Changes in Localization and Orientation of Triads Following Selective Elimination of Cytoskeletal Protein in Rat Skinned Muscle Fiber

Kelly McGRATH*, Tomie NISHIZAWA*,**, Susumu YAMASHITA*, Hiroyuki TAMAKI***, Kazuto SAITO*** and Hiroaki TAKEKURA***

Abstract

We observed the ultrastructural and morphological features of membrane systems (triad) involved in excitation-contraction coupling (E-C) coupling in chemically skinned rat muscle fibres to clarify a question: whether the localization and orientation of triads in a sarcomere are influenced following the selective elimination of cytoskeletal proteins in myofibrils. Basically, the distance between triads and Z-lines depends on the sarcomere length: the distance increase with sarcomere length in the skinned muscle fibres. Three treatments prepared to eliminate the cytoskeletal proteins specifically. One is incubation in a high concentration of KCl, which dissolved myosin filaments. The localization and orientation of triads did not change following the complete elimination of myosin filaments; however, the distance between Z-line and triads become shorter in myosin filaments eliminated skinned fibres. The other is incubation in a treatment with a trypsin, in the presence of ATP, which results in separation of I-Z-I brushes from A-band, presumably due to selective digestion of a-connectin (also called titin) filaments. The localization and orientation of the triads did not change following the elimination of connectin filaments. There was a highly significant ($p<0.01$) positive correlation between sarcomere length and the distance between triads and Z-line in the trypsin-treated skinned muscle fibres. The other treatment was in a high concentration (pCa = 4.0) of Ca^{2+}-rigor solution. The Z-lines are clearly degraded by the incubation in the high concentration of Ca^{2+}-rigor solution for 4 hours. Most of triads were swollen, and vacuolated by the treatment of the high concentration of Ca^{2+}-rigor solution. The membrane vesicles recognizable as parts of triads due to their dense content are observed in proximity of the Z-lines, elongated profiles, presumably remnants of the transverse tubules. There is some direct connections between triads and myofibrils in the sarcomere to hold the triads at the A-I junctional region with transverse orientation.

KEY WORDS: *excitation-contraction coupling, triad, connectin, transverse tubule,* Ca^{2+}

Introduction

Excitation-contraction (E-C) coupling is the signaling process in muscle fibers by which membrane depolarization leads to force development[2, 12, 21]. The E-C coupling depends on appropriate interaction between the junctional domains of the sarcolemma/transverse (t-) tubules and those of the sarcoplasmic reticulum (SR). The interaction between SR and t-tubules occurs at specialized junctions named triads those are internal junctions comprised of two terminal cisternae (TC) of SR and a t-tubule[5, 6, 19].

The initial formation of triads depends on an association with myofibrils, and proper triads are formed in adult mammalian skeletal muscle fibers as the junctions become associated with the transverse orientation region at the A band-I band (A-I) junctional region[6, 23]. The triads have strong

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structural profiles that enable them to withstand a single mechanical stress: the width of junctional gap between the t-tubule and the SR-TC, as well as the orientation of the triads, does not change, even when a sarcomere length is stretched over 5 mm. We observed that the distance between the triads and Z-lines depends on the sarcomere length: the distance increases with the increases of sarcomere length, and there is a highly significant positive correlation[23]. These results indicate that there may be some connections between the triads and the myofibrils[26].

Since the t-tubules show little or no specific association with the myofibrils until the transverse organization of the triads occurs, it is possible that their specific localization at the A-I junctional region may be mediated by the junctional SR[3]. However, it is still unclear what components of the myofibrils have a role of holding the triads at the A-I junctional region with a transverse orientation. While some of myofibril-membrane bridges have been reported at the Z-line regions[18, 26], no specific components has been observed at the A-I junctional region. The specific aim of the present study is to determine whether the triads' localization and orientation in sarcomere are changed by the selective elimination of cytoskeletal proteins in myofibrils.

Materials and Methods

Animal care and sampling

This study was conducted with male Wistar rats (CLEA Japan, Tokyo, Japan, 10 weeks old) of body weight of approximately 210-250g. The rats were housed, two per each cage, in our animal facility under a 12 hour:12 hour light:dark cycle at a room temperature of 23±2℃ and humidity of 50±5%. They were maintained on a diet of rodent chow (CE-2, CLEA Japan) and water ad *libitum*. All procedures using animal experiments were performed in accordance with the guidelines presented in the *Guiding Principles for the Care and Use of Animal in the field of Physiological Sciences*, published by the Physiological Society of Japan.

The extensor digitorum longus (EDL) muscle was surgically dissected under anesthesia (intraperitoneal injection of sodium pentobarbital, 50 mg/kg body weight). Small bundles (less than 50 muscle fibers) were dissected from the EDL muscles in an ice-cold relaxing solution for mammals (120mM KCl, 0.5 mM EGTA, 4 mM $MgCl_2$, 10 mM piperazine-N, N'-bis (2-ethanesulphonic acid)(PIPES), 10 mM ATP; pH 7.0), and chemically skinned by adding 60 μg/ml saponin to the relaxing solution. The rats were euthanized with an overdose of sodium pentobarbitone after the EDL muscles were taken. The chemically skinned muscle bundles were washed several times by relaxing solution and used for the following observations.

Selective elimination of muscle proteins

In order to eliminate the selective muscle proteins (myosin, connectin (titin), and α-actinin (Z-lines)), the chemically skinned muscle bundles were incubated in the following solutions: for myosin, 600 mM KCl, 5.1 mM $MgCl_2$, 4.0 mM EGTA, 10 mM PIPES, 5.3 mM ATP, pH 7.0 at 20℃, for 5 and 10 minutes[23]; for connectin (titin), 117.8 mM KCl, 5.1 mM $MgCl_2$, 4.0 mM EGTA, 10 mM PIPES, 5.3 mM ATP, 0.25 μg/ml trypsin (Type III, Sigma Chemical Co., St Louis, USA), pH 7.0 at 20℃, for 5 and 10 minutes[7, 27]; for α-actinin (Z-lines), 138.9 mM KCl, 1.0 mM $MgCl_2$, 4.0 mM EGTA, 10 mM PIPES, 4.1 mM $CaCl_2$, pH 7.0 at 20℃, for 4 and 8 hours[1].

Electron microscopic observation

The EDL skinned muscle bundles were fixed in 2.5 to 3.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.25) at room temperature for 2 to 3 hours for thin electron microscopic observation. They were then washed in the same sodium cacodylate buffer without glutaraldehyde for 60 minutes (20 minutes each, 3 times). The skinned muscle bundles were post-fixed in 2% OsO_4 (in 100mM sodium cacodylate buffer) for 2 to 3 hours, and stained en bloc with saturated aqueous uranyl acetate for 4 hours at 60℃. Tissues were dehydrated in graded ethanol and acetone, infiltrated with an Epon-acetone mixture (1:1) overnight and embedded in Epon. Thin sections (<50 nm thickness) that were stained with both uranyl acetate and Sato lead, were examined under an electron microscope at 80kV

(JEM-2000EX, JEOL, Tokyo, Japan). We took pictures of each muscle fibre at 5000× magnification, and used to measure the distance between the triads and Z-lines at different sarcomere length. We did not measure the distance between the triads and Z-lines when the triads are lying between two misaligned myofibrils. We measured these distance only when the triads are lying within myofibrils. In the case of the triads did not completely parallel with the Z-lines, we measured the nearest and farthest distances between the triads and Z-lines and averaged.

Statistical analysis

The quantitative data were evaluated statistically using the unpaired Student's t-test or multivariance analysis of variance (ANOVA) to determine the significance of differences. $P<0.05$ was taken as significant.

Results

All triads located at the A-I junctional region with a transverse orientation in adult mammalian skeletal muscle fibres (arrows, Figs. 1A and B). The SR was well developed not only at the A-I junctional region to form triads with t-tubules, but also at M-line regions. These are morphological features of the Ca^{2+} release units directly concerned with E-C coupling in matured mammalian skeletal muscle fibers.

Myosin filaments dissociate incubated in a high concentration of KCl solution, and the most of myosin filaments were eliminated with 5 minutes treatment (Fig. 2A), and were completely eliminated with 10 minutes treatment (Fig. 2C). The localization and orientation of triads did not change in sarcomere by the elimination of myosin filaments (arrow, Figs. 2A and C); however, the distance between the triads and Z-lines seemed to become shorter in the myosin filaments eliminated muscle fibres (Figs. 2C and D). We measured the distance between the triads and Z-lines at the different length of sarcomere in trypsin treated muscle fibres, and observed a significant ($p<0.01$) positive correlation (Fig. 3, $y=0.059x-0.133$, $r=0.391$, $n=83$).

Following trypsin treatment in relaxing solution, the sarcomere is separated into A-band region and I-Z-I brushes (Fig. 4A). All triads has still localized at the A-I junctional region with transverse orientation after the trypsin treatment, and these morphological features has not been changes in response to changes in the sarcomere length. Other ultrastructural features of the triads, such as width of junctional gap and feet, in trypsin treated muscle fibres were similar to the features of triads in the muscle fibres observed in the normal muscle fibres (Fig. 4B). We also measured the distance between the triads and Z-lines at the different length of sarcomere in trypsin treated muscle fibres, and observed a significant ($p<0.01$) positive correlation (Fig. 3, $y=0.273x-0.275$, $r=0.791$, $n=90$). This significant and positive correlation was very similar to the positive correlation which observed in control skinned muscle fibres (Fig. 3, $y=0.253x-0.123$, $r=0.959$, $p<0.01$, $n=95$).

Incubation in solutions containing 4.1mM Ca^{2+} for 4 and 8 hours caused structural alterations of the Z-lines probably due to activate of the CANP. The

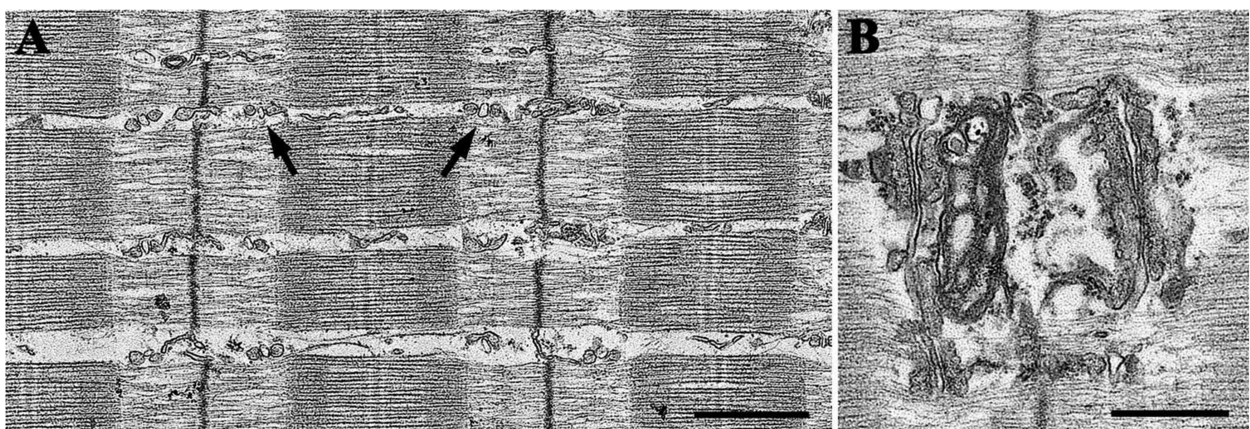

Figure 1. Electron micrographs of the longitudinal section of skinned muscle fibres. All triads locate at the A-I junctional region with transverse orientation (parallel to the Z-lines, arrows in A). Scale bars; 1.0 μm in A, 0.5 μm in B.

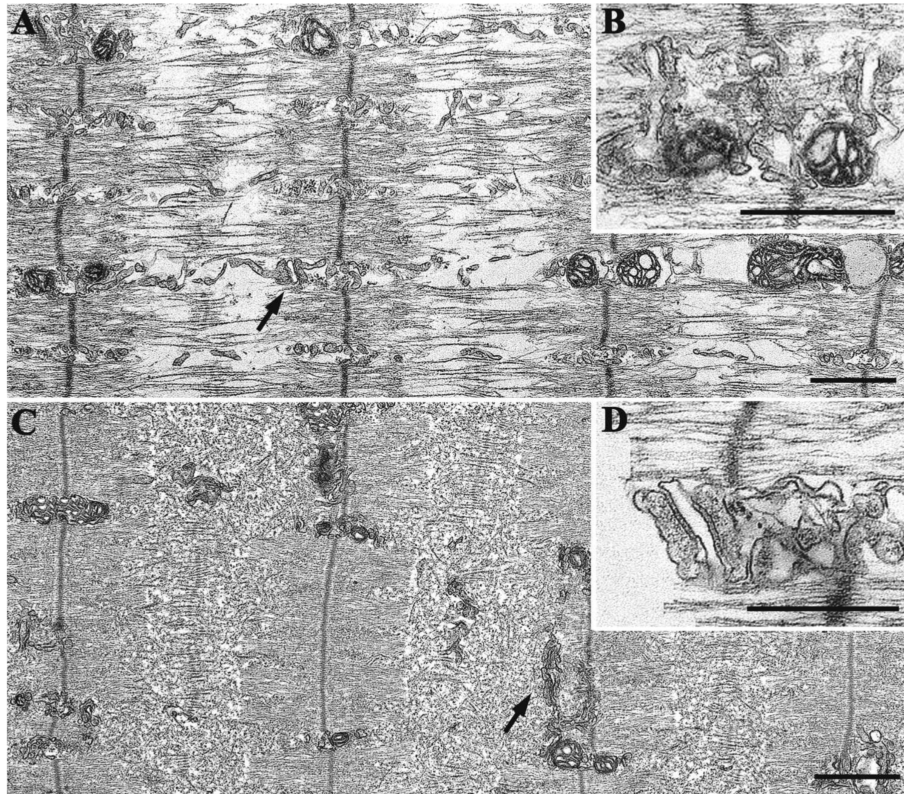

Figure 2. Electron micrographs of the longitudinal section of skinned muscle fibres treated with a high concentration (600 mM) of KCl for 5 (A and B) or 10 (C and D) minutes. Most of myosin filaments were eliminated by 5 minutes of incubation. All triads (arrows, A, see also B) were located at A-I junctional region with transverse orientation. Myosin filaments were completely eliminated for 10 minutes incubation. Despite the cytoskeleton in the skinned muscle fibres being broken, the localization and orientation did not change (arrow in C, see also D); however, the distance between triads and Z-lines seemed to be shortened when the myosin filaments were completely eliminated. All scale bars; 1.0 μm.

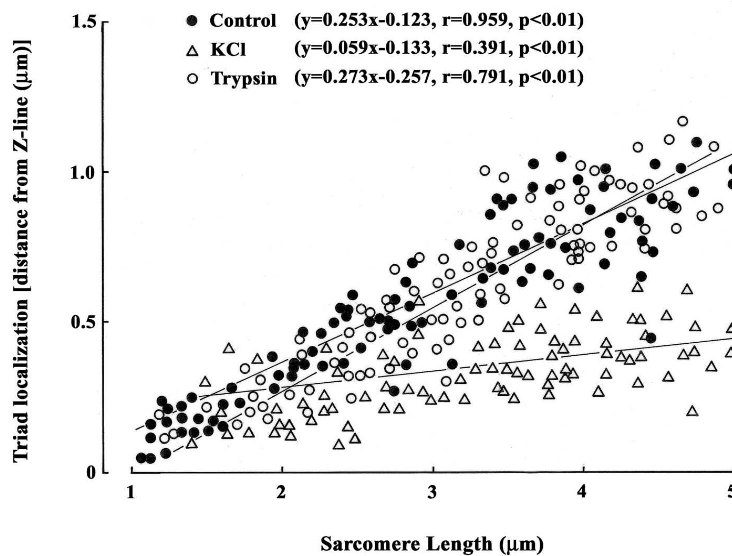

Figure 3. Relationship between sarcomere length and the distance of triads to Z-lines in skinned muscle fibres. Significant ($p < 0.01$) positive relationships between sarcomere length and the distance of triads to Z-lines were observed in the control, KCl treated, and trypsin treated skinned muscle fibres.

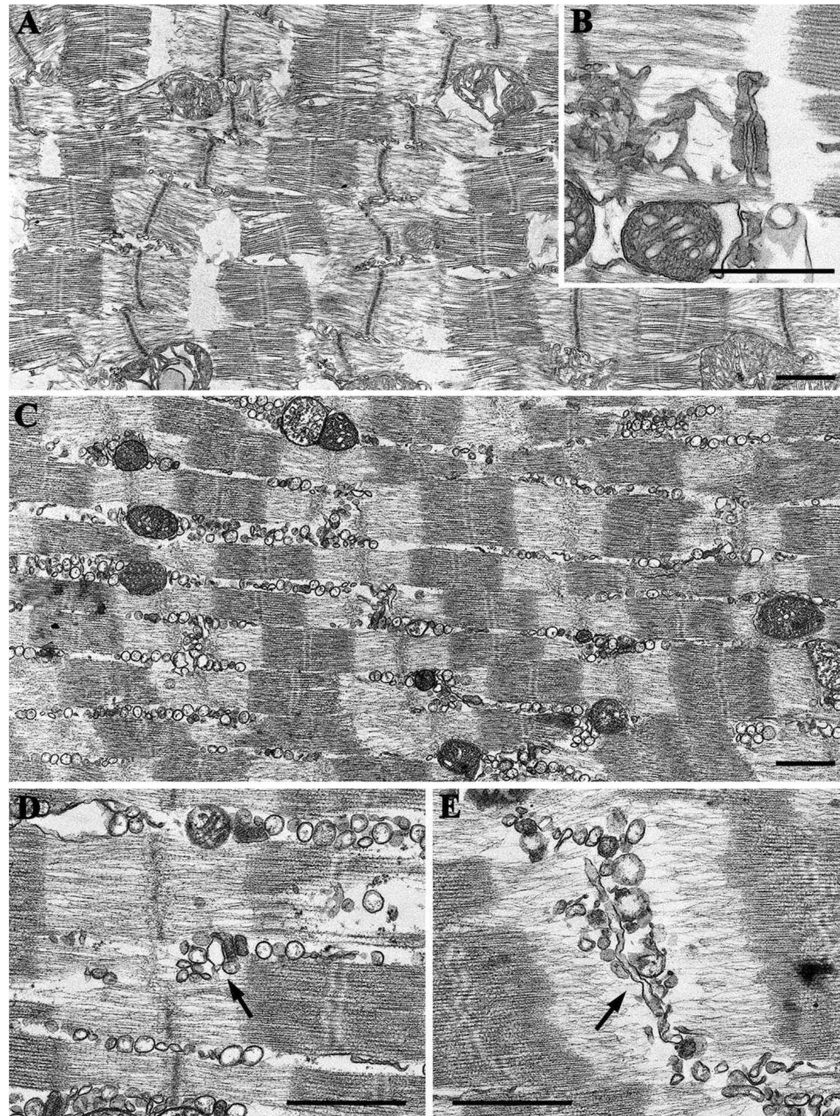

Figure 4. Electron micrographs of the longitudinal section of skinned muscle fibres treated with a trypsin solution (A and B) or treated with a high concentration (4.1 mM) of Ca^{2+} solution for 4 (C and D) or 8 (E) hours. Following trypsin treatment in relaxing solution, the sarcomere is separated into A-band region and I-Z-I brushes (A). All triads has still localized at the A-I junctional region with transverse orientation after the trypsin treatment (B). The Z-lines are clearly degraded at 4 hours (C and D) of incubation and completely eliminated following 8 hours (E) incubation with a high concentration of Ca^{2+} solution. The triads are vacuolated and hard to observe 8 hours after the incubation in the high concentration of Ca^{2+} solution (E). All scale bars; 1.0 μm.

Z-lines are clearly degraded at 4 hours of incubation (Fig. 4C and D) and completely eliminated following 8 hours (Fig. 4E) treatment in a high concentration of Ca^{2+} solution. The remainder of the myofibrils, however, is seemed to structurally intact. Unfortunately the SR is vacuolated and the triads are hard to observe in muscle fibres incubated in the solution containing a high concentration of Ca^{2+} for 4 hours (arrow, Fig. 4D). However, the elongated tubules which may be remnants of the t-tubules, and vesicles with a dense content, probably remnants of junctional SR are new clustered of the level of the Z-line (arrow, Fig. 4E). Small pieces of broken triads, especially t-tubules, were also observed at that part which used to be the Z-line there before in myofibrils, and these t-tubules did not always show the transverse orientation in myofibrils.

Discussion

The triads of matured mammalian skeletal muscle fibres display a highly regular organization and have a

specific localization at the A-I junctional regions with a transverse orientation. These morphological features of triads are completed during myogenesis, before and after birth[4, 6, 20, 22, 24]. The SR develop before the t-tubule appearance, and associates with the Z-lines and the I-band region in myofibrils[3, 4, 22]. The t-tubules seem to appear suddenly, and immediately form randomly localized triads with the SR. All triads move to the A-I junctional region, and rotate their direction from longitudinal to transverse orientation[22, 24]. It has been reported that the width of junctional gap between t-tubule and TC of SR was more highly conserved than the morphological architecture of actin and myosin filaments[5, 18, 23]. The width of junctional gap (9.80 ± 1.33 nm) is not influenced by the sarcomere length, even when sarcomere length is stretched to no overlap[23]. The width of junctional gap in the myosin filaments eliminated skinned muscle fibres was almost consistent with the values of previous report[23]. The interaction of junctional domains of t-tubules and TC is mechanically strong and does not change under normal E-C coupling cycles in skeletal muscle fibres. The distance between triads and Z-lines becomes shorter following extraction of the myosin filaments; however, the tendency for movement away from the Z-lines when the sarcomere is stretched remains. There is a possibility that a specific protein responsible for tethering the triads at the A-I junctional region is interrupted either because it is normally connected to the myosin filaments, or because it is extracted by the conditions that dissociate myosin filaments[18, 23].

Connectin (α-connectin), also called titin, is a giant elastic protein in skeletal muscle fibres (MW: approximately 3000kD). A single connectin molecule runs from Z-line, through the I-band and binds onto myosin filament up to M-line[14, 15, 16]. The connectin is not only responsible for maintenance of cytoskeleton of muscle fibres but also for a resting tension development[11]. Trypsin digestion brakes connectin filaments close to the A-I junctional regions. Connectin (titin) filaments which connect myosin filaments to Z-lines in myofibrils[15, 16], exist as α-connectin in striated muscle, and their proteolytic is β-connectin[14]. Separation of myosin and actin filaments was frequently observed at the A-I junctional region, these results are indicating that the connectin filaments in this region eliminated by incubation in relaxing solution containing trypsin[27]. These authors also found that trypsin treatment resulted in the decrease in density of Z-lines, due to the release of a-actinin from the Z-lines during the trypsin digestion of myofibrils[8, 27]. The localization and orientation of triads were not changed after the trypsin treatment. It is thought that a specific interaction between triads and components of myofibrils, is responsible for the localization of triads at the A-I junctional region. We found previously that the distance between triads and Z-lines is completely dependent on the sarcomere length, and that the position of the triads is not uniquely restricted to the A-I junctional region[23]. Then, it has been suggested that there is a significant positive correlation between the distance between triads to Z-lines and the sarcomere length in the control skinned muscle fibres. As the sarcomere lengthens, triads move away from Z-line, but not as far as the A-I junction. Thus we expected that there is no specific interaction involves protein(s). The significant positive correlation observed in trypsin treated (connectin filaments eliminated) muscle fibres was almost same as the correlation in the control muscle fibres. All these results indicate that connectin filaments do not concern with the decision of triads localization and orientation in myofibrils.

Extracellular Ca^{2+}, but not intracellular Ca^{2+}, sequestered in SR, is necessary for activation of the CANP, and it has been shown that the CANP is activated by both mM and mM orders of extracellular Ca^{2+} [13, 25]. The observation that desmin was immediately cleaved CANP activity, indicated that the mechanism of Z-line removal could be explained in terms of CANP effect on a still unknown Z-line protein (possibly desmin at the periphery of the Z-line), followed by the release of a-actinin from the Z-line1). Regardless of the physiological role of the CANP, it is probable that this factor is responsible for the rapid Z-line degradation in muscle strips incubated in Ca^{2+}-containing solution1). Z-lines were completely removed by incubation in rigor-Ca^{2+} solution for 8 hours, these results indicating that the

CANP in muscle fibres was activated by this solution. At the same time, internal membrane systems and mitochondria were enlarged by incubation in the same rigor-Ca^{2+} solution. Since the SR membrane loss its ability to accumulate Ca^{2+} in postmortem muscle fibers[9, 17], it seems probable that the release of bound Ca^{2+} from the SR may activate the SR factor, thereby initiating the Z-line degradation characteristically observed in these fibers[10]. It is still unclear whether the CANP degrades the specific internal membrane systems in muscle fibers. We found that, when fiber bundles incubated in the rigor-Ca^{2+} solution, triads were disrupted and parts of disrupted internal membranes aggregated in the areas that used to the Z-lines. Small pieces of disrupted triads, in particular t-tubules were also observed in these areas before being observed in myofibrils and these t-tubules did not always show a transverse orientation in myofibrils.

It is still not clear whether these results were brought about by the direct action of the CANP in degrading the triads, and/or by the indirect action of the CANP in degradating the Z-lines. If the unknown protein(s), reported previously by Walker et al.[26] and Nunzi and Franzini-Armstrong[18], holds the triads at the A-I junction region in a transverse orientation, it would seem that this protein is also degraded by the activation of the CANP, and that the localization and orientation of the triads in the sarcomere are deranged. However, these results do provide indirect evidence that the localization and orientation of triads in the sarcomere are related in some way to the existence of Z-lines.

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ラット骨格筋 skinned fiber における細胞骨格蛋白質の選択的除去と triad の位置と方向の変化

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要　約

骨格筋細胞の細胞骨格を形成している蛋白質の選択的除去が，興奮収縮連関の機能発現に直接関与する筋細胞内膜系複合体（triad）の形態的特徴に及ぼす影響をラット（Wistar 系雄性，10週齢）骨格筋 skinned fiber を用いて検討した。成熟した哺乳類骨格筋細胞において triad は，A-I junction 上に横断方向（収縮蛋白質の走行方向に対して直角の方向）に位置している。細胞骨格蛋白質の除去が，この triad の位置と方向の変化に及ぼす影響を観察した。EDL より温血動物用 relaxing solution 中にて筋束（筋線維10本程度）を摘出し，saponin 処理により chemical skinned fiber を作製，化学処理を施して細胞骨格蛋白質を選択的に除去した後，電子顕微鏡観察を用いて triad の微細形態を観察した。Triad と Z 線間の距離はサルコメア長に比例し，サルコメア長の伸展に伴いその距離も増大した。細胞骨格蛋白質の選択的除去は，３種類の化学処理によって行なった。ラット skinned fiber を高濃度（600mM）の KCl で処理し，ミオシンフィラメントを除去した。ミオシンフィラメントを完全に除去しても triad の位置と方向に変化は認められなかったが，triad と Z 線間の距離が同じサルコメア長のコントロール skinned fiber に比較して短縮する傾向が観察された。次に，ATP を含む trypsin 溶液で skinned fiber を処理し，コネクチンフィラメントを選択的に除去した。コネクチンフィラメント除去に伴う triad の形態変化は観察されなかった。Z 線を除去するために，高濃度（pCa＝4.0）の Ca^{2+}-rigor 溶液で skinned fiber を処理した結果，４時間で Z 線は完全に消失した。また，筋細胞内膜系の構造破壊と triad の位置に変化が観察された。大部分の triad は Z 線が存在していた位置に移動し，その方向にもばらつきが観察された。これらの結果は，triad を A-I junction 上に保持すると同時に，横断方向を維持する蛋白質が存在し，その蛋白質は Z 線と結合しており，Ca^{2+} activated neutral protease（CANP）によって分解される可能性を示唆している。

KEY WORDS: *excitation-contraction coupling, triad, connectin, transverse tubule,* Ca^{2+}

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