Subcellular distribution of ryanodine receptors in the cardiac muscle of carp (Cyprinus carpio)

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Chugun, Akihito, Kazumi Taniguchi, Takashi Murayama, Tsuyoshi Uchide, Yukio Hara, Kyosuke Temma, Yasuo Ogawa, and Tai Akera. Subcellular distribution of ryanodine receptors in the cardiac muscle of carp (Cyprinus carpio). Am J Physiol Regul Integr Comp Physiol 285: R601–R609, 2003. First published June 12, 2003; 10.1152/ajpregu.00419.2002.—We examined the subcellular localization of ryanodine receptors (RyR) in the cardiac muscle of carp using biochemical, immunohistochemical, and electron microscopic methods and compared it with those of rats and guinea pigs. To achieve this goal, an anti-RyR antibody was newly raised against a synthetic peptide corresponding to an amino acid sequence that was conserved among all sequenced RyRs. Western blot analysis using this antibody detected a single RyR band following the SDS-PAGE of sarcoplasmic reticulum (SR) membranes from carp atrium and ventricle as well as from mammalian hearts and skeletal muscles. The carp heart band had slightly greater mobility than those of mammalian hearts. Although immunohistochemical staining showed evident striations corresponding to the Z lines in longitudinal sections of mammalian hearts, clusters of punctate staining, in contrast, were distributed ubiquitously throughout carp atrium and ventricle. Electron microscopic images of the carp myocardium showed that the SR was observed largely as the subsarcomelial cisternae and the reticular SR, suggesting that the RyR is localized in the junctional and corbular SR.

anti-ryanodine receptor antibody; immunolocalization; sarcoplasmic reticulum

THE SARCOPLASMIC RETICULUM (SR) plays an important role in the contraction of mammalian cardiac muscle. Depolarization of the cell membrane causes Ca2+ influx via the L-type Ca2+ channel, which, in turn, triggers Ca2+-induced Ca2+ release from the SR. This amplified enhancement in myoplasmic Ca2+ results in cardiac muscle contractions (14, 15). However, in the cardiac muscle of fish, which has been reported to have only a minor SR, muscle contraction is assumed to be produced primarily by the influx of Ca2+ across the cell membrane (10, 12, 37, 43). Some fish hearts, in contrast, have a well-developed SR, suggesting that Ca2+ released from the SR is likely to be involved to some extent in the cardiac contraction (10, 12, 36, 43).

With the use of isolated ventricular muscle preparations of carp heart, we observed the negative staircase phenomenon characteristic of the rat heart (42) and ryanodine-sensitive postrest contractions (8), which are believed to be related to Ca2+ release from the SR (4, 6, 39, 41). In addition, Ca2+-dependent [3H]ryanodine binding has been found in SR membrane preparations from the fish hearts (8, 44), indicating the presence of the ryanodine receptor (RyR) as the Ca2+ release channel in the SR (14, 15). These findings suggest that the SR and RyR play important roles in cardiac muscle contractions, even in the carp heart.

Morphological observations have shown a lack of the T tubule in fish cardiac muscles and variable distribution of the SR among species of fish (5, 23, 35, 37). The SR can be classified into the following four categories: subsarcolemmal cisternae, circular tubules, longitudinal tubules, and reticular SR (36). To gain insights into the roles of the RyR in fish cardiac muscle contraction, it is critically important to determine the localization of the protein in the heart.

Only a few references to date have reported the localization of the RyR in fish cardiac muscle. One of the major reasons for this seems to be the lack of antibodies that adequately react with the RyR of fish cardiac muscle. In preliminary experiments, we observed that two commercially available monoclonal anti-RyR antibodies (C3–33 and 34C) failed to react with the RyR from the carp heart. In the present study, we prepared a new antibody that can react with all preparations of carp heart, we observed the negative staircase phenomenon characteristic of the rat heart (42) and ryanodine-sensitive postrest contractions (8), which are believed to be related to Ca2+ release from the SR (4, 6, 39, 41). In addition, Ca2+-dependent [3H]ryanodine binding has been found in SR membrane preparations from the fish hearts (8, 44), indicating the presence of the ryanodine receptor (RyR) as the Ca2+ release channel in the SR (14, 15). These findings suggest that the SR and RyR play important roles in cardiac muscle contractions, even in the carp heart.

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servation, our results suggest that the RyR exists largely in the subsarcolemmal and corbular SR in the carp heart.

**MATERIALS AND METHODS**

All protocols in this study were approved by the Institutional Animal Care and Use Committee at the Kitasato University School of Veterinary Medicine and Animal Sciences and were in accordance with the *Guide for Care and Use of Laboratory Animals* in the Institute for Laboratory Animal Research.

**Experimental animals.** Carp of either sex weighing ~1 kg were purchased from the Owada fish farm (Aomori, Japan). Male Wistar rats weighing 250–350 g and male Hartley guinea pigs of 300–400 g were purchased from Gokita Breeding Service (Tokyo, Japan).

**SR membrane preparations.** The membranes of the SR were prepared as described previously (8) with some modifications. Carp kept in chilled water were anesthetized with MS-222 (Sankyo, Tokyo, Japan) at a final concentration of 0.02% in the water tank and then killed by destruction of the spinal cord with a sharp needle, and an incision was made along the abdominal midline to expose the heart. After the blood was completely removed by perfusion with a HEPES buffer solution containing protease inhibitors (20 mg/ml leupeptin, 20 mg/ml antipain, 20 mg/ml aprotinin, 20 mg/ml chymostatin). Muscle tissues (10 g) were homogenized in 3 vol of 10 mM 3-(N-morpholino)-2-hydroxy-propanesulfonic acid (MOPSO)-KOH (pH 6.8) using a Waring blender at the maximum speed for 15 s. This procedure was repeated four times at 20-min intervals. The homogenate was filtered through a filter paper (Whatman no. 41), and microsomal pellets were obtained by centrifugation at 27,000 × g for 20 min. The supernatant was resuspended in a buffer (50 mM KCl, 10 mM MOPSO-KOH, pH 6.8) and sedimented again. The microsomes were resuspended in the same buffer containing 0.3 M sucrose, quick-frozen in liquid nitrogen, and stored at ~80°C until use.

**Anti-RyR antibody preparation.** The anti-RyR antibody was prepared by the method of Murayama and Ogawa (26). A synthetic peptide was designed from a sequence of 14 amino acids, HPASKRSEGKEVR, which corresponds to amino acids 139–152 of the human RyR1 and is well conserved among all sequenced RyR isoforms of vertebrates and invertebrates (Fig. 1). A cysteine residue was added to the COOH terminus of the synthetic peptide to allow linking to hemocyanin via lysine residues using the bifunctional agent m-maleimidobenzoyl-N-hydroxysuccinimide ester. Rabbits were immunized with the synthetic peptide monthly for 5 mo, and the antisemur was collected after the sixth month. The antisemur was affinity purified with peptide-conjugated EAH-Sepharose as described previously (26).

**SDS-PAGE and Western blot analysis.** SDS-PAGE of the SR membranes was performed using 2–12% linear gradient gels by the method of Laemmli (21). The following molecular mass standards were used (in kDa): 205 yosin heavy chain, 116 β-galactosidase, 97.4 phosphorylase b, 66 bovine serum albumin, 45 ovalbumin, and 29 carbonate anhydride. Gels were stained with Coomassie Brilliant Blue.

Western blot analysis was carried out as described previously (25). Briefly, proteins resolved on SDS-PAGE were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (PALL, East Hills, NY) at 50 V for 3 h. The PVDF membrane was blocked for nonspecific reactions with 0.5% casein in Tween-Tris buffer solution (TTBS) (0.05% Tween 20, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.5) for 1 h at room temperature and incubated overnight at 4°C with the anti-RyR antibodies diluted with 0.5% casein-TTBS (1:1,000 dilution). The membranes were washed with TTBS and incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) at 25°C for 90 min. A positive band was detected using an ECL system (Amershamb Bio-sciences).

**Immunohistochemical studies.** The hearts of carp, rats, or guinea pigs were removed, frozen in isopentane chilled with liquid nitrogen, and divided into atrial and ventricular muscles. They were cut into 10-μm-thick sections with a cryostat (Leica, CM3050). The sections were fixed with 4% paraformaldehyde and 4% sucrose in 100 mM phosphate buffer (pH 7.4) for 60 min and washed three times with a solution containing 0.05% Tween 20, 10 mM phosphate buffer (pH 7.4), and 500 mM NaCl (TPBS) for 5 min. The sections were protected for nonspecific binding with Block-ace (Dainihon

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Fig. 1. Amino acid (aa) sequence alignment of various ryanodine receptors (RyRs) around the epitope for the anti-RyR antibody. Amino acid sequences near the NH₂ terminal of 9 RyRs from vertebrates and invertebrates were aligned with each other. The number of starting and ending residue is indicated in (left and right margins, respectively. The identical residues are marked by the box. Note that the epitope sequence (14 residues) for antibody production is identical among all the RyRs indicated, hRyR1–3, human RyR1–3 (hRyR1 (46), hRyR2 (45), hRyR3 (27)); rRyR1 and 3, frog RyR1 (33) and RyR3 (33); RyR3, chicken RyR3 (32); mRyR1, blue marlin RyR1 (16); dmRyR, Drosophila melanogaster RyR (40); cErRyR, Caenorhabditis elegans RyR (34).
Pharm, Tokyo, Japan) at room temperature for 60 min and washed once with TPBS for 5 min. Subsequently, they were treated with the anti-RyR antibody at 4°C for 3 days in a moisture box, conditions that allowed more intensive staining than the overnight incubation (data not shown). Then, the sections were washed three times with TPBS for 5 min and treated with a secondary antibody, FITC-conjugated goat anti-rabbit IgG (Biomedical Technologies), at room temperature for 1 h. After three washings with TPBS, the sections were enclosed with an antifade reagent (Fluoro-Guard, Bio-Rad). They were observed with a confocal laser-scanning microscope (LSM410, Carl Zeiss) to combine the fluorescent images with the differential interference contrast images. A high-power objective (Carl Zeiss, Plan-Apochromat, ×63/numerical aperture 1.4, oil) was used with standard Zeiss immersion oil. The pinhole size was set to 20 μm.

For double staining of the RyR and actin, specimens were treated first with FITC-conjugated goat anti-rabbit IgG for RyR staining, as described above, and then with 0.1 μM Rhodamine-Phalloidin (Sigma) overnight at 4°C for actin staining.

Preparations of specimens for electron microscopic analysis. Specimens for electron microscopy were prepared according to the procedure described previously (8). Muscle tissues, 1 mm³, were dissected from the atrial muscles of carp or rat hearts and fixed by immersion in a 2.5% glutaraldehyde solution containing 0.1 M cacodylate buffer. The tissues were postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide for 2 h at 4°C, dehydrated in a graded series of ethanols, and then embedded in epoxy resin. Ultrathin sections were double stained with lead citrate and uranyl acetate and examined using a transmission electron microscope (H7000, Hitachi-Seisakusho, Tokyo, Japan).

RESULTS

Detection of the RyR in SR vesicle membranes of the carp heart by Western blot analysis. To determine whether the RyR is present in carp heart muscles, we initially carried out Western blot analysis of the SR membranes prepared from carp hearts. In preliminary experiments, we used two commercially available monoclonal anti-RyR antibodies, 34C (2) and C3–33 (22, 38), but no positive reaction was obtained with either (data not shown). This was thought to be due to loss of reactivity or weak cross-reactivity of these antibodies with the carp heart RyR rather than to the absence of the RyR in the carp heart. We therefore attempted to produce an antibody that could recognize the carp heart RyR. A peptide HPASKQRSEGEKVR was chosen as the antigen, which corresponds to amino acid residues 139–152 of the human RyR1 and is well conserved among all sequenced vertebrate and invertebrate RyRs (Fig. 1). The peptide was used to immunize rabbits, and the collected antisera was affinity purified before use, as described in MATERIALS AND METHODS.

Figure 2 demonstrates the results of Western blot analysis with the newly prepared antibody. The SR vesicles prepared from rat skeletal muscle (lane 1), cardiac muscles of rat (lane 2), guinea pig (lane 3), and carp (lane 4) were separated by SDS-PAGE on a 2–12% polyacrylamide gradient gel and stained with Coomassie Brilliant Blue. Arrowheads and * indicate a protein of a high molecular mass corresponding to that of the RyR. The anti-RyR antibody specifically reacted with the protein band of the high molecular mass in each specimen. Note that the RyR in the carp heart (‡) showed mobility slightly greater than those of mammalian hearts.

Preparation of mammalian muscles (arrowheads). A band with similar size was also found in the carp heart (‡). On Western blots, the antibody clearly and exclusively recognized these bands in both mammalian muscles and carp heart (Fig. 2B). The reacted bands in mammalian muscles corresponded to those recognized by 34C and C3–33 (data not shown). These results indicate that the antibody prepared in this study is highly specific for the RyR and that a RyR homologue is definitely expressed in the carp heart. It should be noted that the RyR in the carp heart showed slightly greater mobility than those in mammalian hearts.

The tissue distribution of the RyR in the carp heart is demonstrated in Fig. 3A. SR vesicles prepared from the whole heart (lane 1), the atrial muscle (lane 2), the compact layer (lane 3), and the spongy layer (lane 4) of the ventricular muscle were subjected to SDS-PAGE, followed by Western blotting. There was no difference in mobility or density of single bands detected by the antibody among whole cardiac muscle, atrium, or ventricle (arrowheads). These results indicate that the RyR is expressed in both atrial and ventricular muscles of the carp heart.

In mammalian vertebrates, the cardiac RyR isoform RyR2 is distinct from those found in the skeletal mus-
muscle, but it failed to react with the RyR in the heart. Note that 34C recognized the 2 RyR bands in the skeletal muscle. The cardiac RyR2 advanced further than the RyR3 homologue of skeletal muscle of carp with the anti-RyR antibody (Fig. 3). The right lane from the heart and skeletal muscle (skeletal m.) of ventricular muscle of carp. Arrowhead indicates the RyR band. Two isoforms of RyR, RyR1 and RyR3 (29). Fish skeletal muscles express the RyR isoforms of carp skeletal muscle or from RyR2 of mammalian and chicken hearts. The heart RyR is immunologically distinct from the two RyR bands in the SR membranes prepared from carp swimming muscle. It also should be noted that these two bands of skeletal muscle were recognized by monoclonal antibody 34C but that the RyR from carp heart failed to react. These form striking contrasts to RyR2 in chicken heart: intermediate mobility between RyR1 and RyR3 and positive reaction with the monoclonal antibody 34C (3). Thus, the carp heart RyR is immunologically distinct from the two RyR isoforms of carp skeletal muscle or from RyR2 of mammalian and chicken hearts.

Immunohistochemical localization. The immunoreactivity of the anti-RyR antibody on a tissue section can be observed as a FITC-positive reaction with a confocal laser microscope (7, 19, 38). The FITC-positive reactions in rat ventricular muscles were observed as regular striations at ~2-μm intervals (Fig. 4A). However, as a negative control, when the anti-RyR antibody was coincubated with the synthetic peptide (HPASKQRSEGEKVR) used to prepare the anti-RyR antibody, no FITC-positive reaction was observed (Fig. 4B). Similar observations, albeit with a lesser intensity, were made in the guinea pig ventricular muscles (data not shown).

The FITC-positive reaction patterns in carp atrial (Fig. 4C) and ventricular [compact layer (Fig. 4E) and spongy layer (Fig. 4F)] muscles, however, were entirely different from those in rats or guinea pigs. The clusters of positive reaction sites were distributed in a punctate manner throughout the myocytes of the atrium or ventricle. TheFITC-positive reactions were completely absorbed and disappeared by incubation of the antibody with the coexisting synthetic peptide (negative control) (Fig. 4D), proving the specificity of the antibody. In the duplicate image of the FITC-positive reaction (green) and differential interference, positive reactions were observed as punctate staining around myofibrils inside muscle cells (Fig. 5A). In carp ventricular muscles, there are fewer myofibrils in the spongy layer than in the compact layer, as has been reported in other types of fish (1, 36), and there may be proportionately fewer FITC-positive reactions (green) in the spongy layer (Fig. 5B).

To pinpoint further the localization of antibody (FITC)-positive sites, double staining experiments in combination with actin labeling with rhodamine-phalloidin (13) were performed (Fig. 6). In longitudinal sections of rat ventricles, striations ascribable to actin (rhodamine positive) and those due to FITC-positive sites were observed in register, but few striations were yellow (Fig. 6A). In the cross sections, bundles of myofibrils of ~15 μm in diameter were surrounded by FITC-positive sites (Fig. 6B). This is probably the reason why the merged colored sites were infrequent. In the carp ventricular compact layer, rhodamine-positive reactions were observed in striation, as in rats, but FITC-positive reactions, in contrast, were observed in a punctate or patchy manner, independent of striations of actin (Fig. 6C). A cross-sectional image showed FITC-positive sites in a punctate or patchy pattern around rhodamine-positive reactions (Fig. 6D).

Electron microscopic observations. Finally, transmission electron microscopic observations were performed to identify directly the localization of the SR in carp and rat heart.

In rat atrial muscles, the SR (diameter: 75–150 nm) was observed near the Z-line, around myofibrils, and immediately under the sarcolemma (Fig. 7A). In carp atrial muscles, the SR (diameter: 50–100 nm) was also observed just near the subsarcolemmal regions around myofibrils but in a lesser density than in rat atrial muscles. They were not restricted to the Z-line regions (Fig. 7B). As shown in Fig. 7C, the reticular SR surrounding myofibrils (arrowhead) was also observed, in addition to the subsarcolemmal cisternae (arrow).

DISCUSSION

To detect the RyR in the carp heart in the present study, we prepared an antibody against a 14-amino acid sequence, HPASKQRSEGEKVR, which is well conserved among all sequenced RyR isoforms. Western...
blot analysis revealed that the antibody exclusively reacted with a single band of high molecular mass in SR membranes prepared from rat skeletal muscle and rat and guinea pig cardiac muscles. The reacted bands were identical to those recognized by commercially available monoclonal antibodies (34C and C3–33). In addition, rat ventricular muscles labeled with this newly raised antibody demonstrated regular striations corresponding to the Z lines where dyad junctions are localized (7, 19). Thus, the designed antibody specifically recognizes RyRs in Western blot analysis and immunohistochemistry. The antibody can react with α- and β-RyRs of fish swimming muscles. Because the epitope sequence is conserved among all sequenced RyRs, it is expected that the antibody can recognize all RyRs, irrespective of isoform.

In the SR membranes from carp heart, the antibody reacted specifically with a high molecular mass band of mobility similar to those of mammalian RyRs. The band was consistently detected in various portions of the SR membranes from carp heart.

Fig. 4. Immunohistochemical localization of the RyR in the carp myocytes. Fluorescent images were obtained after treating frozen sections with a primary antibody (the anti-RyR antibody raised in a rabbit) at 4°C for 3 days and then with a secondary antibody (FITC-conjugated goat anti-rabbit IgG) at room temperature for 1 h. A: rat ventricular muscle. Positive reactions were observed along Z lines of sarcomers. B: negative control. The specimen in A was treated simultaneously with the antigenic synthetic peptide. No positive reaction was observed. C: carp atrial muscles. D: negative control. The specimen in C was treated simultaneously with the antigenic peptide. E: carp ventricle (compact layer) muscles. F: carp ventricle (spongy layer) muscles. A and B: bars = 10 μm; C–F: bars = 20 μm.
the carp heart, atrium, and compact and spongy layers of the ventricle. These results provided evidence for the presence of the RyR in the carp heart. The finding that the anti-RyR monoclonal antibody 34C did not react with the carp heart SR, but did react with the two isoforms of RyRs in the carp skeletal muscle, strongly indicates that the heart RyR has an epitope and/or conformation distinct from those of the two skeletal muscle isoforms. Because the two skeletal muscle RyR isoforms in fish are thought to be homologues of mammalian RyR1 and RyR3 (16), the carp heart RyR may be the RyR2 homologue, as is true with hearts of mammals and chicks.

The mobility of the carp heart RyR was slightly greater than those of mammalian RyR2 and the carp RyR3 homologue. In addition, two commercially avail-

Fig. 5. Immunohistochemical localization of the carp heart RyR by confocal microscopy. Combined fluorescent and differential interference contrast microscopic images for carp atrial (A) and ventricle (B) muscles. Green: (specifically FITC positive) images overlayed differential interference contrast images. B: white line indicates the border between compact and spongy layers. A and B: bars = 10 μm.

Fig. 6. Double-staining images of rat and carp ventricles in the confocal microscope. Fluorescent images were obtained after treating with rhodaminephalloidin at 4°C for overnight. Frozen sections were treated with a primary antibody (rabbit RyR antibody) at 4°C for 3 days and a secondary antibody (FITC-conjugated goat anti-rabbit IgG) at room temperature for 1 h before the rhodamine-phalloidin treatment. A: longitudinal section of rat ventricular muscles. B: cross section of rat ventricular muscles. C: longitudinal section of carp ventricular muscles (compact layer). D: cross section of carp ventricular muscles (compact layer). A-D: bars = 10 μm.
able anti-RyR monoclonal antibodies (34C and C3–33) did not recognize the carp heart RyR, indicating possible alterations of the epitope sequence in the RyR. It is interesting that chicken RyR2 showed an intermediate mobility on SDS-PAGE between the two isoforms of chicken skeletal muscle and reacted positively with 34C. Thus, it is quite probable that the carp heart RyR may have the primary structure distinct from the mammalian and chicken RyRs and that these differences might underlie their different functional properties. Further studies of molecular cloning of the fish heart RyR will clarify this matter.

In immunohistochemical experiments using this new anti-RyR antibody, the specific reactions of the rat cardiac muscle RyR were found to be localized in striation around the Z-line area. This observation is consistent with previous reports (7, 18, 20, 22, 38). Moreover, the FITC-positive reaction in guinea pig cardiac muscle appeared weaker than that of rat cardiac muscle. This difference may be due to the scarcity of the RyR in the guinea pig heart, as was suggested by a physiological functional study using isolated heart muscle preparations (6).

In the carp heart, in contrast, the FITC-positive reaction did not show striations, and the immunoreactive sites were not restricted within such a specified region as the Z line but were scattered in a punctate manner throughout the sarcoplasm. This characteristic distribution was observable not only in longitudinal sections but also in cross sections, as evidenced by FITC-positive reactions enclosing rhodamine-positive sites in double-staining experiments of actin and the RyR. Santer (36) reported in an electron microscopic study that fish SR could be classified into four categories: 1) subsarcolemmal cisternae, existing right under the sarcolemma, separated from myofibrils, and connected to other structures; 2) circular tubules, surrounding myofibrils in a circle; 3) longitudinal tubules, with the SR running longitudinally; and 4) reticular SR, with the SR covering myofibrils in a hexagonal grid-like pattern. The present electron microscopic study indicates much less circular tubules or longitudinal SR in carp cardiac muscles. As reported by Santer (36), in fish such as rainbow trout, the reticular SR is dominant, with a well-developed compact layer. Because the carp heart has a well-developed compact layer, it can be assumed that the reticular SR is abundant in the carp heart (Fig. 7C). In this case, the RyR will exist as corbular SR, because of no or scanty T tubules in the carp heart. Because the cardiac cells of fish are generally smaller in diameter than those of mammals, the T tubules will not always be necessary for rapid internal propagation of excitation (36). The subsarcolemmal cisternae are reasonably thought to perform functions such as dyads do in developing myotubes to release Ca\(^{2+}\) from RyRs on the excitation of cell membranes (17). In fact, electron microscopic images of carp cardiac muscles indicated that the subsarcolemmal SR was observed right under the cell membrane in greater abundance than in rats. It is quite reasonable to assume that the RyR occurs in the junctional surface of the SR.

In conclusion, the biochemical and intracellular distributions of the RyR in carp heart muscle cells are different from those of mammalian RyRs. However, the carp RyR should have an important role in contraction in atrial muscle and in the compact and spongy layers of ventricular muscle.

![Image](https://www.ajpregu.org)
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