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Electron Microscope Studies on the Structural Complex of Transverse Tubules and Sarcoplasmic Reticulum in Scorpionfish Swimbladder Muscles

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Abstract: To clarify the distribution of transverse tubule-sarcoplasmic reticulum (T-SR) complexes in the swimbladder muscle (SBM) of the scorpionfish (*Sebastiscus marmoratus*), electron microscope observations were performed. In the anterior part of the SBM, Z-type triads were exclusively observed, in contrast to the regular distribution of AI- and Z-type triads with pentads and heptads in the posterior part of the SBM reported in our previous paper. Various body muscles of scorpionfish contained only Z-type triads. Furthermore, SBM and body wall muscles (BWM) in other sound-producing fishes each contained only one type of triadic contacts. In the posterior fibers of scorpionfish SBM, feet on the junctional SR membrane formed a square lattice with three or more vertical lines and numerous horizontal rows. Isolation and identification of ryanodine receptor (RyR) revealed that αRyR and βRyR coexist in the SBM and BWM of scorpionfish. Anti-αRyR and βRyR antibodies were produced in rabbits, and immunoelectron microscopy with these antibodies demonstrated that both αRyR and βRyR form parallel lines respectively on the same junctional membrane as AI- and Z-type triads. The results are discussed in connection with the anatomical features and function of the swimbladder.
Keywords: swimbladder muscle, fish body muscles, distribution of triadic contact, feet array, coexistence of α and β ryanodine receptor isoforms

Introduction

Triadic contacts between the transverse (T) tubule and the sarcoplasmic reticulum (SR) are very significant for ensuring excitation-contraction (E-C) coupling in the skeletal muscles[1-4]. Generally, two types of triadic contact are found in the skeletal muscles of vertebrates[5]. One is the AI-type triad located around the boundary between the A- and I-bands of the sarcomere, and the other is the Z-type triad found around the Z-band of the sarcomere. Furthermore, it has been believed that each muscle fiber contains only one type of triadic contact[5]. However, in 2003, the authors found newly that, in scorpionfish swimbladder muscles (SBM), two types of triadic contact together with the other T-SR complexes (pentads and heptads) are distributed regularly within single fibers[6]. The previous study also clarified that the SBM is anatomically constructed with the anterior and posterior parts connected by a layer of tendon at the middle of the muscle, as also reported for the toadfish SBM[7], and the new finding on the distribu-

tion of triadic contacts was made in the posterior part of the SBM. In the present study, electron microscope observations were performed to examine whether the unique distribution of triadic contacts is also found in the anterior part of the SBM and other body muscles of scorpionfish, and in the BWM and body muscles of other sound-producing fish.

It has been believed that, in the E-C coupling of skeletal muscles, the signal transmission from T tubules to SR is completed by the membrane proteins located respectively in those junctional membranes, namely dihydropyridine receptor (DHPR) in T tubules and ryanodine receptor (RyR) in SR, and that the RyR corresponds to feet found electron microscopically in triadic junction[8]. In the present study, a two-dimensional array of feet was analyzed by measuring their dimensions and distances.

It is also well known that, in some amphibian and fish skeletal muscles, two kinds of RyR isoform, αRyR and βRyR, are arrayed alternatively in the junctional membrane of the terminal cisternae of SR to ensure efficient Ca release for contraction[9]. Since little information is available at present on the RyR isoform in the scorpionfish SBM, biochemical isolation and identification of RyR and immunoelectron microscopy using anti-RyR antibodies produced from the identified RyR were performed. To give an ultrastructural basis for the analysis of RyR isoform distribution, the foot considered to be the real structure of RyR was observed by electron microscope, and the dimensions and factors of the two-dimensional array were measured. Furthermore, to identify the Ca-binding protein in the T-SR complex of the scorpionfish SBM, additional immunoelectron microscopy using anti-calsequestrin (CSQ) antibody was performed.

Materials and Methods

Muscle fiber preparation

Adult scorpionfish, *S. marmoratus* (body length 16-20 cm), were collected at Sagami Bay or obtained from a commercial source, and kept in a natural seawater tank at 17ºC. Other sound-producing fish for comparative studies, gurnard (*Chelidonichthys spinosus,* body length ~40 cm) and white croaker (*Argyrosomus argentatus*, body length ~30 cm), were obtained at Odawara Fish Market. The procedure for preparing the scorpionfish SBM was described precisely in our previous paper[6]. Briefly, a bundle of 3-5 fibers (*in situ length*) with tendons was dissected from the anterior and posterior parts of the SBM in a fish Ringer's solution. They were mounted horizontally in an acrylic chamber filled with the Ringer's solution; both ends were mechanically fixed with pins on a silicone rubber plate put on the bottom of chamber. In the case of various body muscles used for swimming, fiber bundles were also prepared, and fixed mechanically with pins by using tendons. For the gurnard and white croaker, fiber bundles of SBM and body wall muscles (BWM) were prepared in the same way.

Electron microscopy

For conventional electron microscopy, muscle fibers of *in situ* length were fixed with a 2.5% glutaraldehyde solution, post-fixed with a 1% osmium tetroxide solution, dehydrated, and embedded in Quetol 812. To observe the distribution of triadic contacts in the anterior part of the scorpionfish SBM, the single fiber was divided into 12 longitudinal segments prior to the embedding. In the body muscles of scorpionfish and various muscles of gurnard and white croaker, no segmentation was performed before embedding. Ultrathin sections of the fixed preparations were cut on an Ultracut N ultramicrotome (Reichert, Vienna, Austria), and stained with uranyl acetate and lead citrate. Sections were examined by using a transmission electron microscope (JEM2000EX: JEOL, Akishima, Tokyo, Japan).

For immunoelectron microscopy, the muscle fibers were fixed with a 4% paraformaldehyde solution, and treated with 50 mM glycine solution to neutralize the aldehyde groups[10, 11]. After dehydration, they were embedded in Lowicryl K4M resin, and polymerized by illumination with ultraviolet light at -20ºC. Ultrathin sections treated previously with 20 mM glycine and 1% bovine serum albumin (BSA) were immunostained with anti-αRyR antibody, anti-β RyR antibody and anti-CSQ antibody. Then they were stained secondary with AuroProbe EM goat anti-rabbit IgG conjugated with colloid gold particles of diameter 5 nm for anti- αRyR antibodies or diameter 10 nm for anti- βRyR antibodies and anti-CSQ antibodies. Anti-RyR antibodies were produced in the present study as described later, and anti-CSQ antibody was obtained from a commercial source.

Immunostained sections were observed with the same electron microscope as described above.

Production of anti-RyR antibodies and their specificities

Purification and characterization of α and β RyR and their antibodies from SBM and BWM of scorpionfish were conducted as described by Murayama and Ogawa (1992)[12]. Scorpionfish muscles were homogenized and centrifuged at 4,700 *g* for 20 min. The SR fractions were obtained from the pellet of centrifugation after centrifuging twice at 27,000 *g* for 70 min. The fractions were then solubilized in 4% CHAPS solution, and centrifuged at 100,000 *g* for 30 min. The supernatant was layered onto tubes of 5-20% linear sucrose density gradient, and centrifuged at 69,000 *g* for 20 h. The resulting fractions (5 ml each) were analyzed for protein composition by SDS-PAGE and for [^{3}H] ryanodine binding by means of scintillation counting.

To identify RyR isoforms, SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting were performed. In SDS-PAGE, SR fractions of *Xenopus laevis* were also used as a marker, together with the standard marker obtained from a commercial source. Proteins separated by SDS-PAGE were treated with western blotting, using a pan-RyR primary antibody[13] and the secondary antibody (peroxidase conjugated goat anti-rabit IgG antibody).

Polyclonal antibodies against RyR (α and β) purified from the scorpionfish body muscles were produced in rabbits. Rabbit serum was collected after five immunizations with the emulsion (including complete/incomplete Freund's adjuvant) by intradermal injection at two-week intervals. Antigens separated by SDS-PAGE were electro-phoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. Using these membranes as a purification tool by reacting with anti-serum, anti-BWM RyR (α and β) antibodies and anti-SBM RyR (α and β) antibodies were obtained.

Results

Distribution of various triadic contacts in fish muscles

To confirm the distribution of triadic contacts or T-SR complexes, SBM and body muscles of scorpionfish and two kinds of sound-producing fishes were examined by electron microscopy. Longitudinal sections of 12 segments divided from the anterior part of scorpionfish SBM fibers were observed. In contrast with the diversified distribution of T-SR complexes in the posterior part[6], the T-SR complex almost exclusively contained the Z-type triad in the anterior SBM fibers, as shown in Fig. 1. Only one triadic contact (pentad and/or heptad) was excep-

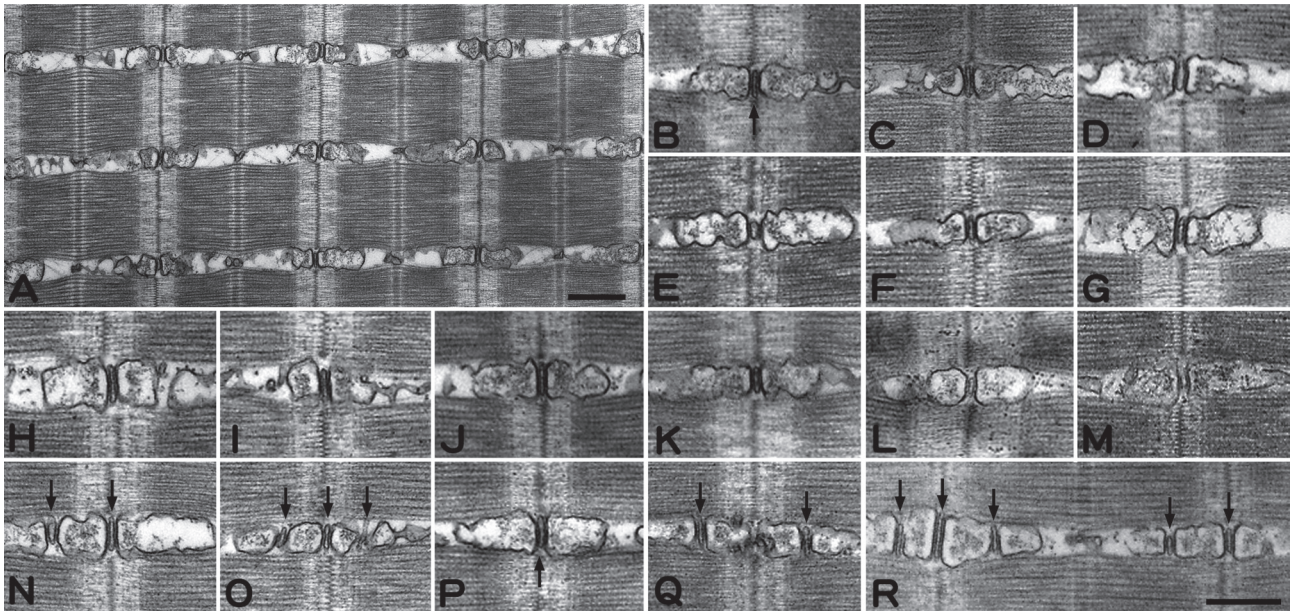

Fig. 1. Triadic contacts of scorpionfish SBM. A. Typical electron micrograph of myofibrils in the anterior part of SBM, showing Z-type triads. Scale bar, 1 μm. B-M. Z-type triads in 12 segments (the 1st ~ the 12th) of anterior SBM fibers. N and O. Pentad and heptad rarely found in the 5th segment. P. Typical Z-type triad in both end regions of posterior SBM fibers. Q. AI-type triad in the central region of posterior SBM fibers. R. Heptad and pentad in the mixture region between the both fiber ends and its central region of posterior SBM fibers. Arrows indicate T tubules. Scale bar, 0.5 μm (B-R).

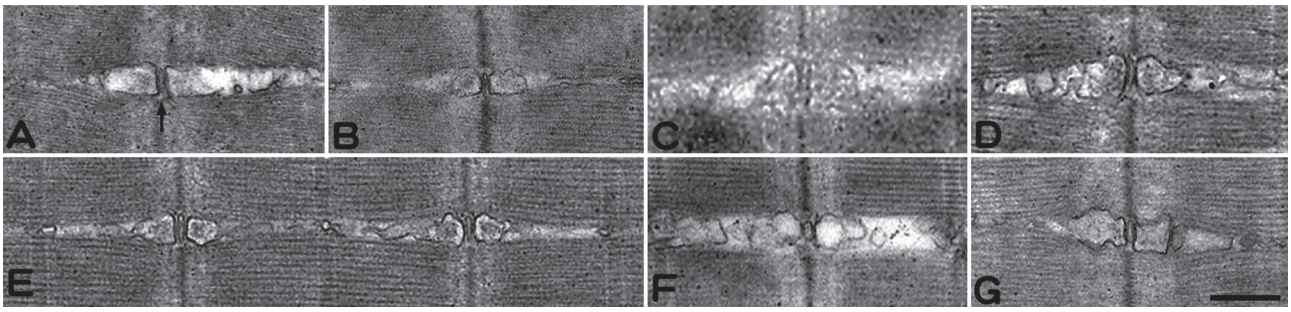

Fig. 2. Triadic contacts of scorpionfish body muscles. Electron micrographs of Z-type triads in supracarinalis posterior muscle (A), infracarinalis posterior muscle (B), abductor superficialis muscle (C), inclinator analis muscle (D), arrector dorsalis pelvicus muscle (E), hypochordal longitudinalis muscle (F), and interradialis muscle (G). Arrow indicates T tubule. Scale bar, 0.5 μm.

tionally observed in the 5th segment of the anterior part (Fig. 1M, O). In contrast, the diversified distribution of T-SR complexes[6] was re-confirmed by observing 12 segments of the posterior SMB fibers.

For comparative morphology, seven body muscles of scorpionfish were observed: supracarinalis posterior, infracarinalis, posterior, abductor superficialis, arrector dorsalis pelvicus, inclinator analis, hypochordal longitudinalis, and interradialis. All triadic contacts in these muscles were the Z-type triad (Fig. 2).

The SBM and body wall muscles of two other sound-producing fishes which are different from the scorpionfish were also observed. In gurnard, the triadic contacts were the AI-type triad in SBM (Fig. 3A), and the Z-type in the pectoral fin muscle and the BWM (Fig. 3B). In white croaker, the triadic contacts were the Z-type triad in SBM and BWM (Fig. 3C, D).

Two-dimensional array of feet

The feet of triads were observed in the posterior part of scorpionfish SBM fibers. In longitudinal sections cut parallel to the myofibril axis, feet were detected as a row with a regular interval along the triadic junction (Fig. 4A, C). On the other hand, in transverse sections cut in perpendicular to the myofibril axis, feet were arrayed as a square lattice (Fig. 4B, D), constructed with three or more vertical lines and numerous horizontal rows. The overall shape of each foot was a square with a concave center on the side. These structural views of feet were not fundamentally different between AI-type and Z-type triads. Various dimensions and distances of the arrayed feet were measured. The measured values were summarized in Table 1.

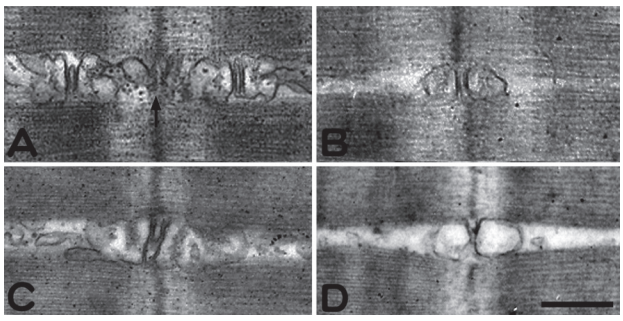

Fig. 3. Triadic contacts observed in muscles of sound-producing fishes (gurnard and white croaker). A. A-I type triads in gurnard SBM. B. Z-type triads in gurnard pectoral fin muscles. C and D. Z-type triads in SBM and BWM of white croaker. Arrow indicates T tubule. Scale bar, 0.5 μm.

In longitudinal sections, the inner and outer diameters of T tubules were approximately 24 and 45 nm, respectively. There was no difference in the measured values between AI- and Z-type triads. The gap distance of T-SR junctions in AI-type triads was 11.6 nm, and slightly wider than that measured in Z-type triads. The long and short diameters of feet found in the junctional gap were approximately 16.5 and 9.5 nm in AI- and Z-type triads, respectively. The center-to-center distance between feet was 33.7 nm in AI-type triads, and larger than the value measured in Z-type triads (32.5 nm).

In transverse sections, the center-to-center distance between feet was approximately 36.5 nm along the vertical lines in AI- and Z-type triads. Along the horizontal rows, it was 39.3 nm in AI type triads, and shorter than that measured in Z-type triads (42.7 nm). The side lengths of each foot found in AI- and Z-type triads were 27.7 and 29.3 nm respectively.

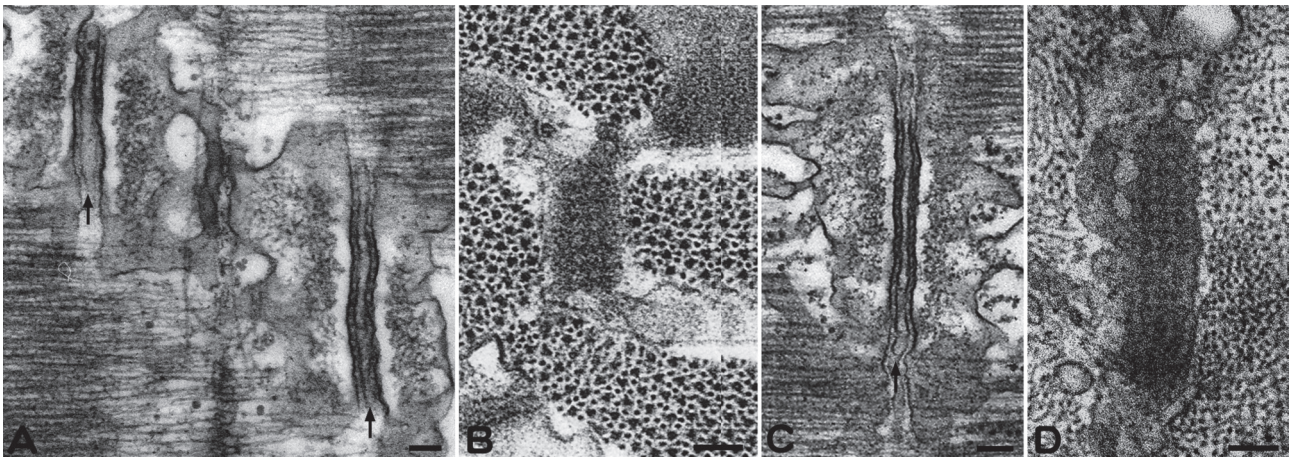

Fig. 4. Feet array in triadic contacts of scorpionfish SBM. A and B. Cross-sectional views of A-I type triads, showing a feet row with regular intervals (A) and a square lattice of feet (B). C and D. Cross-sectional views of Z-type triads, showing a feet row with regular intervals (C) and a square lattice of feet (D). Arrows indicate T tubule. Scale bars, 100 nm.

Table 1. Dimensions and distances of structural compoents of triadic contacts in scorpionfish swimbladder muscles

#	Structural components	AI-type triad nm	(n=)	Z-type triad nm	(n=)	Level of significance
A	Inner diameter of T tubule	24.1 ± 7.8	(51)	24.4 ± 6.9	(52)	＞0.5
	Outer diameter of T tubule	45.1 ± 10.0	(51)	44.6 ± 8.6	(52)	＞0.5
	Junctional gap distance	11.6 ± 1.7	(88)	11.0 ± 1.3	(111)	＜0.001
	C-C D* between feet	33.7 ± 4.0	(129)	32.5 ± 2.3	(121)	＜0.001
	Long diameter of foot	16.7 ± 3.4	(159)	16.3 ± 3.0	(318)	＞0.5
	Short diameter of foot	9.7 ± 1.8	(159)	9.6 ± 1.2	(117)	＞0.4
B	C-C D* between horizontal rows	36.3 ± 3.6	(32)	36.8 ± 3.7	(33)	＞0.5
	C-C D* between vertical rows	39.3 ± 2.4	(18)	42.7 ± 4.1	(27)	＞0.01
	Side length of foot	27.7 ± 2.3	(31)	29.3 ± 2.6	(30)	＞0.01

A, B: Measurements in EM images of longitudinal (A) and transverse (B) sections.
C－C D*: Center-to-center distance.
Values are the mean ± SD.

Production of anti-RyR antibodies

To purify RyR (α and β) of scorpionfish muscles, the solubilized SR from SBM and BWM was fractionated by sucrose density-gradient centrifugation. The resulting 16 fractions were analyzed for composition by SDS-PAGE and for [^{3}H]ryanodine-binding activity. The 12th and 13th fractions from SBM and BWM strongly revealed the presence of proteins corresponding to RyR used as the standard marker, and [^{3}H] ryanodine-binding activity.

SDS-PAGE for SR fractions of scorpion fish muscles indicated two bands corresponding to the α RyR and β RyR bands exhibited in SR fractions of *Xenopus* (Fig. 5A). Western blotting for these two bands using anti-RyR antibodies derived from synthetic peptide demonstrated strong binding activity (Fig. 5B), indicating that scorpionfish SBM and BWM

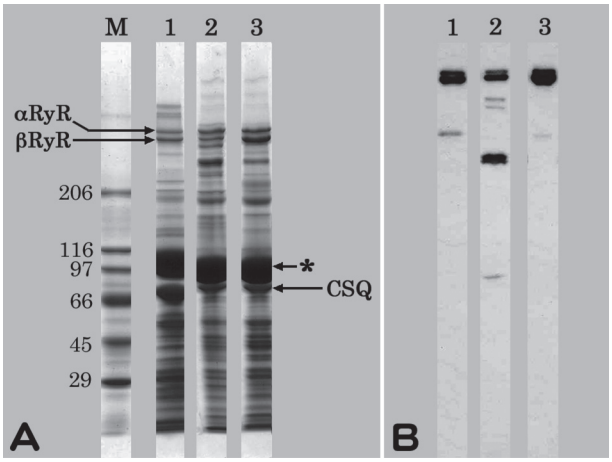

Fig. 5. Analysis of SR fractions of scorpionfish muscles on SDS-PAGE (A) and western blotting using anti-RyR antibodies derived from synthetic peptide (B). Lane M, markers. Lane 1, SR fractions of *Xenopus*. Lane 2, scorpionfish SBM. Lane 3, scorpionfish BWM. *, Ca-ATPase.

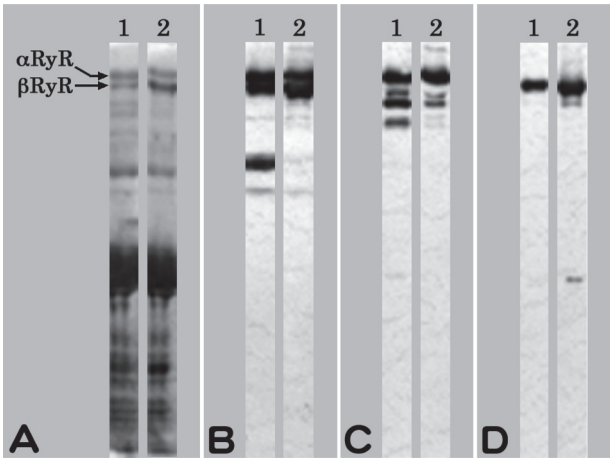

Fig. 6. Specificity of the isoform-specific antibodies against α RyR and β RyR. A. Purified α RyR and β RyR from SR fractions of scorpionfish SBM (Lane 1) and BWM (Lane 2), separated by SDS-PAGE, transferred electrophoretically onto a PVDF membrane, and stained with Coomassie Brilliant Blue. B-D. Immuno- blotting against RyR (shown in A) by using the anti-RyR antibody derived from synthesized peptide (B), the anti-SBW α RyR antibody (C) and the anti-SBM β RyR antibody (D). Lane 1, scorpionfish SBM. Lane 2, scorpionfish BWM.

contain both α RyR and β RyR. In SDS-PAGE, bands derived from Ca-ATPase and CSQ were also detected.

The specificity of the isoform-specific anti- bodies derived from rabbit serum against α RyR and β RyR of SBM and BWM was examined. As shown in Fig. 6, RyR proteins on the PVDF membrane were immunostained well, indicating the successful production of anti-SBM and BWM RyR (α and β) antibodies.

Immunoelectron microscope observation

The distribution of α RyR and β RyR in the scorpionfish SBM was examined by immunoelectron microscopy. To visualize the localization of RyR isoforms, secondary antibodies conjugated with gold particles of diameter 5 and 10 nm were used against the primary anti-αRyR antibody and anti-βRyR antibody, respectively. Typical results are shown in Fig. 7. No gold particles were observed when SBM was treated without the primary antibodies in whole fibers including AI-type (Fig. 7A, E) and Z-type triads (Fig. 7I), indicating that no specific reaction was caused by process of immunostaining. In the sections stained with primary and secondary antibodies, gold particles with both small and large diameters were observed as clusters at triadic junctions, especially on the junctional membrane of SR, in AI-

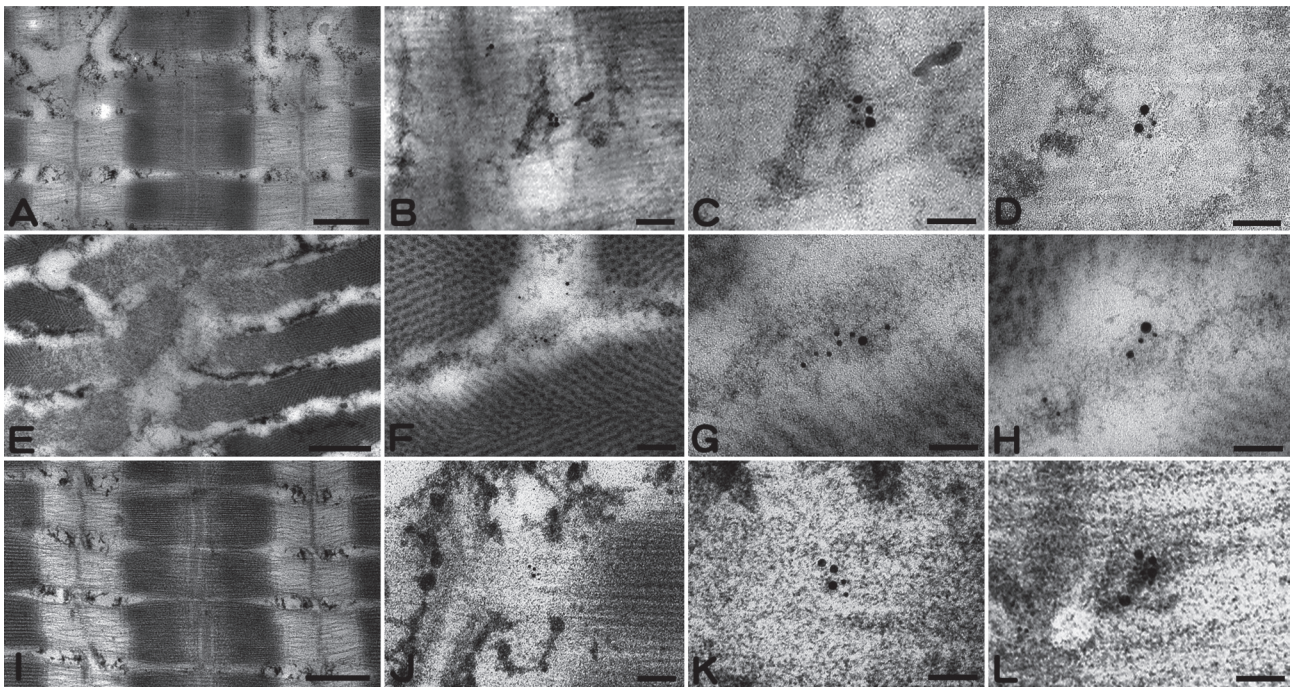

Fig. 7. Immunoelectron microscope images for RyR isoforms labeled with anti-SBM α RyR and β RyR antibodies in scorpionfish SBM. A-H. Cross-sectional views of AI-type triads in longitudinal sections (A-D) and transverse sections (E-H). I-L. Cross-sectional views of Z-type triads in longitudinal sections. Antibodies conjugated with gold particles (small for α RyR and large for β RyR), indicating localization of RyR isoforms, are never detected in sections treated without antibodies (A, E, I), and found together in the junctional region of triadic contact in sections treated with antibodies (B-D, F-H, J-L). C, G and K are enlarged views of B, F and J, respectively. Other enlarged views are also shown in D, H and L. Note the array of gold particles. Scale bars, 500 nm (A, E, I); 100 nm (B, F, J); 50 nm (C, D, G, H, K, L).

type triads (Fig. 7B, C, D, F, G, H) and Z-type triads (Fig. 7J, K, L). In Fig. 7C showing the longitudinal view of AI-type triads, three large particles are arrayed linearly with roughly equal spacing along the junctional membrane, and four small particles are arrayed in parallel with the row of large particles. In Fig. 7G showing the transverse view of AI-type triads, seven small particles are arrayed roughly linearly with one large particle. In Fig. 7K showing the longitudinal view of Z-type triads, three large particle and two small particles appear closely side-by-side. In the observations of particle arrays, small and large particles were never found along the same lines; they were segregated from each other to form the respective lines.

To consider the arrangement of RyR isoforms on the junctional SR membrane of triads, center-to-center distances between gold particles were measured. In the A-I type triad, distances were 19.2 ± 9.6 nm (n=66) between small particles, 24.8 ± 14.2 nm (n=55) between large particles, and 21.2 ± 7.6 nm (n=96) between small and large particles. In the Z-type triad, distances were 29.0 ± 22.1 nm (n=40) between small particles, 36.3 ± 21.7 nm (n=43) between large particles, and 30.2 ± 16.9 nm (n=58) between small and large particles.

Imunoelectron microscopy was also applied to detect Ca-binding protein, CSQ, in the scorpionfish SBM. Gold particles were detected in the terminal cisternae of SR (Fig. 8), as suggested previously by the existence of the CQS band on SDS-PAGE (Fig. 5A).

Discussion

Distribution of triadic contacts

As revealed in the previous study, in posterior SBM fibers, AI-type triads and Z-type triads are regularly located respectively in the middle and both ends of the fiber[6]. In contrast, precise observations clarified that the triadic contacts were only Z-type triads in the anterior SBM fibers, although other triadic contact (pentad and/or heptad) was observed exceptionally. Furthermore, the triadic contacts were Z-type triads exclusively in seven kinds of body muscles. In addition, SBM and body muscles of other sound-producing fish respectively contained only one type of triadic contacts. The present results reconfirmed that the coexistence of two different types of triad in the posterior SBM of scorpionfish is an extremely rare example among vertebrates, and suggested that the different distribution of triadic type between anterior and posterior parts in SBM is meaningful for sound production in the swimbladder.

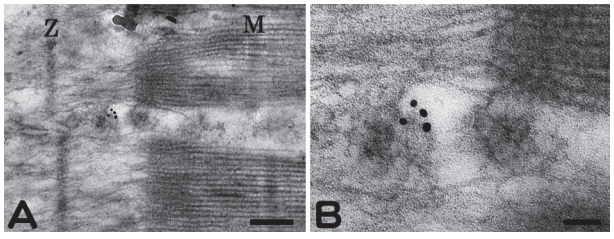

Fig. 8. Immunoelectron microscope images for CSQ labeled with anti-CSQ antibodies in scorpionfish SBM. Antibodies conjugated with gold particles, indicating localization of CSQ, are found in the region of SR terminal cisternae. Scale bars, 200 nm (A); 50 nm (B).

Two-dimensional array of feet

The measured dimensions of feet and the center-to-center distance between them in longitudinal sections coincided well with values reported in previous papers[8, 14, 15]. In transverse sections, feet were arrayed as a square lattice on the junctional SR membrane as found in the SBM of toadfish[14]. The lattice array was generally constructed with a few vertical lines and numerous horizontal rows. Since they were observed in the same plane, it was difficult to identify whether the outside lines corresponded to the row of parajunctional feet proposed in another paper[15]. The side lengths of each foot comprising the array were approximately 28 nm and 29 nm in AI- and Z-type triads respectively, agreeing well with the established values[16].

The center-to-center distances of feet located on the vertical lines in lattice arrays were approximately 36 nm in AI- and Z-type triads, and shorter than the diagonal length (~40 nm) of square feet with 25 nm side length. This result means that feet are arrayed at an angle to the vertical axis in the lattice array; the angle was estimated to 5~8 degrees by means of geometric figure analysis on papers. In contrast, the center-to-center distances of feet located on the horizontal rows in lattice arrays were approximately 39 and 43 nm in AI- and Z-type triads, respectively, and the values roughly corresponded to the diagonal length (~40 nm) of the square foot. However, if the respective foot is arranged on the vertical line of the lattice at 5~8° angles to the line axis, horizon-

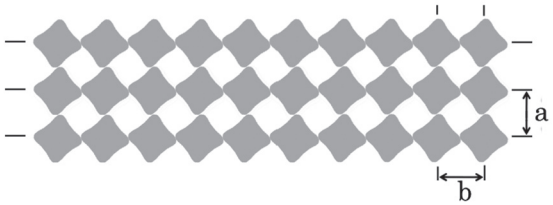

Fig. 9. Schematic drawing of feet array in scorpionfish SBM. Numbers of horizontal lattice rows and vertical lattice lines are not real. Center-to-center distances between feet (a = ~40 nm, b = ~36 nm).

tal rows may form with a space of 3~4 nm between them, as shown in Fig. 9. This fact may support the orthogonal array of feet proposed previously[15, 17].

Localization and two-dimensional array of α RyR and β RyR

Isolation and identification of SR membrane proteins revealed that, in scorpionfish, two kinds of RyR isoforms (α and β) are contained not only in SBM but also in BWM. Immunoelectron microscope observations demonstrated their localization on the junctional SR membrane in both AI- and Z-type triads of SBM. Regarding the existence of RyR isoforms, the present results contrasted with the case of toadfish; it has been reported that the SBM of toadfish contains only α RyR[18]. The discrepancy may be due to specific differences among fish and/or muscle type.

The center-to-center distances between gold particles suggested that RyR were roughly arrayed on the junctional membrane. However, using only this measurement, it is difficult to know the precise arrangement on the square lattice expected from the section view as shown in Fig. 4, because the length of IgG antibody and particle size may disturb the real position of membrane proteins. On the other hand, the pattern of the particle array was significant. Although small and large particles were arrayed linearly, they were never observed along the same line. This means that each RyR isoform forms an inherent line, excluding other isoforms, and supports a similar idea of segregated array proposed elsewhere[15]. Since α RyR is believed to be the electrically-sensitive Ca-release channel (foot)[9], it seems likely that the line including αRyR occupies the central position in the square lattice of feet, exactly opposing the T tubule membrane including DHPR.

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