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DISTRIBUTION OF CONNECTIN (TITIN) AND TRANSVERSE TUBULES AT MYOTENDINOUS JUNCTIONS

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Abstract

The ends of muscle fibers form many longitudinal projections which are further divided into numerous processes and attach to the collagen fibrils of tendons to form myotendinous junctions (MTJs). Immunocytochemical and electron microscopic observations on pectoralis muscles of the chicken revealed the presence of an elastic filamentous protein, connectin (titin), within the terminal sarcomere on the side adjacent to the terminal Z bands, and the absence of connectin and myosin and the presence of actin at the apical sarcoplasmic region of MTJ processes between the terminal Z band and the MTJ sarcolemma. Intermediate voltage electron microscopy showed that T tubules in the terminal sarcomere were absent at the level of the A-I junction on the MTJ side in the rat vastus intermedius, and at the level of the terminal Z band or under the MTJ subsarcolemmal densities in the chicken pectoralis.

Key Words: Muscle-tendon junction, connectin, titin, immunocytochemistry, lanthanum nitrate, T system, intermediate voltage EM

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Introduction

Contractile force is transmitted from muscle to tendon at the myotendinous junction (MTJ). In this region, skeletal muscle fibers exhibit characteristic surface specialization in the form of cylindrical folds to amplify their interface with tendon (Maruyama and Shimada, 1978; Ishikawa et al., 1983; Trotter et al., 1985; Saito and Ikenoya, 1988). Recently, the localization and behavior of a giant myofibrillar protein, connectin (titin), within myofibrils have been studied by immunofluorescence and immunoelectron microscopy (Maruyama et al., 1985, 1989; Fürst et al., 1988). Further, the three-dimensional distribution of transverse (T) tubules in muscle fibers has been clarified by high voltage electron microscopy (Ishikawa and Tsukita, 1977; Franzini-Armstrong and Peachey, 1982; Peachey and Franzini-Armstrong, 1983). However, there has been very limited information on the immunocytochemistry of this protein and the structure of T tubules at these functionally important areas. In this article, we describe our recent observations of these problems (see also Sonoda et al., in press).

Materials and Methods

All materials were fixed at a physiological length in the initial fixative, and then small strips containing MTJ areas were excised and fixed again in the same fixative.

Scanning Electron Microscopy (SEM)

Rectus abdominis muscles of the mouse were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 1% unbuffered OsO_4. They were then treated with 8 N HCl for 25-35 minutes at 60°C to remove connective tissue components and basal laminae (Desaki and Uehara, 1981). Tissue specimens were further immersed successively in 1% OsO_4, 1% tannic acid and 1% OsO_4 (Murakami, 1973). After washing, dehydration and drying with the t-butyl alcohol freeze-drying method (Inoué and Osatake, 1988), the samples were sputter-coated with gold-palladium (thickness: 2-4 nm) and examined with a field-emission type scanning electron microscope (Hitachi S-800) operated at 3 kV.

Transmission Electron Microscopy (TEM)

Pectoralis muscles of the chicken were fixed in 4% formaldehyde in 0.1 M phosphate buffer. They were then treated with 0.1% saponin in the same buffer to enhance the contrast of filamentous and other cytoskeletal structures by washing out soluble proteins from the sarcoplasm. They were postfixed with 1% OsO_4 in the cacodylate buffer, dehydrated and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1200 EXII electron microscope operated at 80 kV.

Fluorescence and Immunoelectron Microscopy

The following polyclonal (pAbs) and monoclonal antibodies (mAbs) were used: anti-connectin mAbs 4C9 (Matsuno et al., 1989) and SM1 (Itoh et al., 1988), and goat anti-connectin pAb against chicken 1200 KDa peptide of α-connectin (P1200) (Matsuura et al., 1991).

Pectoralis muscles of the chicken were fixed in 4% formaldehyde in 0.1 M phosphate buffer. They were infused with 2.3 M sucrose in phosphate buffered saline (PBS), and then rapidly frozen in liquid N_2. Semi-thin frozen sections mounted on glass slides were treated with the primary antibody and then with the second antibody (fluorescein isothiocyanate [FITC]-labeled goat anti-mouse or rabbit anti-goat IgG). They were subsequently stained with tetramethylrhodamine (rho)-labeled phalloidin. Ultrathin frozen sections were treated with the primary antibody and then incubated with biotinylated anti-mouse or anti-goat IgG, followed by treatment with 15 nm colloidal gold-streptoavidin. They were then fixed with 1% glutaraldehyde in PBS and negatively stained with 2% ammonium molybdate.

For fluorescence microscopy, sections were examined with a Zeiss standard microscope equipped for epifluorescence using appropriate filters for FITC or rho. Electron microscopic specimens were observed under a JEOL 1200 EXII at 80 kV.

Fig. 1. Scanning electron micrograph of the end of a mouse rectus abdominis muscle fiber treated with HCl. At the MTJ, the conical end of a muscle fiber was characterized by formation of many longitudinal projections, processes and invaginations. Bar = 5 μm.

Intermediate Voltage Electron Microscopy (IVEM)

Vastus intermedius muscles of the rat and pectoralis muscles of the chicken were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. They were then postfixed in 1% OsO_4 in 0.1 M cacodylate buffer containing 0.8% potassium ferrocyanide, washed in the same buffer, and incubated in 1% lanthanum nitrate in 0.1 M cacodylate buffer. They were dehydrated in ethanol and embedded in Epon 812. Thick sections (0.8-1.0 μm) without further

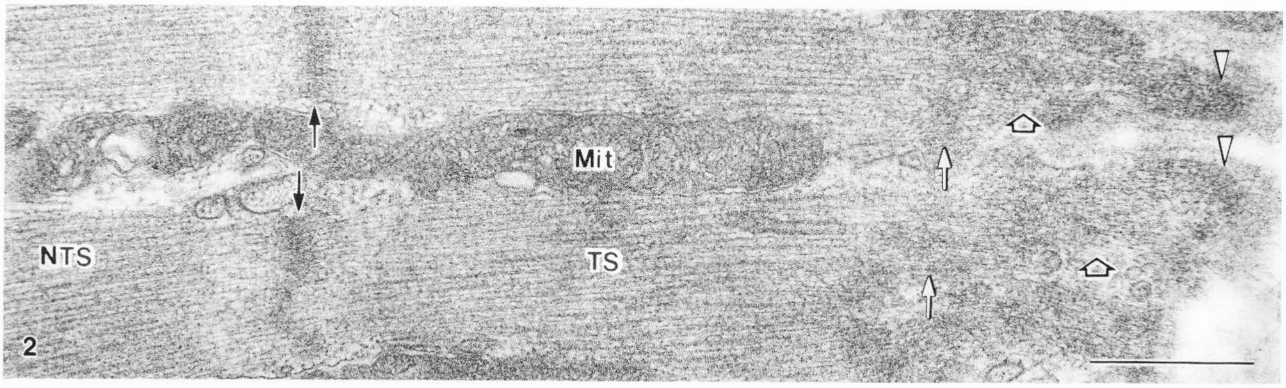

Fig. 2. Thin-section electron micrograph of a chicken pectoralis muscle fiber. Thin filaments from the terminal sarcomere (TS) converged on terminal Z bands (white arrows). Fibrillar material (open arrows) extended from terminal Z bands to dense subsarcolemmal material (white arrowheads) at the apices of the MTJ processes. Non-terminal Z bands and sarcomeres are indicated by black arrows and NTS, respectively. Mitochondria, mit. Bar = 0.5 μm.

staining were examined under a JEOL 4000 EX at 400 kV. Stereo pairs of IVEM micrographs were made by tilting the specimen stage by ±7 degrees.

Results and Discussion

Surface Features of the MTJ

In the specimens where intramuscular connective tissue components and basal lamina were adequately removed, the true surface of the muscle fibers was exposed. In confirmation with previous works of TEM reconstruction of serial thin-sections (Saito and Ikenoya, 1988) and SEM (Trotter et al., 1985), the ends of muscle fibers were seen to taper abruptly, forming many longitudinal projections. The ends of these projections were further divided into numerous processes, with the diameter gradually decreasing and finally terminating in a cone shape. Deep indentations were found between the projections and/or processes (Fig. 1). Raminification of the ends of muscle fibers has been reported to differ in degree between its proximal and distal portions (Saito and Ikenoya, 1988) and among animal species and muscle fiber types (Ishikawa et al., 1983).

Fine Structure of the MTJ

Conventional electron microscopy of longitudinal sections of saponin-treated chicken pectoralis muscle fibers showed that, as a myofibril approached the MTJ, it broke up into several thinner myofibrils which extended into digit-like processes. Thin filaments of the terminal sarcomere converged on dense bodies traversing at the root of the processes (terminal Z bands; Tidball, 1987; Tidball and Lin, 1989). Apical regions of the processes beyond the terminal Z bands contained an assemblage of longitudinally-oriented filaments (Fig. 2). These portions appeared to contain actin, because these filaments bind heavy meromyosin (Maruyama and Shimada, 1978) and were reactive with phalloidin (see below).

Immunocytochemistry of Connectin at the MTJ (Figs. 3 and 4)

Immunofluorescence microscopy of longitudinal sections of the breast muscle of the chicken double-stained with phalloidin and anti-connectin (mAbs 4C9 and SM1, and pAb P1200) showed periodic transverse banding. Staining with 4C9 and SM1 formed "doublets" flanking Z bands. The width of these "doublets" revealed with 4C9 was wider than that with SM1 (Fig. 3b,d). Staining with pAb P1200 appeared to form single bands on Z bands (Fig. 3f). Electron microscopic observation of sections immunogold-labeled for connectin showed that mAb 4C9 stained the edges of the A band, mAb SM1 the center of the I band, and pAb P1200 the regions immediately lateral to the Z band. Thus, at the fine structural level all of these antibody stainings were seen to form "doublets" flanking Z bands (Fig. 4a-c).

Near the ends of muscle fibers, only single bands of fluorescent staining ("singlets") were seen with all of these antibodies (Fig. 3b,d,f). Electron microscopy revealed that these "singlets" appeared to be located within the terminal sarcomere on the side adjacent to the terminal Z bands (Fig. 4a-c). The levels of the labeling of the "singlets" with each antibody on myofibrils were the same as those seen on respective halves of "doublets" observed on the opposite side of the same terminal sarcomere and on the other sarcomeres.

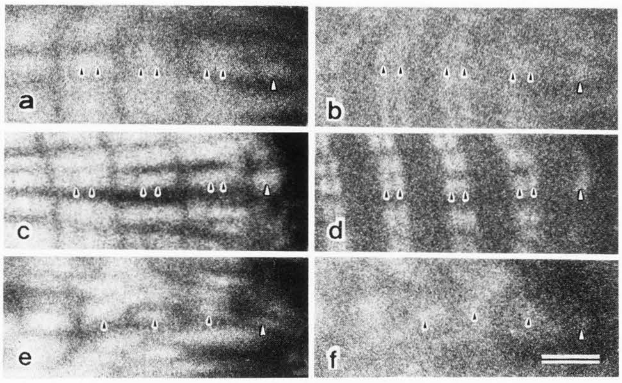

Fig. 3. Fluorescence micrographs of chicken pectoralis muscles double stained with rho-phalloidin (**a**, **c**, **e**) and FITC-anti-connectin (mAb 4C9, **b**; mAb SM1, **d**; pAb P1200, **f**). "Doublets" and "singlets" formed by the antibodies are indicated by black and white arrowheads, respectively (**b**, **d**, **f**); the corresponding levels of these bands on phalloidin-stained myofibrils are also shown by black and white arrowheads, respectively (**a**, **c**, **e**). Bar = 2 μm.

The wedge-shaped apical sarcoplasmic regions of MTJ processes were reactive with phalloidin but not with anti-connectin (Fig. 3a-f). Electron microscopy showed no anti-connectin labeling in the areas between the terminal Z bands and the MTJ sarcolemma (Fig. 4a-c). No myosin filaments seem to be present in these regions (Fig. 2), although actin filaments exist (Maruyama and Shimada, 1978). Thus, the finding that connectin is absent from the apical sarcoplasmic area where myosin is absent but actin is present supports the notion that connectin is a myosin-associated protein (Maruyama, 1986). Further, although the terminal Z bands seem to be different from non-terminal Z bands with regard to reactivity to antibody against smooth-muscle-type α-actinin (Tidball, 1987), connectin filaments seem to link the former bands to myosin filaments at the terminal sarcomere on the MTJ side in the same manner as in other areas of the sarcomeres.

IVEM on T tubules at the MTJ

Lanthanum nitrate selectively stains T tubules of muscle fibers (Franzini-Armstrong and Peachey, 1982; Ishikawa and Tsukita, 1977; Peachey and Franzini-Armstrong, 1983). In the present lanthanum stained muscles, the electron dense tracer was found in the extracellular space (in the deep infoldings of the sarcolemma between the digit-like processes at MTJs and at the lateral cell surface), the subsarcolemmal caveolae and the T tubules (Figs. 5 and 6). T tubules ran transversely at the level of the A-I junction in the rat vastus intermedius (Fig. 5) or the Z band in the chicken pectoralis (Fig. 6) to form planes of continuous networks.

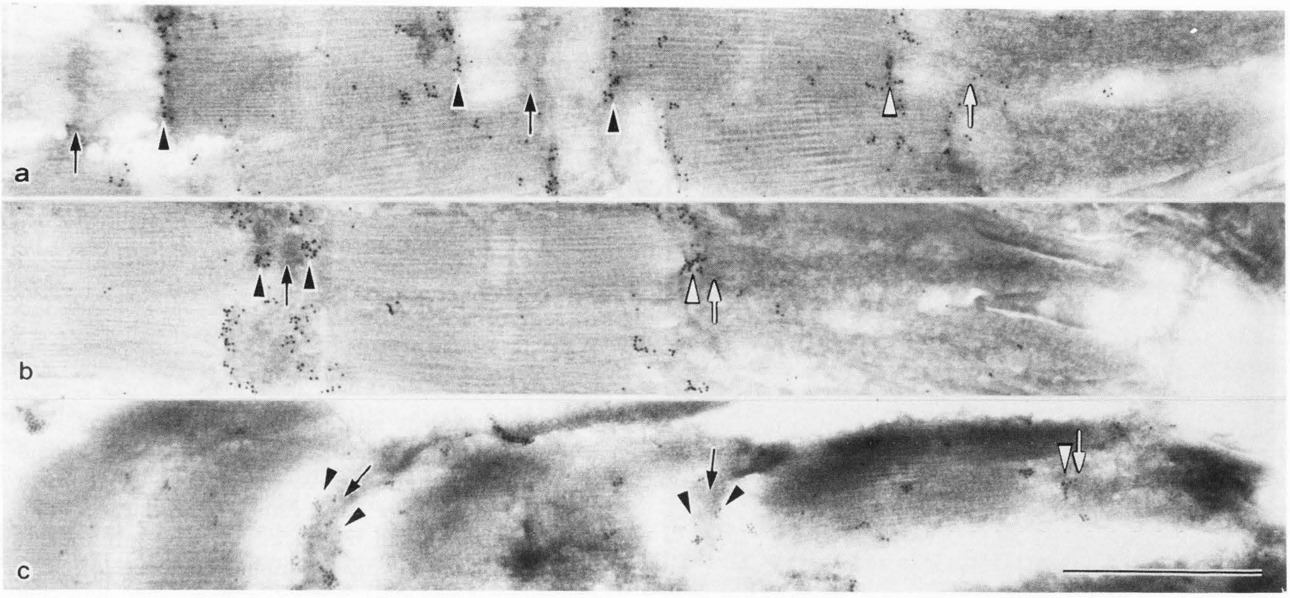

Fig. 4. Electron micrographs of chicken pectoralis muscles immuno-gold labeled with anti-connectin (mAb 4C9, **a**; mAb SM1, **b**; pAb P1200, **c**). All of these antibody labelings formed "doublets" (black arrowheads) flanking non-terminal Z bands (black arrows). Near the MTJ, anti-connectin formed "singlets" (white arrowheads) within the terminal sarcomere on the side of the terminal Z band (white arrows). Bar = 1 μm.

Two adjacent planes of networks were often connected with longitudinal tubules.

Within the projections and processes of MTJs, T tubules were often dilated or formed tangles or coils (Figs. 5 and 6). Similar structures have been seen in muscles of myogenic diseases (Oguchi and Tsukagoshi, 1980; Miike et al., 1984a) and regenerating/developing muscles (Ezerman and Ishikawa, 1967; Ishikawa, 1968; Kelly, 1971; Miike et al., 1984b; Chan et al., 1990). Thus, such morphological alterations of T tubules can occur not only in myonecrotic or regenerating/developing processes, but are in fact normally present in the sarcoplasm, which is unique for MTJs. Since MTJ areas are sites where myofiber elongation and myofibril assembly take place when the muscle is stretch-hypertrophied (Dix and Eisenberg, 1990), the possibility exists that such special structures of T tubules might represent a quiescent state waiting for growth.

T tubules were seen to open at the deep bottom of MTJ infoldings by their longitudinal portions and at the lateral wall of these infoldings by their transverse portions (Figs. 5 and 6), in addition to their opening at the lateral surface of the myofibers by transverse portions as noted previously (Franzini-Armstrong and Porter, 1964; Bertaud et al., 1970). They appear to assist inward diffusion of an activating substance at such areas where planes of T tubule networks are interrupted by the presence of MTJ infoldings of the sarcolemma.

Of particular interest was the observation that T tubules were absent at the following levels: at the final A-I junction of the terminal sarcomere in proximity to the MTJ sarcolemma in the rat vastus intermedius (Fig. 5), and at the terminal Z bands or under the subsarcolemmal densities into which thin filaments of the terminal sarcomere were inserted (Fig. 6). It is possible that thin filaments of the terminal sarcomere on the MTJ side can slide with respect to the terminal thick filaments by calcium release from the sarcoplasmic reticulum which formed couplings with the side wall of the invaginated sarcolemma and/or with the longitudinally oriented tubules in the MTJ processes. However, further studies are required to clarify this problem.

The results obtained from the present observations are illustrated schematically in Figs. 7 and 8.

Acknowledgments

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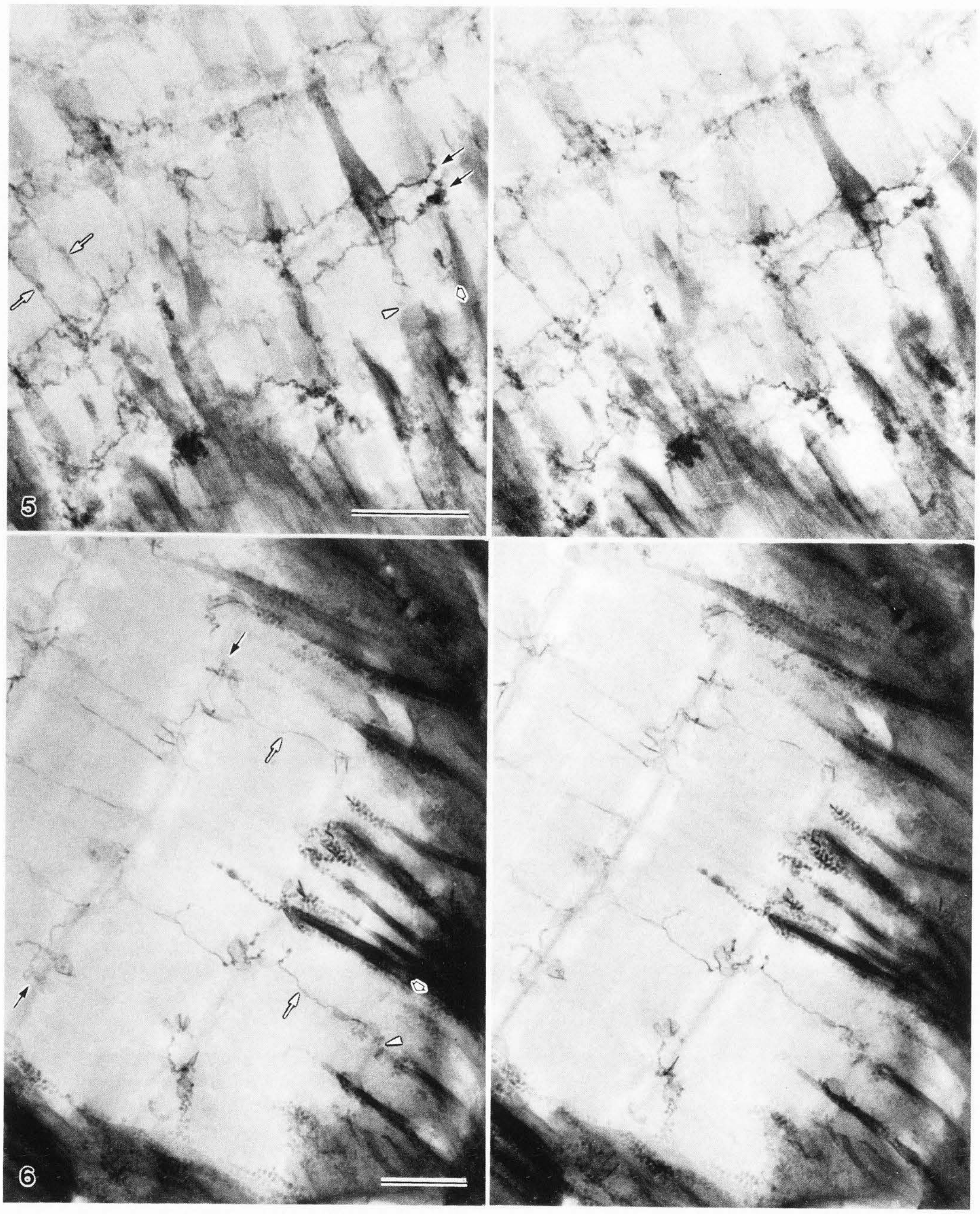

Figs. 5 and 6. Stereo pairs of IVEM micrograph of thick sections of the rat vastus intermedius (Fig. 5) and the chicken pectoralis (Fig. 6) stained with lanthanum nitrate. T tubules formed planes of continuous networks (black arrows) at the level of A-I junctions (Fig. 5) and Z bands (Fig. 6). These networks were absent at the terminal sarcomeric half adjacent to the MTJ sarcolemma (open arrows). Within MTJ processes, T tubules often formed dilatations and coils. Longitudinal tubules connected neighboring T tubule networks (white arrows) and opened to the bottom of infoldings (white arrowheads). Bar = 1 μm.

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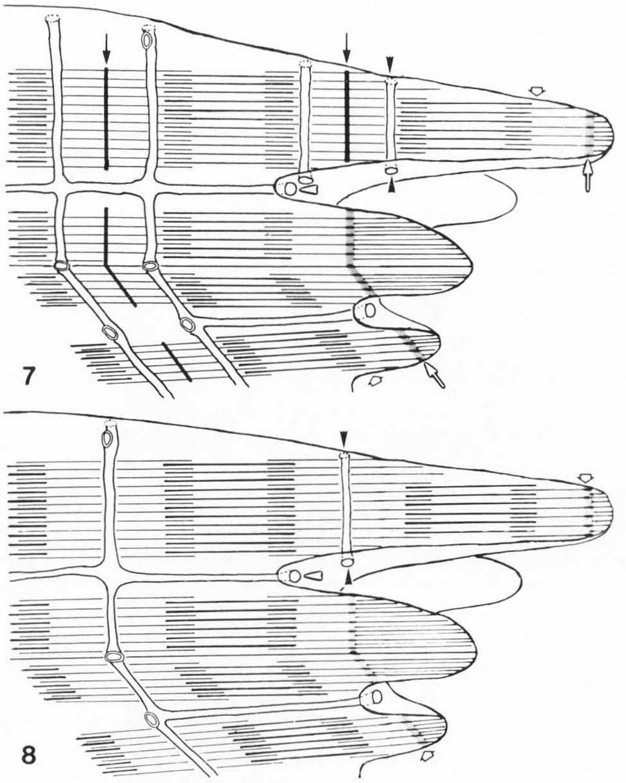

Figs. 7 and 8. Schematic illustrations of T tubules at the MTJ areas of rat vastus intermedius (Fig. 7) and chicken pectoralis (Fig. 8). T tubules are absent at the levels indicated by open arrows. T tubules open at the lateral wall (black arrowheads) and at the bottom of MTJ infoldings (white arrowheads). Terminal and non-terminal Z bands are indicated by white and black arrows, respectively (Fig. 7).

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Discussions with Reviewers

H. Ishikawa: You described that thin filaments of the terminal sarcomere are seen to converge on a dense body resembling the Z band at the MTJ. I wonder if you are justified to call the dense body as the terminal Z band. There is no evidence of the definitive Z band ultrastructurally and immunochemically.

D.A. Fischman: I can't see in the micrographs the structures identified as "terminal Z bands," and it is unclear to me why the authors talk about them as if they were clearly identifiable morphological features. Rather, it seems to be the case that the terminal actin filaments are associated into a bundle with increased electron density. There seems to be no justification for calling the most distal part of that bundle the "terminal Z band."

Authors: Although the structures described in this article as "terminal Z bands" are not clearly identifiable as those of non-terminal Z bands, we followed the term used by Tidball (1987, 1989) for convenience sake. Concerning the immunocytochemistry of the terminal Z band, see the article by Tidball (1987). It is not known if the terminal thin filaments in the terminal half sarcomere are longer than 1 µm and whether they extend directly into the apical regions of the digit-like processes with increased electron density to terminate at the MTJ sarcolemma.

H. Ishikawa: It is stated that at MTJs T tubules were often dilated or formed tangles or coils. Such features may not be unique to MTJs in mature muscles besides regenerating and developing portions. Have you examined the T tubules in the middle portions of muscle fibers? Are there any differences in the form of T tubules among different fiber types?

Authors: Our impression was that such morphological features were more numerous in the MTJ areas than in the middle portions of muscle fibers. We have not examined differences in the structure of T tubules among different fiber types.