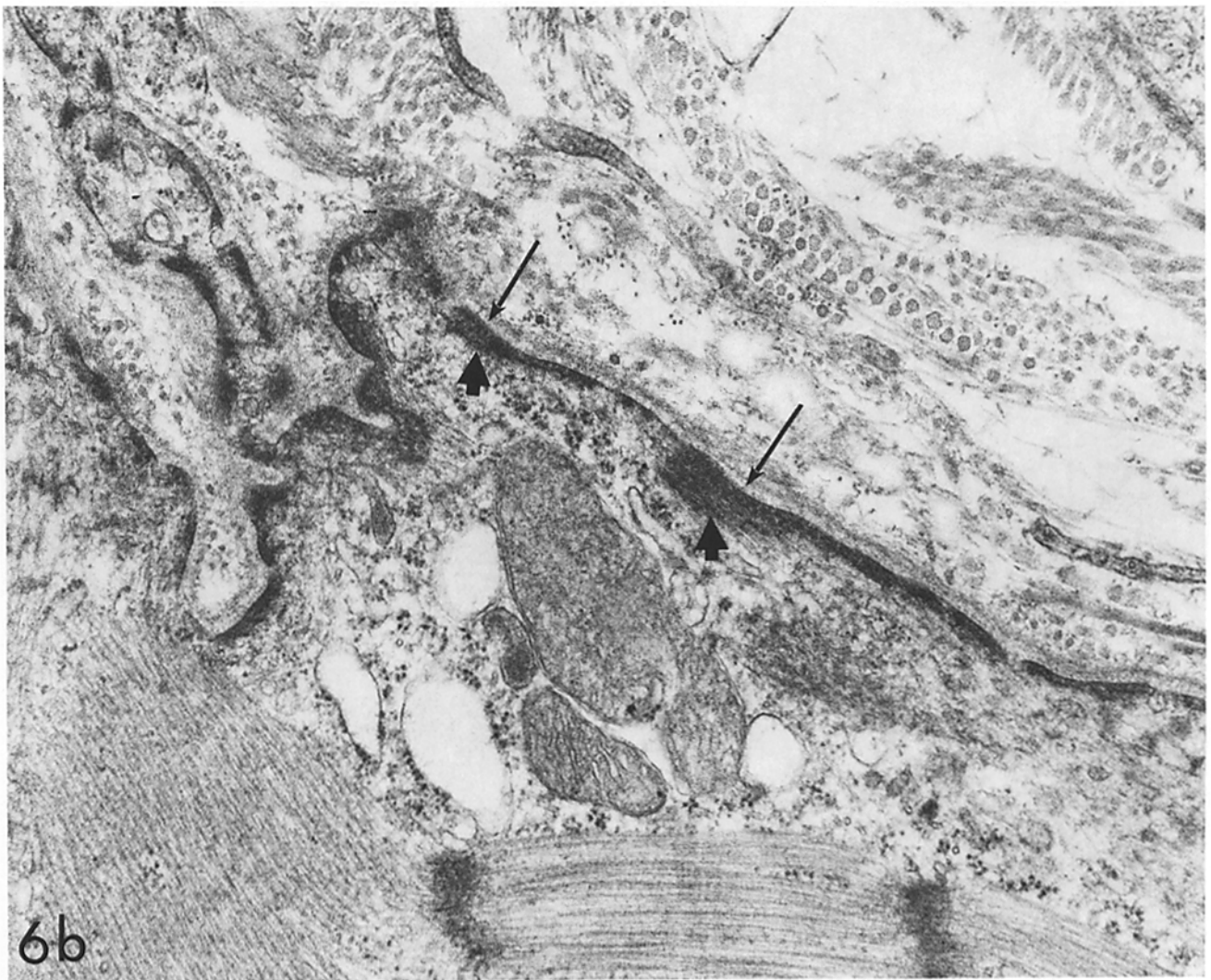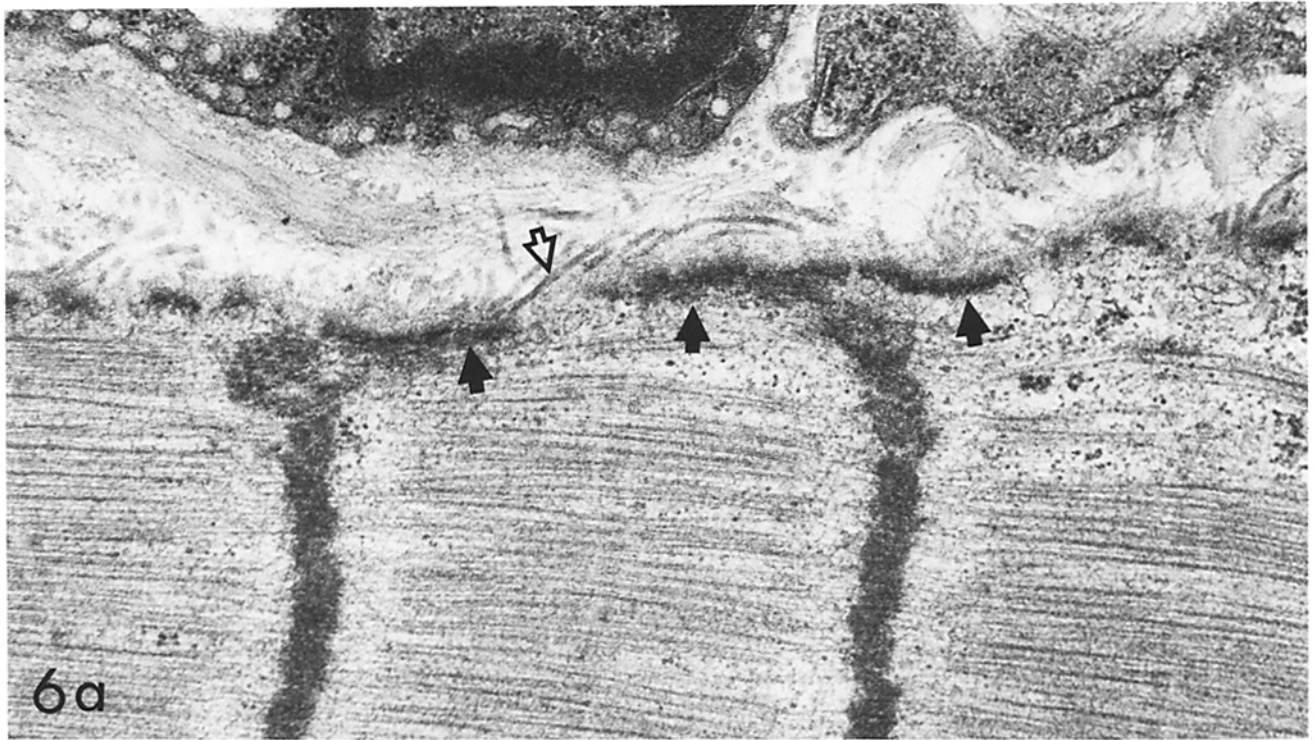
FIGURE 5   A longitudinal section shows a twitch ALD muscle fiber. Subsarcolemmal densities are not seen. Endomysial collagen fibers (closed arrows) and the lamina densa (open arrows) are indicated. × 40,000.
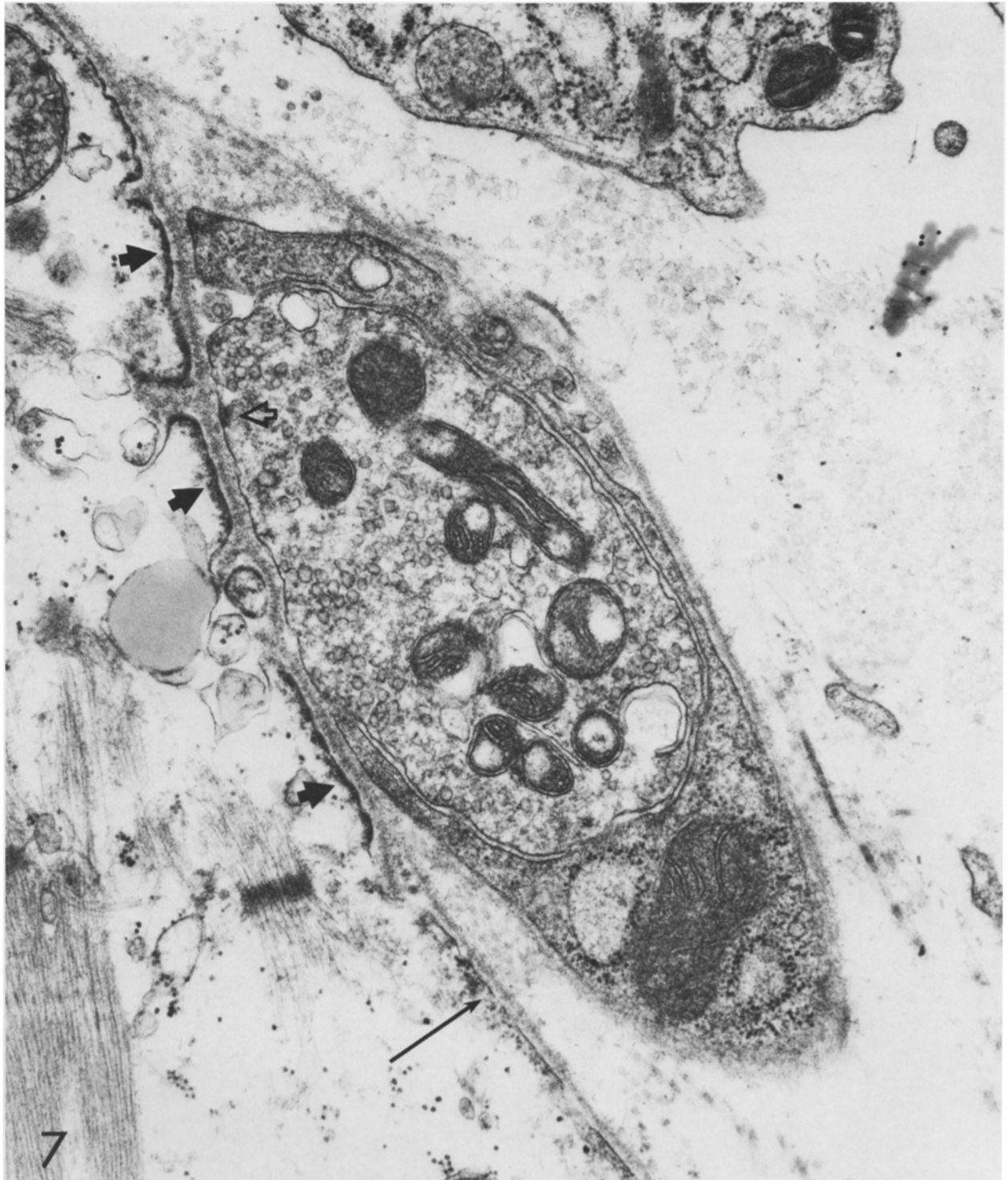
6a

6b

FIGURE 7   Neuromuscular junction of a twitch PLD muscle fiber treated with saponin and tannic acid. Subsarcolemmal densities (large solid arrows) are confined to the postsynaptic membrane and do not occur along the extrajunctional basal lamina (thin solid arrow). In twitch myofibers, the neuromuscular and the myotendinous junctions are the only regions that have subsarcolemmal densities (compare Figs. 5 and 6). The smaller density associated with the presynaptic membrane (open arrow) probably represents an active zone. × 39,000.

FIGURE 6   (a) Longitudinal section of a twitch ALD muscle fiber near the myotendinous junction. In twitch muscle fibers the subsarcolemmal densities (large arrows) are seen at the myofiber ends and the neuromuscular junction (Fig. 7) but not elsewhere (compare Fig. 5). Endomysial collagen fibers (open arrow) often appear more closely applied to the basal lamina near the subsarcolemmal densities. A distinct lamina rara interna cannot be seen. × 55,200. (b) Longitudinal section of a tonic ALD muscle fiber within the myotendinous junction. The subsarcolemmal densities are seen (closed arrows). The lamina rara interna (small arrows) has many thin filamentous structures associated with the subsarcolemmal densities. Caveolae are seen in many of the regions that lack subsarcolemmal densities. × 52,000.
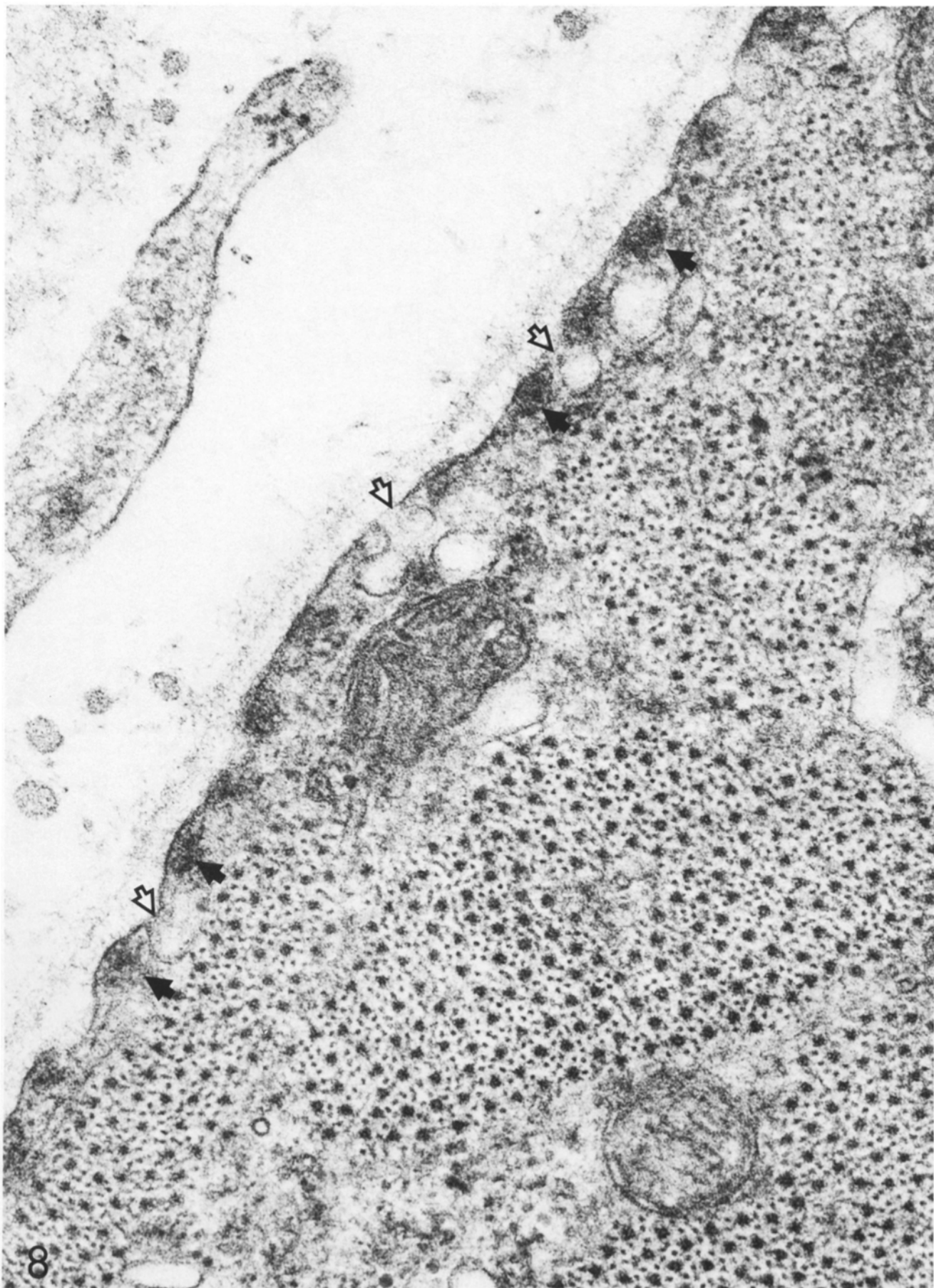
FIGURE 8 Transverse section from the central region of a tonic ALD muscle fiber. The subsarcolemmal densities are present along most of the membrane (solid arrows) except where caveolae are found (open arrows). Filamentous structures within the lamina rara interna are more numerous where they overlie the subsarcolemmal densities. × 169,000.
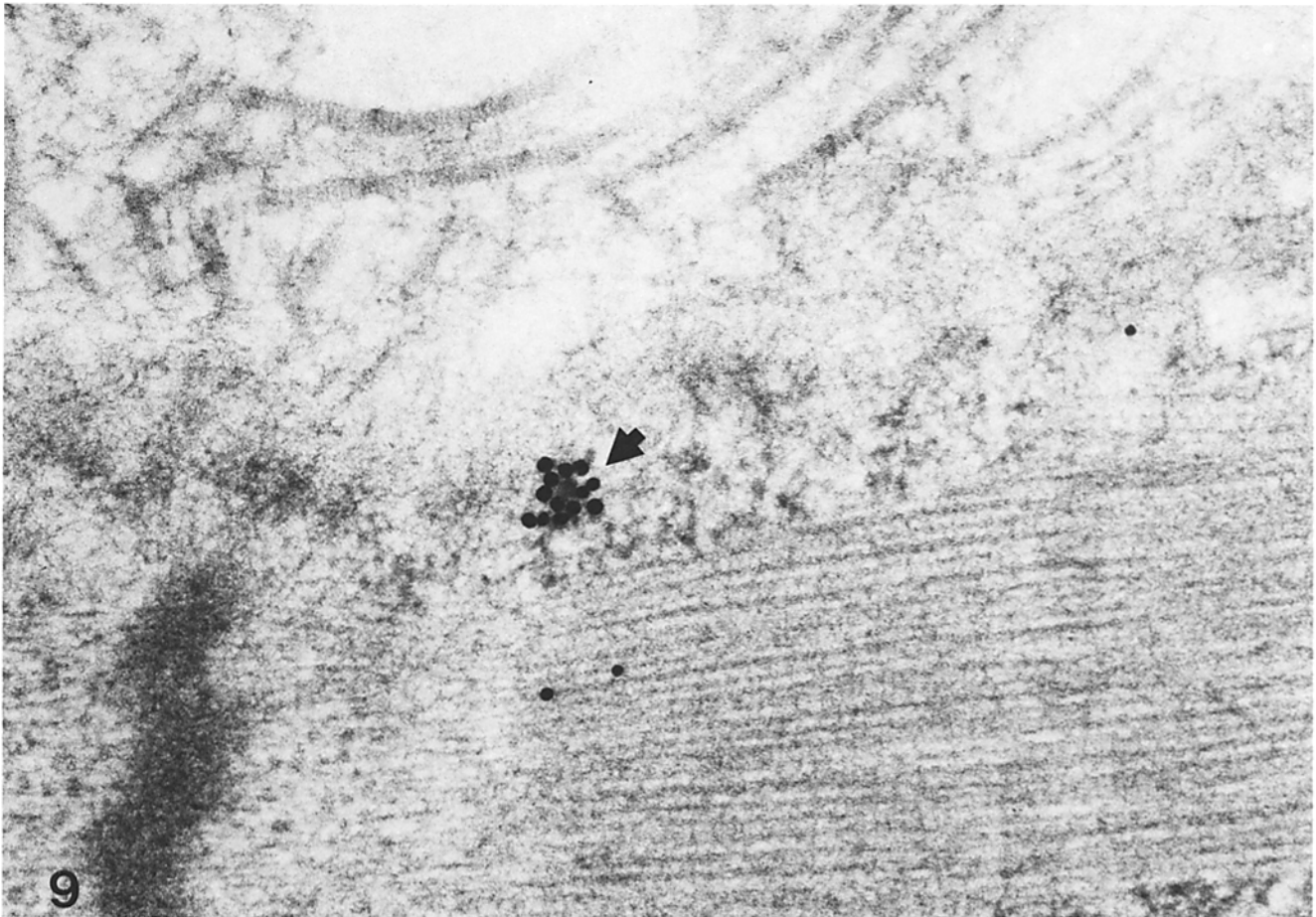
FIGURE 9 Longitudinal section of a tonic ALD muscle fiber etched with periodate and treated first with antivinculin antibody (12 μg/ml) and then with protein A coupled to gold particles of ~20 nm diam. A subsarcolemmal density labeled with colloidal gold is indicated (arrow). Staining is limited to electron-dense structures that underlie the sarcolemma. × 105,000.

jacent sarcomeres. In myofibers from adult muscles, fixed at resting length, subsarcolemmal densities occurred along 60 ± 25% of the sarcolemma overlying the I-band but only 25 ± 17% of the sarcolemma overlying the A-band. Because of the variability from one sarcomere to the next in the overlap of the densities with the A-bands, the relationship between the densities and the myofibrillar banding pattern cannot be determined precisely from any one micrograph. In addition, some isotonic fiber contraction that occurred before or during fixation may account for the presence of some subsarcolemmal densities over the A-bands in otherwise normal muscles (see below; compare Figs. 4, 10, and 11 a). The fact that subsarcolemmal densities are more frequently observed over the I-band agrees with the results obtained in immunofluorescence observations of longitudinally sectioned muscle (Fig. 3).

To investigate the relationship of the sarcolemma and the subsarcolemmal densities to the contractile apparatus of the muscle fiber, we fixed the ALD and PLD muscles at a shortened length. These muscles were then treated with glutaraldehyde, tannic acid, and saponin, and postfixed in 1% OsO₄ (29). We examined the central regions of the myofibers with the electron microscope (Figs. 10 and 11, a and b). The sarcolemma of the shortened myofibers treated in this fashion were thrown into regular folds. Street (47) reported a similar finding in shortened frog skeletal muscle and termed the folds

"festoons." In tonic myofibers treated with tannic acid and saponin the folds appeared to be attached to the underlying contractile apparatus at every Z-disk (Figs. 10 and 11 a). Regular sarcolemmal infolding was also observed in shortened twitch myofibers in the ALD (not shown) and PLD (Fig. 11 b). Unlike the tonic myofibers, however, the infolding of the sarcolemma of the shortened twitch myofibers occurred at every Z-disk and M-line. Despite these extensive attachments, subsarcolemmal electron dense plaques were seldom seen in twitch muscle fibers (Fig. 11 b).

The stable association of the sarcolemma with the Z-disk in tonic fibers suggests that there may be a structure connecting them. Pierobon-Bormioli (37) reported the presence of such connections in muscle that she first pinned transversely and then fixed with a mixture of osmium tetroxide and zinc iodide. We repeated her procedure with the ALD and PLD muscles and found that the fixative caused extensive damage and significant change to the muscle ultrastructure. We therefore repeated the experiment, but we combined Pierobon-Bormioli's transverse pinning with Maupin and Pollard's fixation protocol (29). In this case (Fig. 11 a, inset) we observed irregular filamentous connections between the Z-disks and the subsarcolemmal densities adjacent to the Z-disk in tonic ALD myofibers. We saw no such filaments in twitch muscle fibers. Connections between the Z-disks and M-lines to the sarcolemma in twitch fibers appeared to involve the
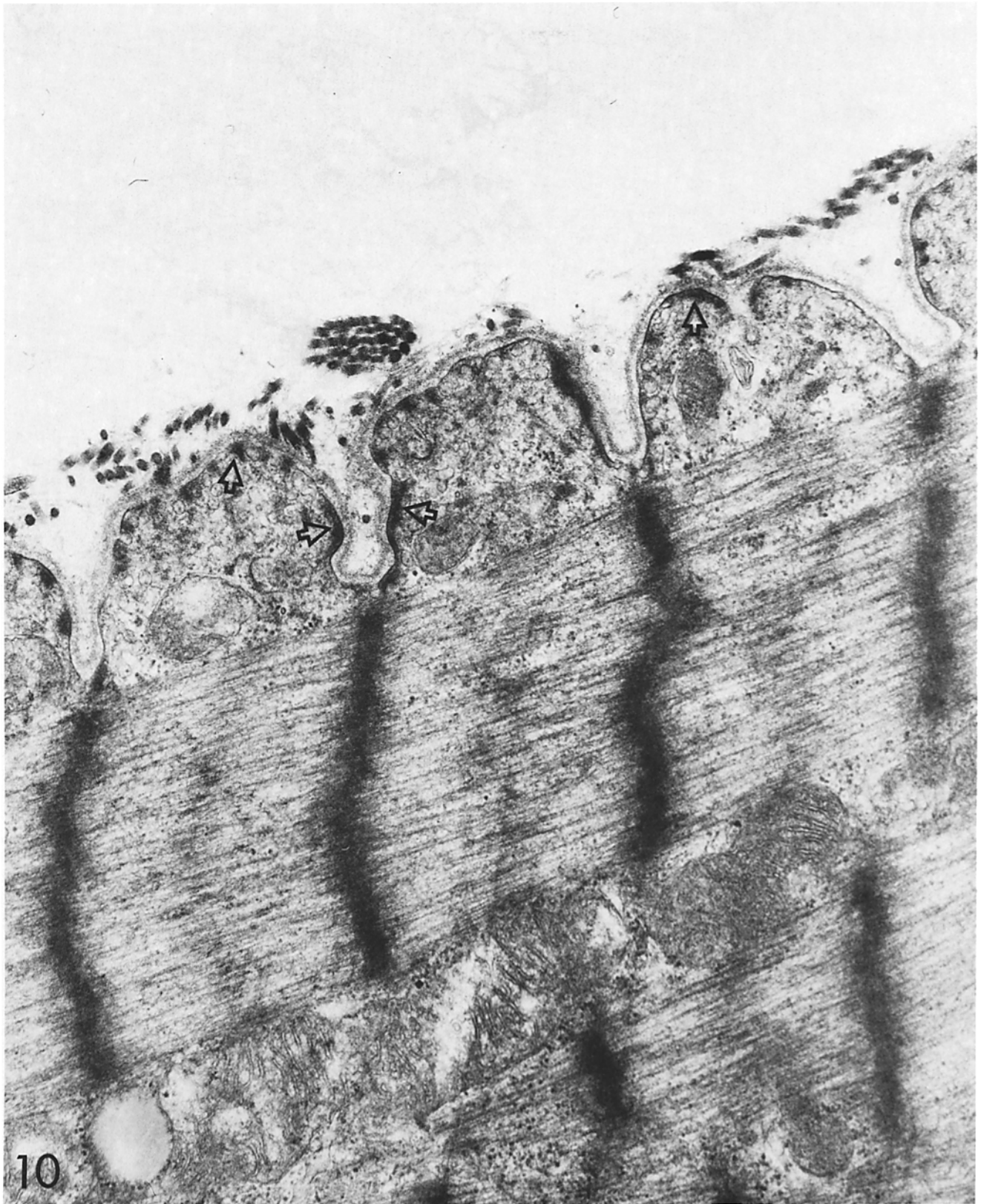
FIGURE 10 Longitudinal section of a shortened tonic ALD muscle fiber that shows festooning of the sarcolemma. In shortened tonic fibers, the sarcolemma appears closely applied to the Z-disk at all sarcomere lengths (compare Fig. 11 a). Densities are indicated by open arrows. × 45,000.

fenestrated collar or other membranous elements associated with the sarcoplasmic reticulum (Fig. 11 b).

EXTRACELLULAR MATRIX: Vinculin in other cells is

associated with sites of insertion of microfilaments and extracellular matrix into the plasmolemma (14, 46). Above we described some intracellular structures associated with densi-
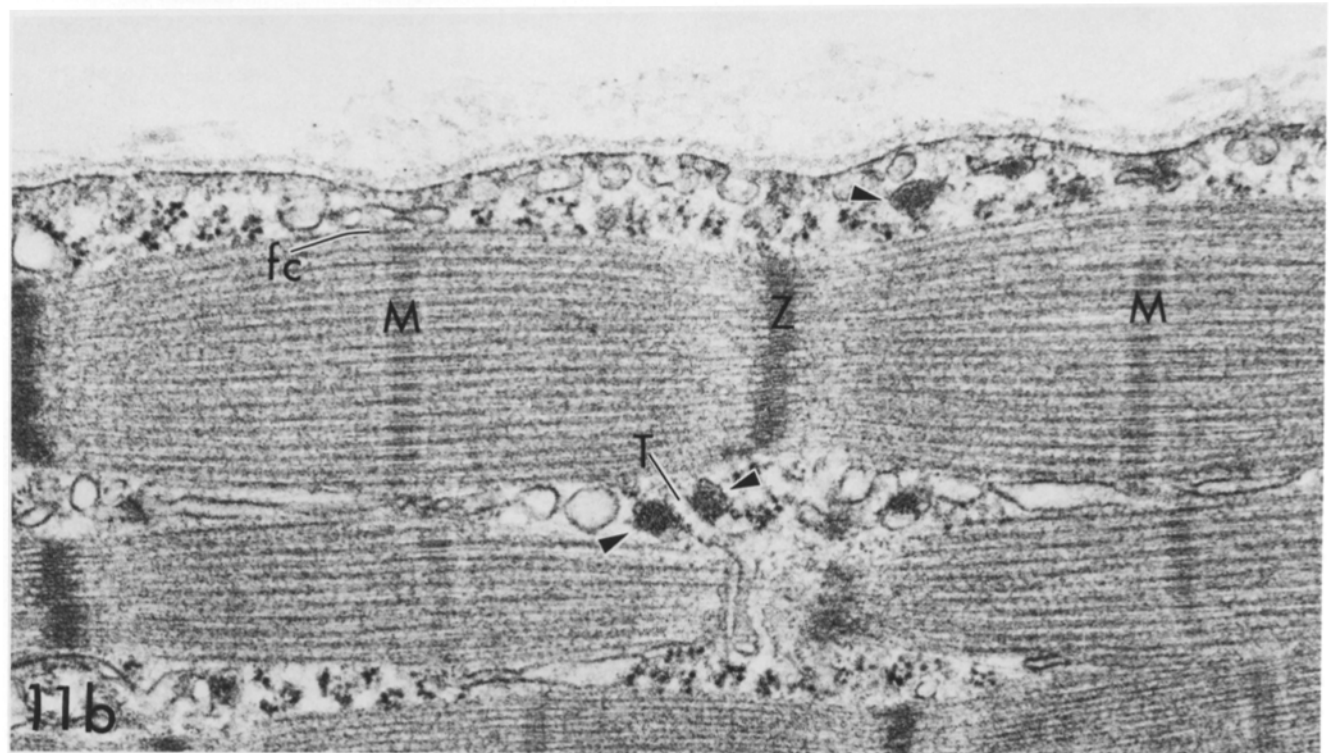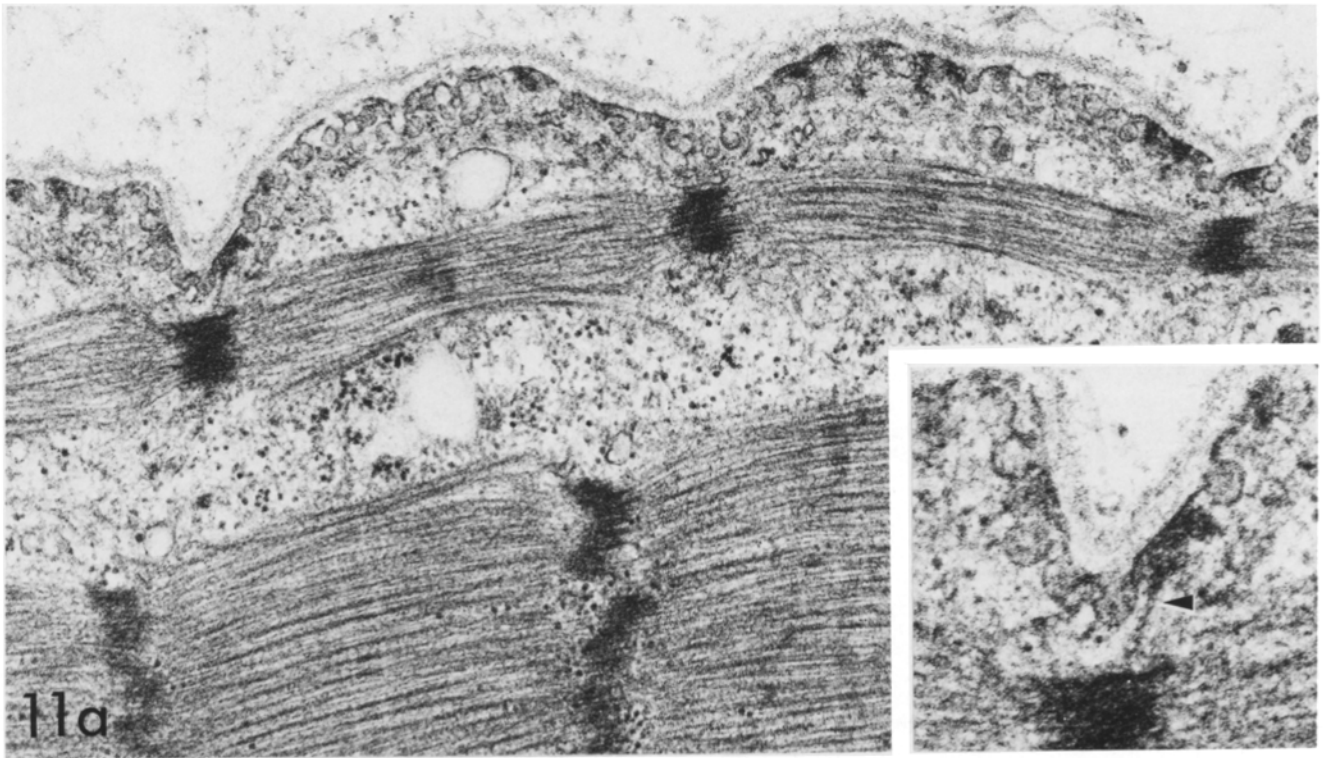
FIGURE 11 (a) Longitudinal section of partially shortened tonic ALD muscle fiber treated with saponin and tannic acid and held both longitudinally and transversely during fixation. Cables of irregular thickness (inset, arrow) are seen between the subsarcolemmal densities and the Z-disk in myofibers prepared in this fashion. Note that the sarcolemma is not closely applied to the sarcomere at the M-line region of the subjacent sarcomere. × 49,000. inset, × 99,000. (b) Longitudinal section of a shortened twitch PLD muscle fiber treated as above. Subsarcolemmal densities are absent. The sarcolemma appears more closely applied to the contractile apparatus at both the Z-disk (Z) and M-line (M) regions of the sarcomere. The membrane-bound dense structures represent profiles of terminal cisternae (arrowheads) that belong to the sarcoplasmic reticulum (SR). Flattened saccule-like profiles (fc) represent the fenestrated collar of the SR and are usually seen between the M-line and the sarcolemma. A T-tubule (T) and associated terminal cisternae (arrowheads) are also indicated. Compare the pattern of festooning in this twitch myofiber to that of the tonic myofibers in a and in Fig. 10. × 67,000.

ties. We also examined the subsarcolemmal densities to learn if they were associated in any unique way with extracellular material. In muscles treated with standard and 2.5% glutaraldehyde and postfixed with 1% OsO₄, the extracellular material appeared enriched in the lamina rara interna that immediately overlay membrane regions associated with subsarcolemmal densities at all their loci (Fig. 8). In myofibers from the central region of the ALD muscle (i.e., the belly) this extracellular material in the lamina rara interna was filamentous and appeared to form trabeculae connecting the sarcolemma to the lamina densa. The trabeculae were ~35 to 50 nm long and 5 to 6 nm across and approached the membrane almost perpendicularly (Fig. 8). Sites of their apparent insertion into the sarcolemma were not associated with membrane specializations other than the subsarcolemmal densities. Trabeculae were less apparent in saponin-tannic acid–treated muscles.

We have been unable to detect as clear an association of lamina densa and subsarcolemmal densities at the postsynaptic region of the neuromuscular junction as we have at other regions along the fiber. At the neuromuscular junction, the lamina rara interna was always thinner and the filamentous connections were not as distinct as at other regions along the sarcolemma (Fig. 7).

Collagen fibers often appeared more enriched in the endomysium near subsarcolemmal densities than elsewhere (Figs. 4, 6, and 10). We have not, however, quantified this observation.

Immunoblotting

Two laboratories recently reported that smooth and cardiac muscles of the chicken have a second protein, termed metavinculin, which reacts with antibodies prepared against purified chicken gizzard vinculin (13, 45). To determine if such a protein might be responsible for the subsarcolemmal localization of vinculin antibodies, we solubilized segments from the belly of lyophilized ALD and PLD muscles and analyzed them by SDS PAGE and immunoblotting. Silver-stained gels, containing an equivalent amount of material (dry weight) from each muscle, showed that a band that co-electrophoresed with purified gizzard vinculin was more abundant in ALD than in PLD muscles (not shown). When electroblotting of this material was performed and the resulting blots were stained with antivinculin antibody, we found that only those bands that co-electrophoresed with purified vinculin were labeled (Fig. 12). More immunoreactive material was present in extracts of ALD than of PLD muscles. We conclude that, at least in muscle extracts denatured with SDS, antivinculin reacted preferentially with vinculin and showed little or no reaction with other polypeptides. These results also support the inference from our morphological work that the ALD muscle contains more vinculin than does the PLD muscle.

DISCUSSION

Subsarcolemmal Densities

Using light microscopic and electron microscopic immunocytochemical methods we have studied the distribution of the protein vinculin (14) in adult chicken skeletal muscle. One of our basic findings is that vinculin is enriched at discrete regions within the myofibers. In the twitch ALD and PLD muscle fibers, vinculin is present at apparently high density
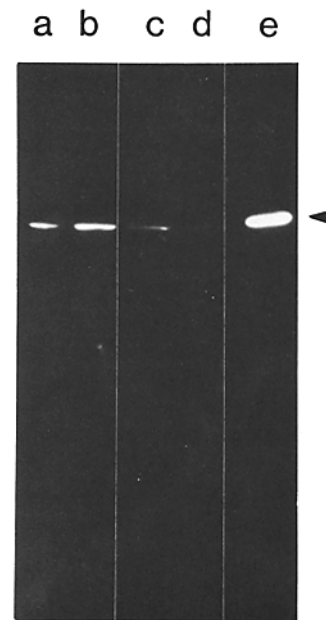

FIGURE 12 Antivinculin reacts only with vinculin in extracts of ALD or PLD. ALD and PLD muscles were frozen and lyophilized. Fragments from the central region were excised, weighed, dissolved in a boiling SDS solution, and analyzed by SDS PAGE. After electrophoresis, material was transferred electrophoretically onto a nitrocellulose sheet. The sheet was then stained with antivinculin and radioiodinated protein A. Lane a, ALD muscle, 50 μg dry wt. Lane b, ALD muscle, 100 μg dry wt. Lane c, PLD muscle, 100 μg dry wt. Lane d, PLD muscle, 50 μg dry wt. Lane e, freshly purified vinculin. The arrowhead points to the position of intact vinculin. The bands visualized in the autoradiogram of extracts of ALD and PLD co-electrophorese with purified vinculin. No other proteins react with antivinculin. Note that the PLD extract contains significantly less vinculin than does the ALD extract.

at both neuromuscular and myotendinous junctions but not in the remaining central regions of the fibers. In contrast, in the tonic myofibers of the ALD muscle, we have found high levels of vinculin at the periphery of the myofibers along their entire length. These differences in vinculin enrichment between tonic and twitch fibers were not remarked by Pardo et al. (35), although they examined both types of muscle. In a more recent report (36), however, the same group showed clear differences in fluorescent antivinculin staining intensity between nearby regions of bovine cardiac muscle. In these studies, "costameres" and tubular structures stained much less intensely than did the intercalated disk and the periphery of Purkinje fibers.

In this report we have concentrated on the regions of chicken skeletal muscle that we found to be particularly rich in vinculin. At every such region, we found structures that we have termed subsarcolemmal densities. Using immunocytochemical staining for vinculin at the ultrastructural level, we showed that at least some of the subsarcolemmal densities in the tonic ALD myofibers contained considerably more vinculin than did other sites in the muscle fiber. The variability of antivinculin staining of periodate-etched thin sections makes it impossible to conclude from these experiments alone that all, or even most, of the subsarcolemmal densities contain vinculin. Nevertheless, the close correlation between subsarcolemmal densities and sites of antivinculin immunofluores-

cence labeling, coupled with our finding that some densities do indeed contain vinculin, leads us to suggest that, where it is enriched in either twitch or tonic myofibers, vinculin is present largely within subsarcolemmal densities. This suggestion agrees with the results of Geiger, Tokuyasu, et al. (15, 48), who showed that vinculin was enriched at the fascia adherens of the intercalated disk of cardiac muscle and at the dense bodies of smooth muscle. When viewed with the electron microscope, these structures appear as dense, membrane-associated structures, perhaps analogous to the subsarcolemmal densities we have identified in tonic and twitch muscle fibers.

Another significant property of the subsarcolemmal densities is their distinctive relationship to other sarcolemmal structures. In the central regions of the fibers (i.e., the myofiber belly), and also near the myotendinous junction, the densities are found only between caveolae. At the postsynaptic region of the neuromuscular junction, the densities are continuous and almost unbroken, and few caveolae are seen. Thus, the densities may define the plasmolemmal domains that may or may not invaginate. Equally striking, the densities are associated on their extracellular aspect with fine, filamentous structures that extend from the sarcolemma through the lamina rara interna to the lamina densa. In both tonic and twitch myofibers, the extracellular threadlike structures lie within the lamina rara interna almost perpendicular to the sarcolemma above subsarcolemmal densities. Even at the neuromuscular junction, we occasionally observed threadlike connections within the lamina rara interna between the postsynaptic membrane and the lamina densa, although the additional thickness of the lamina densa, characteristic of this region, obscured much of the fine structural detail within the lamina rara interna. Such associations between the postsynaptic sarcolemma and the lamina densa have been reported previously in skeletal muscles from other species (see, for example, reference 20).

Other workers have described densities, similar to those described in the present study, in a wide variety of striated muscles, including those from guinea pig (18), rat (26), frog (31), lamprey (30), hagfish (26), and man (8). Nakao's observations (30) on lamprey skeletal muscle are particularly relevant. In lamprey muscle, the subsarcolemmal densities and their associated threadlike connections to the lamina densa of the basal lamina are found at the myotendinous ends of both tonic and twitch fibers, but at parts of the myofiber away from the tendon the densities persist only in the tonic fibers. The chick skeletal myofibers described here demonstrate the same characteristics. At the myotendinous junction in mouse twitch skeletal muscle, Trotter et al. (49) reported filamentous "microspike"-like structures in the lamina rara interna at the ends of the myofibers but not elsewhere along the basal lamina. In that study, the sarcolemma still transmitted force to the tendon after detergent treatment. The microspike-like structures described by Trotter et al. appear identical to the filamentous structures we have observed in the lamina rara interna, at both the center and the myotendinous junction of the tonic muscle fibers.

Relationship to the Contractile Apparatus

According to the sliding filament theory of muscle contraction (for review see reference 22), arrays of interdigitating thick and thin myofilaments form the cores of the myofibrils.

As no other tension-bearing cellular components have yet been described, it is generally accepted that the myofibrils form unbroken columns running from end to end of the muscle fibers, generate tension, and transfer it to the tendons through the terminal arrays of thin myofilaments that insert on the myotendinous junction. Until recently, direct evidence for tension transmission through structures other than the myotendinous junction was scarce (for review see reference 5). However, the work of Faulkner et al. (11) and Street (47) provides strong evidence that lateral transmission of both active and resting muscle tension across intact fibers to the extracellular connective tissue layer occurs in many skeletal muscles.

Several groups have been investigating the nature of the structures that connect the contractile apparatus to the cell surface. Nelson and Lazarides have proposed that such connections may be mediated in part by the cytoskeletal protein, spectrin (32, 33), which is enriched around the periphery of myofibers, particularly at the Z-disk. Pardo et al. (35, 36), on the other hand, have proposed that vinculin is localized at the sarcolemma in structures called costameres which flank the Z-disk. Many of our results agree with those of Pardo et al. (35). Immunofluorescence of frozen longitudinal sections clearly shows an enrichment of vinculin over the I-bands, identical to the costameres described by Pardo et al. (35). Electron microscopy shows that the sarcolemma of the tonic muscle fibers overlying the I-band region has more than twice the number of associated electron dense plaques than is seen over the A-bands of the same myofiber.

Pardo et al. (35, 36) have suggested that vinculin is one of the elements of a sarcolemmal lattice and serves to integrate the contractile apparatus with the sarcolemma during lengthening and shortening of the muscle fiber. Street (47) and others (see reference 35 for a discussion) have argued that connections between the contractile apparatus and the sarcolemma can be inferred from the pattern of cell surface bulges, or "festoons," seen when the muscle is highly contracted. In contracted frog twitch muscle, for example, the plasma membrane bulges or festoons between the Z-disk and the M-line but remains closely associated with the underlying contractile apparatus at these two distinct regions. We have observed that the festoons of shortened tonic ALD muscle fibers are attached to the myofiber membrane by distinct but irregular cables only at the Z-disks, with no close connections elsewhere in the sarcomere. Such connections are seen only when the muscles are treated with saponin and tannic acid, held at a constant length, and pinned transversely during fixation. Since the subsarcolemmal densities we described are usually associated with the I-band, and oblique cables are sometimes seen between the densities and the Z-disks, it is possible that, in tonic fibers, these densities attach the membrane to the myofibrillar contractile apparatus.

Connections between the sarcolemma and the contractile apparatus in the central regions of twitch muscle fibers clearly differ from those in tonic fibers. Although the connections observed at the Z-disk in festooned fibers may involve vinculin, they cannot involve vinculin organized into subsarcolemmal densities.

Possible Functions for Vinculin in Densities

Whereas vinculin in subsarcolemmal densities may be involved in linking the plasma membrane to the nearby con-

tractile apparatus, which runs parallel to the membrane, it is almost certainly involved in reinforcing the attachment of the contractile apparatus to the cell periphery where it inserts at sharp angles into the membrane. This anchoring function is most clearly illustrated by the intercalated disk of cardiac muscle and by the subsarcolemmal densities of the myotendinous junction of skeletal muscle. Such anchoring probably involves linking intracellular structures to extracellular objects or lattices. Presumably, force transmission from the contractile apparatus to the tendon, in the case of the myotendinous junction, or to the neighboring cells, in the case of the intercalated disk, occurs through these densities.

The role of the densities is probably different in the belly of the ALD and at the neuromuscular junction.[3] In the belly of the ALD, attachment of the contractile apparatus to the sarcolemma appears to occur only at the Z-disk (see above). If the extracellular matrix transmits some of the contractile tension along the length of the muscle fiber (e.g., reference 47), attachment of the extracellular matrix to the sarcolemma would help to distribute the tension more uniformly around the fiber periphery, relieving the strain at the interface between the Z-disk and the sarcolemma. A similar role could be proposed for vinculin-enriched subsarcolemmal densities linked by intracellular filaments to the Z-disk.

At the neuromuscular junction, contractile tension is presumably transmitted more weakly to the cell surface. Vinculin in subsarcolemmal densities at the junction may be involved in stabilizing postsynaptic structures and reducing any deformation caused by contraction. The neuromuscular junction has a distinctive extracellular matrix (e.g., references 1, 40, and 41) which is connected to the postsynaptic membrane by numerous filaments (e.g., reference 20; see also Fig. 7) and which influences the organization and stability of postsynaptic structures (references 2, 9, and 28; Bloch, R. J., J. H. Steinbach, J. P. Merlie, and S. Heinemann, manuscript submitted for publication). Vinculin in subsarcolemmal densities could anchor this material and thereby reinforce the postsynaptic region.

The role we have proposed for vinculin in subsarcolemmal densities is consistent with its location and suggested functions in muscle and in other tissues. Vinculin is associated with sites of cell-to-cell and cell-to-substrate contact (14), often where "stress fibers" insert into the plasma membrane. In cultured hamster fibroblasts, Singer (46) showed that vinculin is enriched at the cell membrane subjacent to a regular array of extracellular filaments containing fibronectin. In the acetylcholine receptor clusters of cultured rat myotubes, vinculin is preferentially enriched at membrane domains involved in cell-substrate contact (6). These domains contain filamentous material that extends from the lipid bilayer to the substrate (D. Pumplin, personal communication). In cardiac muscle, vinculin is especially enriched at the intercalated disk (36, 48), which, like the myotendinous junction of skeletal muscle, transmits much of the force produced by contractile activity. These examples are consistent with the idea that sites of vinculin enrichment near the sarcolemma are usually associated with extracellular structures, which they anchor and to which they can, in some cases, transmit force.

---

[3] In this report we have referred to subsarcolemmal densities without distinguishing among the densities present at the several locations we have studied in the ALD and PLD. We recognize, however, that the different roles we propose for the densities at these locations may require that they be composed of different macromolecules associated with vinculin.

Note Added in Proof: Recently, B. R. Eisenberg and R. L. Milton (1984, Am. J. Anat. 171:273–284) reported similar specializations in the lamina rara associated with subsarcolemmal densities at the myotendinous junction in frog sartorius muscle.

REFERENCES

1. Anderson, M. J., and D. M. Fambrough. 1983. Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparan sulfate proteoglycan on the surface of skeletal muscle fibers. J. Cell Biol. 97:1396–1411.
2. Bader, D. 1981. Density and distribution of α-bungarotoxin binding sites in postsynaptic structures of regenerated rat skeletal muscle. J. Cell Biol. 88:357–370.
3. Bendayan, M., and M. Zollinger. 1983. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. 31:101–109.
4. Bendayan, M., J. Roth, A. Perrelet, and L. Orci. 1980. Quantitative immunocytochemical localization of pancreatic proteins in subcellular compartments of the rat acinar cells. J. Histochem. Cytochem. 28:149–160.
5. Bennett, P. 1979. Myofibrillar connection. Nature (Lond.). 278:602–603.
6. Bloch, R. J., and B. Geiger. 1980. The localization of acetylcholine receptor clusters in areas of cell-substrate contact in cultures of rat myotubes. Cell. 21:25–35.
7. Bloch, R. J., and Z. W. Hall. 1983. Cytoskeletal components of the vertebrate neuromuscular junction: vinculin, α-actinin, and filamin. J. Cell Biol. 97:217–223.
8. Bonilla, E. 1983. Ultrastructural study of the muscle cell surface. J. Ultrastruct. Res. 82:341–345.
9. Burden, S. J., P. B. Sargent, and U. J. McMahan. 1979. Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of nerve. J. Cell Biol. 82:412–425.
10. Burnette, W. N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195–203.
11. Faulkner, J. A., D. R. Claflin, K. K. McCully, and D. A. Jones. 1982. Contractile properties of bundles of fiber segments from skeletal muscles. Am. J. Physiol. 243 (Cell Physiol. 12):C66–C73.
12. Fedde, M. R. 1969. Electrical properties and acetylcholine sensitivity of singly and multiply innervated muscle fibers. J. Gen. Physiol. 53:624–637.
13. Feramisco, J. R., J. E. Smart, K. Burridge, D. M. Helfman, and G. P. Thomas. 1982. Coexistence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. J. Biol. Chem. 257:11024–11031.
14. Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. Cell. 18:193–205.
15. Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc. Natl. Acad. Sci. USA. 77:4127–4131.
16. Ginsborg, B. L. 1960. Some properties of avian skeletal muscle fibres with multiple neuromuscular junctions. J. Physiol. (Lond.). 154:581–598.
17. Gutmann, E., V. Hanzlikova, and E. Holeckova. 1969. Development of fast and slow muscles of the chicken in vivo and their latent period in tissue culture. Exp. Cell Res. 56:33–38.
18. Hanak, H., and P. Bock. 1971. Die Feinstruktur der Muskel-Sehnenverbindung von Skelett- und Herzmuskel. J. Ultrastruct. Res. 36:68–85.
19. Hess, A. 1970. Vertebrate slow muscle fibers. Physiol. Rev. 50:40–62.
20. Hirokawa, N., and J. E. Heuser. 1982. Internal and external differentiations of the postsynaptic membrane at the neuromuscular junction. J. Neurocytol. 11:487–510.
21. Hnik, P., I. Jirmanova, L. Vyklicky, and J. Zelena. 1967. Fast and slow muscles of the chick after nerve cross-union. J. Physiol. (Lond.). 193:309–325.
22. Huxley, A. F. 1980. Reflections on muscle. Princeton University Press, Princeton. 111 pp.
23. Jirmanova, I., and J. Zelena. 1970. Effect of denervation and tenotomy on slow and fast muscles of the chicken. Z. Zellforsch. Mikrosk. Anat. 106:333–347.
24. Jirmanova, I., and J. Zelena. 1973. Ultrastructural transformation of fast chicken muscle fibres induced by nerve cross-union. Z. Zellforsch. Mikrosk. Anat. 146:103–121.
25. Koenig, J., and M. Fardeau. 1973. Etude histochemique des muscles grands dorseaux antérieur et postérieur du poulet et des modifications observées après dénervation ou réinnervation homologue ou croisée. Arch. Anat. Microsc. 62:249–267.
26. Korneliussen, H. 1973. Ultrastructure of myotendinous junctions in Myxine and rat. Specializations between the plasma membrane and the lamina densa. Z. Anat. Entwicklungsgesch. 142:91–101.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature (Lond.). 227:680–685.
28. McMahan, U. J., and C. R. Slater. 1984. Influence of basal lamina on the accumulation of acetylcholine receptors at synaptic sites in regenerating muscle. J. Cell Biol. 98:1453–1473.
29. Maupin, P., and T. Pollard. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. J. Cell Biol. 96:51–62.

30. Nakao, T. 1975. Fine structure of the myotendinous junction and "terminal coupling" in skeletal muscle of the lamprey, *Lampetra japonica. Anat. Rec.* 182:321–338.
31. Nakao, T. 1976. Some observations on the fine structure of the myotendinous junction in myotomal muscles of the tadpole tail. *Cell Tissue Res.* 166:241–254.
32. Nelson, W. J., and E. Lazarides. 1983. Expression of the β-subunit of spectrin in nonerythroid cells. *Proc. Natl. Acad. Sci. USA.* 80:363–367.
33. Nelson, W. J., and E. Lazarides. 1983. Switching of subunit composition of muscle spectrin during myogenesis in vitro. *Nature (Lond.).* 304:364–368.
34. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified, ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361–363.
35. Pardo, J. V., J. D. Siliciano, and S. W. Craig. 1983. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. *Proc. Natl. Acad. Sci. USA.* 80:1008–1012.
36. Pardo, J. V., J. D. Siliciano, and S. W. Craig. 1983. Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. *J. Cell Biol.* 97:1081–1088.
37. Pierobon-Bormioli, S. 1981. Transverse sarcomere filamentous systems: "Z- and M-cables." *J. Muscle Res. Cell Motil.* 2:401–413.
38. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80:585–592, and erratum 83:336.
39. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208–213.
40. Sanes, J. R. 1982. Laminin, fibronectin, and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. *J. Cell Biol.* 93:442–451.
41. Sanes, J. R., and Z. W. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.* 83:357–370.
42. Shear, C. R., and G. Goldspink. 1971. Structural and physiological changes associated with the growth and development of avian fast and slow muscle. *J. Morphol.* 135:351–372.
43. Shear, C. R. 1978. Cross-sectional myofibre and myofibril growth in immobilized developing skeletal muscle. *J. Cell Sci.* 29:297–312.
44. Shear, C. R., S. L. Sankey, W. G. Resneck, and R. J. Bloch. 1984. Heterogeneity of the chicken ALD muscle: evidence for a minor, twitch fiber type. *Exp. Neurol.* 85:506–522.
45. Siliciano, J. D., and S. W. Craig. 1982. Meta-vinculin—a vinculin-related protein with solubility properties of a membrane protein. *Nature (Lond.).* 300:533–535.
46. Singer, I. I. 1982. Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. *J. Cell Biol.* 92:398–408.
47. Street, S. F. 1983. Lateral transmission of tension in frog myofibers: a myofibrillar network and transverse cytoskeletal connections are possible transmitters. *J. Cell. Physiol.* 114:346–364.
48. Tokuyasu, K. T., A. H. Dutton, B. Geiger, and S. J. Singer. 1981. Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy. *Proc. Natl. Acad. Sci. USA.* 78:7619–7623.
49. Trotter, J. A., K. Corbett, and B. P. Avner. 1981. Structure and function of the murine muscle-tendon junction. *Anat. Rec.* 201:293–302.
50. Zelena, J., and I. Jirmanova. 1973. Ultrastructure of chicken slow muscle after nerve cross union. *Exp. Neurol.* 38:272–285.