Cloning and characterization of fiber type-specific ryanodine receptor isoforms in skeletal muscles of fish

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Franck, Jens P. C., Effery Morrissette, John E. Keen, Richard L. Londraville, Mark B. Beamssley, and Barbara A. Block. Cloning and characterization of fiber type-specific ryanodine receptor isoforms in skeletal muscles of fish. Am. J. Physiol. 275 (Cell Physiol. 44): C401–C415, 1998.—We have cloned a group of cDNAs that encodes the skeletal ryanodine receptor isoform (RyR1) of fish from a blue marlin extracellular muscle library. The cDNAs encode a protein of 5,081 amino acids with a calculated molecular mass of 576,302 Da. The deduced amino acid sequence shows strong sequence identity to previously characterized RyR1 isoforms. An RNA probe derived from a clone of the full-length marlin RyR1 isoform hybridizes to RNA preparations from extracellular muscle and slow-twitch skeletal muscle but not to RNA preparations from fast-twitch skeletal or cardiac muscle. We have also isolated a partial RyR clone from marlin and toadfish fast-twitch muscles that shares 80% sequence identity with the corresponding region of the full-length RyR1 isoform, and a RNA probe derived from this clone hybridizes to RNA preparations from fast-twitch muscle but not to slow-twitch muscle preparations. Western blot analysis of slow-twitch muscles in fish indicates the presence of only a single high-molecular-mass RyR protein corresponding to RyR1. [3H]ryanodine binding assays revealed the fish slow-twitch muscle RyR1 had a greater sensitivity for Ca2+ than the fast-twitch muscle RyR1. The results indicate that, in fish muscle, fiber type-specific RyR1 isoforms are expressed and the two proteins are physiologically distinct.

Fiber types

The process of excitation-contraction (EC) coupling in muscles involves the release of Ca2+ from sarcoplasmic reticulum (SR) stores. This release is mediated by the SR Ca2+ release channel, a large tetrameric protein complex of ~2.2 x 106 Da. The affinity of the SR Ca2+ release channel for the plant alkaloid ryanodine has provided a tool for the purification and biochemical characterization of this protein common referred to as the ryanodine receptor (RyR) (22, 23). Vertebrate RyRs identified to date are all similar-sized homotrimeric proteins composed of four polypeptide subunits with molecular masses of 500–600 kDa (37). mRNAs for the three known isoforms of the RyR gene family have been cloned and sequenced. The primary sequences for these isoforms were first characterized from mammalian tissues, including RyR1 from rabbit and human skeletal muscle (39, 42), RyR2 from rabbit cardiac muscle (29), and RyR3 from rabbit brain (20).

Giannini et al. (19) extensively surveyed the expression of the three RyR isoforms in various marine tissues. With the use of both RNA antisense probes and isoform-specific antisera, a much wider tissue distribution of the mammalian isoforms of the RyRs emerged.

Three homologous isoforms of the RyR have also been identified in nonmammalian tissues based on molecular, biochemical, immunologic, and physiological results (1, 25, 28, 30, 31). In contrast to adult mammals that predominantly express only the RyR1 isoform in skeletal muscle, nonmammalian skeletal muscle expresses two isoforms, originally described as α- and β-RyRs, due to unknown homologies to mammalian isoforms (1, 25, 28). The cloning and characterization of the two skeletal RyR isoforms from bullfrog have revealed the α- and β-RyRs are homologous to the mammalian RyR1 and RyR3 gene products, respectively (30, 31). Partial sequence data indicate that in lower vertebrates the homolog of the RyR2 gene family is also present in cardiac muscle (J. Keen and B. A. Block, unpublished data). In Western blot analysis of nonmammalian skeletal muscles, RyR1 and RyR3 occur as discrete high-molecular-weight bands, with RyR3 having a slightly higher mobility. Exceptions to this expression pattern exist in certain nonmammalian muscles. In specialized muscle fibers of nonmammals, such as the super-fast-contracting swim bladder muscle of toadfish, immunoblotting experiments have determined that RyR1 is the sole isoform expressed, making the muscle a pure source of the nonmammalian RyR1 isoform (3, 25). The ryanodine binding characteristics and single-channel conductance of the fish RyR1 channel indicate it is the functional homolog of the mammalian RyR1 protein (9, 32, 38). A second exception to the two RyR isoform expression pattern of nonmammalian skeletal muscles is the slow-twitch muscle fibers in fish. In a preliminary report of the results in this paper, we demonstrated that fish slow-twitch muscles do not express RyR3 (17).

Recent physiological studies of fish skeletal muscle fiber Ca2+ release kinetics indicate distinct Ca2+ transients are present in the slow- and fast-twitch muscles of fish (34). Studies in mammalian skeletal muscles also indicate that the mechanism of Ca2+ release differs between muscle fiber types (12, 13, 18, 36). The most prominent difference between fiber types is the slow course of intracellar Ca2+ transients. In addition, different sensitivities of the RyR Ca2+ release mechanism (in fiber and SR vesicle preparations) to known modulators of the RyR channel, such as Ca2+, Mg2+, ATP, caffeine, doxorubicin, and ruthenium red, indicate distinct differences between slow- and fast-twitch vesicle...
The molecular basis for the distinct Ca\(^{2+}\) transients has also been attributed to the presence of two isoforms of Ca\(^{2+}\)-ATPase in skeletal muscles of tetrapods (5) as well as differences in the troponin off-rate of Ca\(^{2+}\) (34). In mammals and fish, slow fibers have longer Ca\(^{2+}\) transients and more sensitive force-pCa relationships. In this paper, we present evidence for two distinct fiber type-specific SR Ca\(^{2+}\) release channels in the skeletal muscles of fish that may also be contributing to the physiological differences between fiber types (34).

The discrete anatomic separation of fast- and slow-twitch muscle fibers in fish provides an unparalleled system for studying the biochemical and molecular components of different muscle fiber types. One of the richest sources of slow-twitch muscle is found in the large open ocean fishes (marlin and tunas) that use this muscle type to power endurance swimming across ocean basins as well as for thermogenic purposes (2). The slow-twitch (red) muscles of tuna and marlin are composed of 100% slow-twitch fibers, and the fast or white muscles are 99% pure sources of fast-twitch fibers (40). In this study, we constructed a cDNA library from the superior rectus muscle to characterize the message for the fish RyR1 isoform. Our interest in the superior rectus muscle stems from studies on the thermogenic potential of eye muscles in marlin (3). We report the cloning and characterization of a unique vertebrate RyR1 message from blue marlin (Makaira nigricans) eye muscle (a mixed fiber type muscle) that is also expressed in slow-twitch muscles of fish. We have also cloned and sequenced partial RyR1 messages from cDNA libraries derived from super-fast-contracting toadfish swim bladder muscle and fast-twitch muscle of marlin. Hybridization of RNA probes from these three sources reveals a fiber type-specific distribution of the fish RyR1 isoforms, indicating the presence of both slow and fast RyR1 messages. Ryanodine binding assays with heavy SR vesicles isolated from slow- and fast-twitch muscle fibers reveal distinct sensitivities of the Ca\(^{2+}\) dependence of the ryanodine receptor proteins.

**MATERIALS AND METHODS**

cDNA cloning and sequencing. Total RNA was extracted from blue marlin (M. nigricans) tissues by either the guanidium isothiocyanate/cesium chloride method of Chirgwin et al. (8) or by using Tri-reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from 10 µg total RNA using an oligo(dT) primer. Reaction conditions for PCR included 200 ng template, 1 µM of each primer, 200 µM dNTPs, and 0.5 U Taq DNA polymerase (Promega Biotech, Madison, WI). First-strand cDNA synthesized from superior rectus muscle of blue marlin was used to amplify an ~750-bp PCR product with primers RyR24 (5'-AAGGCATCAATGACTACGACC-3') and RyR25 (5'-CTGTACATCAGACGAGGCC-3'). The PCR product corresponds to nucleotides 14056–14819 in the rabbit RyR1 open reading frame (ORF) (42). This PCR product was used to screen a commercially prepared oligo(dT)random-primed cDNA library derived from the superior rectus eye muscle of blue marlin (Stratagene, La Jolla, CA). For the initial screening and all subsequent library screenings, probes were labeled by random priming with [\(\alpha\)-\(^{32}\)P]dCTP according to Feinberg and Vogelstein (15) or with the Ready-to-Go random-priming kit (Pharmacia Biotech). The initial screening yielded clone \(\lambda\)BMRR1 (ORF 14,044–15,332; Fig. 1).

The cDNA library was also screened using a monoclonal antibody, Ab34C (28), which yielded clone \(\lambda\)BMRR2 (ORF 5133–8127). For the immunoscreening procedure, the library was plated at a density of 50,000 pfu/plate and incubated for 3.5 h at 42°C. The recombinant clones were induced to express by placing nylon membranes impregnated with 10 mM isopropyl-\(\beta\)-thiogalacto-pyranoside (Hybond-N, Amer sham, Arlington Heights, IL) on the plates and continuing incubation at 37°C for an additional 4 h. The membranes were preblocked in 2% dried milk and 1× Tris-buffered saline and 0.2% Tween (TBST) for 1 h followed by a wash for 10 min in 1× TBST. The primary antibody was diluted 1:1,000 in TBST and incubated with the membranes for 2 h. After the primary antibody incubation, the secondary antibody conjugate was diluted three times for 10 min each in 1× TBST. The membranes were subsequently incubated with the secondary antibody/alkaline phosphatase conjugate diluted 1:1,000 (goat anti-mouse) for 1 h. All incubations were performed at room temperature.
After the secondary antibody incubation, the membranes were washed three times for 10 min each in TBST. Positive clones were detected colorimetrically with the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical, St. Louis, MO). The human-specific RyR primer pair RR61FX (5′-ATCTCTAGATCCAAAAGCTGGGA-GCGAGCGAGG-3′) and RR20RX (5′-ATCTCTAGATGATG-GCCCAATGCTGGTCTG-3′) amplified an approximate 1,200-bp PCR product from blue marlin first-strand cDNA derived from the superior rectus muscle. This PCR product was subsequently used to screen the random/oligo(dT)-primed superior rectus muscle cDNA library, yielding two clones, λBMRR3 and λBMRR4 (ORF 10,003–11,685). Screening with this PCR product also yielded clones λBMRR5 (ORF 11,700–13,685) and λBMRR6 (ORF 11,375–11,725). The gap between clones λBMRR4 and λBMRR6 was closed by PCR amplification from first-strand cDNA with primers specific to the two clones. The PCR product pBMRR1 was cloned into the vector pGEM and sequenced, which determined its identity to clones λBMRR4 and λBMRR7 in overlapping regions. An approximate 800-bp PCR product amplified from the 3′-terminus of clone λBMRR2 was used to screen the random/oligo(dT)-primed library, resulting in the isolation of clone λBMRR7 (ORF 6800–8647). Clone λBMRR8 (ORF 2879–6000) was isolated from the library after screening with an ~380-bp PCR product derived from the 5′-end of clone λBMRR2. Two of the clones used in the assembly of the final RyR1 sequence were isolated from a primer extension library. An 18-mer primer complementary to nucleotide residues 3030–3047 in the final fish RyR1 ORF was used to prime first-strand synthesis from 2.5 µg poly(A)− RNA isolated with a Poly(A) Quik mRNA isolation kit from the superior rectus muscle of M. nigricans (Stratagene). After second-strand synthesis was completed, the cDNAs were blunt ended by adding 2 U Pfu DNA polymerase according to the manufacturer’s protocol (Stratagene). EcoR I adaptors were blunt-end ligated to the cDNAs and kinased using 10 U T4 polynucleotide kinase. Removal of excess adaptors and size fractionation of the cDNAs were performed by centrifugation through a 5-50% Sephacryl column according to the manufacturer’s instructions. The entire aliquot of the size-fractionated cDNA was ligated to the EcoR I vector arms and packaged using the Gigapack III Gold packaging extract. The primer extension library was screened with a PCR product derived from the 5′-terminus of the λBMRR8 cDNA clone. This screening yielded clone λBMRR9, which corresponds to nucleotides 985–3,191 in the final RyR1 ORF. Clone λBMRR9 was partially restriction mapped, and a 400-bp BamHI/Hind III restriction fragment from the 5′-end of the clone was used to rescreen the primer extension library. This yielded clone λBMRR10, which corresponds to nucleotides 869–3,190 in the final ORF. The clone that codes for the NH2 terminus of the fish RyR1 sequence was isolated from the original oligo(dT)/random-primed cDNA library using an ~350-bp probe amplified from the 5′-end of clone λBMRR10. This screening yielded clone λBMRR11, which contains 65 bp of 5′-untranslated sequence and extends to base 1,100 in the final ORF. PCR-amplified regions of the 3′- and 5′-ends of clones λBMRR7 and λBMRR4 were radiolabeled and used to screen the oligo(dT)/random-primed library, resulting in the isolation of clone λBMRR12 corresponding to nucleotides 8,948–10,374 in the final ORF. Rescreening of the library with a PCR-amplified region from the 5′-end of clone λBMRR12 yielded clone λBMRR13, which corresponds to nucleotides 6,941–8,690. The missing sequence between clones λBMRR12 and λBMRR13 was amplified from first-strand cDNA using a primer derived from the clones. The PCR clone was identical in sequence to the overlapping region of clone λBMRR12 and λBMRR13. The insert from clone pBMRR2 was radiolabeled and used to rescreen the oligo(dT)/random-primed library, which yielded two clones, λBMRR14 and λBMRR15, which correspond to nucleotides 8,736–9,732 and 8,715–9,450, respectively, in the final ORF.

cDNA libraries were constructed from RNA isolated from blue marlin white muscle RNA and toadfish swim bladder RNA using the λZAP kit of Stratagene. Both libraries were screened with a radiolabeled probe derived from the λBMRR1 clone using the primers RyR24 and RyR25 (see above). This screening yielded four clones from the toadfish swim bladder (TFSB) library, named λTFSB1 through λTFSB4, and one clone from the blue marlin white muscle (BMWM) library, named λBMWM1. The TFSB clones encompassed sequence corresponding to nucleotides 13,695–14,950 of the blue marlin ORF, whereas the BMWM clone contained sequence corresponding to nucleotides 13,750–15,150.

Ribonuclease protection assays. The RyR1-specific antisense probe was synthesized from a subcloned region amplified from clone λBMRR8 using U-strand primer RyRIeco (5′-TATGATTCCTCAAGAATGCTG-3′) and L-strand primer RyRko (5′-GATCTGGGCTGCTTTGCTGC-3′). The amplified probe was digested with the restriction enzymes EcoRI and Xho I and unidirectionally cloned into Bluescript SK + digested with EcoRI and Xho I. The subcloned region corresponds to nucleotides 4,075–4,315 in the blue marlin RyRI ORF. The antisense probe was synthesized from the EcoRI linearized clone with T7 RNA polymerase according to the Ambion Maxiscript T7/T3 in vitro transcription kit protocol (Ambion, Austin, TX). An antisense probe was synthesized from a 375-bp region of clone λTFSB1 using U-strand primer (5′-AGGATGATTCTGCTGACTCTG-3′) and L-strand primer (5′-AGGGAGCTCGAGGGCATTGTTA-TTC-3′). The PCR product was digested with the restriction enzymes EcoRI and Xho I and used to rescreen the oligo(dT)/random-primed library, named λTFSB1, yielding four clones from the toadfish swim bladder (TFSB) library, named λTFSB1 through λTFSB4. The antisense probe was synthesized from the TFSB linearized clone with T7 RNA polymerase. Total RNA for the ribonuclease protection assays (RPA) was prepared using TriZol reagent. The assay was performed using RNA according to the protocol of the Ambion Direct Protect RPA kit except that total RNA (20 µg) was used instead of tissue homogenates. All hybridizations were performed at 37°C. Samples were separated on a 6% sequencing gel that was dried and exposed to X-ray film for 24–72 h at –70°C with intensifying screens.

Heavy SR protein preparation. Approximately 10–25 g of blue marlin and tuna fast-twitch muscle, slow-twitch muscle, or toadfish swim bladder muscle were homogenized in 10 vol of homogenization buffer containing 300 mM sucrose, 5.0 mM Na2EGTA, 10.0 mM Na2EDTA, 20 mM K-PIPES, pH 7.3, 1.1 µM diisopropyl fluorophosphate, and various protease inhibitors using a Tekmar tissue homogenizer. Sodium pyrophosphate (25 mM) and 100 mM KCl were added to the homogenization buffer. The slurry was stirred on ice for 45 min to separate myofibrillar and SR proteins from triads. The homogenate was centrifuged for 50 min at 100,000 g in a T150.2 Beckman rotor. The supernatant was discarded, and the pellet was resuspended in homogenization buffer and centrifuged at 2,000 g for 20 min in a Sorval SS34 rotor. The supernatant was passed through two layers of cheese cloth, and the crude microsomes were pelleted by centrifugation at 100,000 g for 50 min. The pellets were resuspended in 300 mM sucrose and 5 mM K-PIPES, pH 7.0. This material was layered onto discontinuous sucrose gradients (6 ml 20%, 8 ml 30%, 8 ml 36%, and 4 ml 40% C403FIBER TYPE-SPECIFIC RYANODINE RECEPTORS IN MUSCLE C403
Fig. 2. Multiple alignment of fish RyR1 amino acid sequence to published RyR1 isoforms. Amino acid sequence deduced from fish RyR1 amino acid sequence was aligned to RyR1 isoform sequences from frog skeletal muscle (31), rabbit skeletal muscle (42), and human skeletal muscle (39). Amino acid positions that are identical to fish residue are indicated by black shading. Gaps have been introduced to permit alignment. Amino acid residue numbers are indicated at right.
Fig. 2—Continued.
Fig. 2—Continued.
RESULTS

Sequence determination of a full-length RyR1 isoform in fish. Screening of an oligo(dT)/random-primed library and a specific primer extension library with DNA and antibody probes resulted in the identification of a series of overlapping clones (Fig. 1). Compilation of the cDNA clones resulted in a 16,313-bp contiguous sequence. The contiguous sequence codes for an ORF of 15,243 bp with the initiation methionine at position 66 and the termination codon (TAG) at position 15,309. A termination codon TAA is found 15 bp upstream of the initiator methionine. The 3'-untranslated region is 1,002 nucleotides long. Polyadenylation signals with the motif AATAAA were located at 961, 892, and 247 bases upstream of the 3'-terminus. The complete fish RyR cDNA sequence encodes a protein of 5,081 amino acids with a deduced molecular mass of 576,302 Da, including the initiator methionine. A multiple alignment of the deduced amino acid sequence to RyR1 sequences of frog, rabbit, and human illustrates that the four sequences share large regions of sequence identity especially toward the highly conserved COOH terminus of the molecule (Fig. 2). The fish sequence is longer than the other three vertebrate RyR1 sequences, which is largely accounted for by two insertions, one of 30 amino acids from amino acid 1,851–1,880 and one of 25 amino acids from amino acids 1,926–1,950. The overall sequence identities between the fish RyR1 sequence and published sequences are given in Table 1. The deduced amino acid sequence from fish shares the greatest sequence identity with the frog RyR1 sequence (77%) and is 72 and 73% identical in pairwise comparisons with human and rabbit RyR1 amino acid sequences, respectively.

The fish RyR1 amino acid sequence was analyzed for conserved motifs for putative regulatory domains (Table 2). The RyR protein is known to be phosphorylated by kinases. With the use of the consensus sequence RXXS/T

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Deduced amino acid sequences were compared in a pairwise fashion, and sequence identities were calculated on a matches/length basis. Values given are percentage figures. Large deletions were counted as single mutational events. Fish ryanodine receptor (RyR1) sequence was compared with amino acid sequences of frog RyR1 (31), human RyR1 (39), rabbit RyR1 (42), rabbit RyR2 (29), chicken RyR3 (30), frog RyR3 (31), and rabbit RyR3 (20).

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Putative modulatory sites for calmodulin-dependent protein kinase (consensus RXXS/T) and nucleotide binding (consensus GXGXXG) (41) were predicted by computer block search of deduced amino acid sequence for full-length fish RyR1 isoform (Fig. 2).
for calmodulin-dependent protein kinase, a computer block search of the deduced amino acid sequence revealed 16 sites. Six of the sites were conserved in all RyR1 sequences, and five were unique to the fish RyR1 sequence. The activity of the RyR is also known to be modulated by adenine nucleotides. With the use of the nucleotide binding site consensus sequence, GXGXXG, seven sites were identified in the fish amino acid sequence. Five of the sites were completely conserved in all RyR1 sequences, and two were unique to the fish sequence. Takeshima et al. (39) in their description of the first RyR isoform characterized from human skeletal muscle (RyR1) identified a putative Ca\(^{2+}\) binding domain between residues 4,253 and 4,499. Site-directed antibodies raised against specific fusion proteins further resolved that the Ca\(^{2+}\) binding domain lies between residues 4,478 and 4,512 (6, 7). Within this region is a PE amino acid repeat motif that is reiterated six times in the mammalian RyR1 isoforms. It was hypothesized that this region or region(s) adjacent to it may be the sites of Ca\(^{2+}\) binding. The multiple alignment of the fish RyR1 sequence to other RyR1 isoforms reveals that the PE repeat motif is not conserved (residues 4,519–4,532). The multiple alignment does, however, reveal a stretch of six amino acids (EPEKAD) adjacent to the PE repeat that is completely conserved between all RyR1 isoforms.

The fish RyR1 sequence was analyzed for potential transmembrane regions using the Predict Protein algorithm (35). Predictions improve with the addition of more evolutionary divergent vertebrate taxa. Transmembrane regions using this algorithm are predicted at a >95% expected accuracy per residue for membrane proteins. Four transmembrane regions were predicted (4,596–4,615, 4,686–4,712, 4,885–4,900, and 4,910–4,931) that correspond closely to the four regions M1-M4 originally predicted by Takeshima et al. (39) for the mammalian RyR1 isoform.

To determine the evolutionary relationship of the RyR isoform characterized from the superior rectus muscle library, the deduced amino acid sequence of the fish RyR isoform was aligned to the published mammalian and amphibian RyR sequences using the CLUSTALW multiple alignment program. The Drosophila RyR sequence was included in the analysis to serve as the designated outgroup. The multiple alignment was analyzed using the protein parsimony algorithm ProtPars of PHYLIP (16). A full-length RyR1 isoform clustered with the RyR1 isoforms of frog, rabbit, and human (Fig. 3). The tree also clustered the RyR3 isoforms of rabbit, frog, and chicken in a separate clade. The RyR2 isoform sequence of rabbit is found on a separate branch and appears to be the most primitive of the three isoforms.

An RPA was performed to qualitatively determine the distribution of the RyR1 message cloned from the marlin eye muscle cDNA library. The message was detected in RNA isolated from marlin eye muscle, marlin slow-twitch muscle, and tuna slow-twitch muscle. The message could not be detected by hybridization in RNA isolated from marlin or tuna fast-twitch muscle (Fig. 4). Longer exposures revealed a very low level of expression in the fast-twitch muscle RNA preparations from marlin and tuna (data not shown).

Identification of fast-twitch muscul specific RyR1 isoforms. To determine if fish express fiber type-specific RyR1 isoforms, we screened cDNA libraries derived from toadfish swim bladder (RyR1 only muscle) and blue marlin fast-twitch muscles (RyR1 and RyR3) (25). Both libraries were screened with a radiolabeled probe.
amplified from the λBMRR1 clone. This screening yielded four clones from the TFSB library named λTFSB1 through λTFSB4 and one clone from the BMWM library named λBMWM1. The TFSB clones generated a contiguous sequence corresponding to nucleotides 13,695–14,950 of the blue marlin eye muscle RyR ORF, whereas the BMWM clone contained a sequence corresponding to nucleotides 13,750–15,150.

The derived amino acid sequences from these two contiguous sequences were aligned to the previously described RyR1 sequence from marlin eye muscle and the RyR2 and RyR3 sequences from rabbit and frog (Fig. 5). The alignment reveals several fast-twitch muscle-specific amino acid residues (conserved in toadfish swim bladder and marlin white muscle but not present in the RyR1 sequence from extracardiac muscle). Phylogenetic comparison of the toadfish swim bladder and marlin fast-twitch muscle sequences to the marlin eye muscle sequence and other RyR sequences clustered the two fast-twitch sequences together to the exclusion of the marlin eye muscle sequence and in a larger cluster with all the RyR1 sequences (Fig. 6).

Tissue distribution of the fast-twitch RyR1 message was determined using RPAs with a probe synthesized from the toadfish swim bladder clone λTFSB1 (Fig. 7). The RNA probe hybridized to RNA preparations from toadfish swim bladder, toadfish fast-twitch muscles, marlin fast-twitch muscle, and tuna fast-twitch muscle. Importantly, the message could not be detected by hybridization, even after long exposures, in marlin or tuna slow-twitch muscle. An RNA probe constructed from the blue marlin fast-twitch muscle clone also hybridized to RNA isolated from the fast-twitch muscles but not the slow-twitch muscles (data not shown).

Ryanodine binding and Western blot analysis. The previous results suggest that two distinct RyR1 isoforms are expressed in fish skeletal muscle in a fiber-type specific manner. [3H]Ryanodine binding was performed to characterize the properties of the RyR isoforms in fish fast- and slow-twitch muscles. [3H]Ryanodine binding to RyR1 in mammalian skeletal muscle SR vesicles displays a classic bell-shaped Ca²⁺ dependency, with activity occurring at micromolar Ca²⁺ concentrations and inactivation occurring at millimolar

Fig. 5. Multiple alignment of marlin white muscle and toadfish swim bladder partial RyR sequence. Partial amino acid sequences from marlin white muscle (BM fast) and toadfish swim bladder muscle (TFSB) were aligned to corresponding regions of rabbit RyR2 isoform (RabRyR2; Ref. 29), frog RyR3 isoform (FrogRyR3; Ref. 31), rabbit RyR3 isoform (RabRyR3; Ref. 20), and RyR isoform characterized from eye muscle (BM slow). Highlighted residues indicate fast-twitch specific (BM fast and TFSB) and slow-twitch specific (BM slow) amino acids. The amino acid alignment is from 4618 to 4955 in the fish RyR1 (Fig. 2).
Ca\textsuperscript{2+} concentrations. \[^{3}H\]ryanodine binding to SR preparations from marlin fast-twitch muscle exhibited this bell-shaped Ca\textsuperscript{2+} dependency with a peak at pCa 4 (Fig. 8A). The SR preparation from marlin slow-twitch muscle also exhibited a bell-shaped Ca\textsuperscript{2+} dependency but with the peak shifted to a lower [Ca\textsuperscript{2+}] of pCa 5 (Fig. 8B). Additional ryanodine binding assays with SR preparations from tuna fast-twitch muscle and tuna slow-twitch muscle confirmed the marlin results (Fig. 8, D and E). The data from the binding curves in Fig. 8 were normalized to show the amount of \[^{3}H\]ryanodine binding relative to the peak for each curve (Fig. 9). The left shift in the Ca\textsuperscript{2+} sensitivity for the slow-twitch preparations is clearly evident. Western blot analysis using the antibody C010, which reacts against an epitope common to all RyRs (25), detected two high-molecular-weight bands in the fast-twitch (white) muscle preparations previously identified as RyR1 and RyR3 (25). Surprisingly, only one band, with a mobility similar to the RyR1 protein of fast-twitch muscles, was found in the slow-twitch muscle SR preparations (Fig. 10). Thus the properties examined in the \[^{3}H\]ryanodine binding assays can be attributed to this one RyR protein expressed in the slow-twitch preparations.

To establish that the differences in \[^{3}H\]ryanodine binding between slow- and fast-twitch muscle SR preparations were not because of the influence of RyR3 in the fast-twitch muscle preparations of marlin, we performed binding with an SR preparation from the superfast toadfish swim bladder muscle, which does not express RyR3 (25). The binding characteristics for the toadfish swim bladder SR preparation were similar to the fast-twitch muscle preparations with a peak of binding at pCa 4 (Fig. 8E). This indicated that the differences observed in the Ca\textsuperscript{2+} sensitivity of fast- and slow-twitch muscle were because of the presence of the distinct RyR1 isoforms and not because of the presence or absence of RyR3 in the preparation.

**DISCUSSION**

The results of this study suggest that fast- and slow-twitch muscles of fish express specific RyR isoforms. The full-length RyR1 sequence presented here represents a novel vertebrate isoform of the RyR1 gene family. The cDNA isolated from the superior rectus muscle library encodes a deduced amino acid sequence that is 77% identical in pairwise comparison to the frog RyR1 isoform. The calculated molecular mass of 576 kDa is also similar to the RyR1 isoforms of mammals and amphibians (31, 39, 42). Molecular phylogenetic analyses also confirm that the cDNA codes for a RyR isoform that is most closely related to the RyR1 isoforms (Fig. 3).

Hybridization of an antisense RNA probe was performed to resolve the tissue distribution of the fish eye muscle RyR1 isoform. The message for the fish RyR1 isoform is preferentially expressed in the slow-twitch (red) muscle of fish (Fig. 4). Surprisingly, this RyR1 message is not expressed in fast-twitch muscles of three fish species examined (marlin, tuna, and toadfish). The message can only be detected in minor abundance if exposure times are significantly increased, and this most likely corresponds to the presence of a small number of slow-twitch fibers in these muscles. The message is detected in the mixed fiber type superior rectus muscle (40) from where the library was originally constructed. The full-length cDNA we have cloned and sequenced is designated RyR1 slow.

The expression results prompted us to determine if a second, fast-twitch muscle RyR1 isoform may also be expressed in fish muscles. We constructed and screened cDNA libraries derived from marlin fast-twitch muscle and toadfish swim bladder muscle and were able to...
obtain partial RyR sequences from both tissues. Previous expression studies based on the presence of isoforms of the Ca\(^{2+}\)-ATPase (SERCA1 and SERCA2) have shown the toadfish swim bladder muscle is composed of only fast-twitch fibers (40). The toadfish swim bladder muscle and marlin fast-twitch muscle sequences share high identity with the RyR1 gene family and are similar but not identical to the RyR sequence derived from the extraocular eye muscle library. Phylogenetic analyses determined that these partial sequences are closely related to the fish slow-twitch isoforms but significantly distinct. Importantly, the marlin fast-twitch muscle and toadfish swim bladder muscle sequences are more closely related to each other than either is to the marlin slow-twitch sequence (Fig. 6). A probe derived from the toadfish swim bladder muscle RyR1 clone hybridizes to messages in swim bladder muscle and fast-twitch muscle fibers of marlin, toad-
Fig. 9. Normalized Ca\(^2+\) sensitivity curves for \([\text{H}]\)ryanodine binding to fast- and slow-twitch muscle. Binding data from Fig. 8 were normalized to show amount of \([\text{H}]\)ryanodine binding relative to peak for each curve. Left shift in slow-twitch muscle preparations is clearly evident. ●, Tuna slow-twitch red muscle; ■, blue marlin slow-twitch red muscle; ▲, tuna fast-twitch white muscle; ◊, blue marlin fast-twitch white muscle; ▲, toadfish swim bladder muscle.

Fig. 10. Western blot analysis with C010 antisera. C010 antisera were reacted against SR protein preparations from toadfish swim bladder (TFSB), blue marlin superior rectus eye muscle (BMSR), blue marlin fast-twitch muscle (BMWM), and blue marlin slow-twitch muscle (BMRM). Antisera recognize a single RyR1 isoform in toadfish swim bladder, blue marlin eye muscle, and blue marlin slow-twitch muscle and react against 2 polypeptides (RyR1 and RyR3) in SR preparation of blue marlin fast-twitch muscle.

Fish, and tuna, but not to slow-twitch muscle from marlin and tuna (Fig. 7). These results indicate that fish express fiber type-specific RyR1 isoforms.

Although several splice variants of the mammalian RyR1 message have been described (33, 43), the current study represents the first evidence of unique RyR1 gene products in fast- and slow-twitch muscle fibers. The two partial sequences isolated from the fast-twitch fish muscles share significant amino acid substitutions (shared derived characters) when compared with the slow-twitch isoform. This indicates that within the channel region for which the limited comparative sequence data exist, there are significant differences between the fast and slow isoforms that represent evolutionary divergences in the RyR1 isoforms. The fact that fiber type-specific RyR1 isoforms have not been characterized in mammals or amphibians may be associated with a loss of one of the isoforms in higher vertebrates, but it could also be attributed to the bias of selecting fast-twitch muscles for the construction of cDNA libraries, since these muscles contain a high content of SR (26, 31, 39, 42). Londraville et al. (unpublished data) have recently shown that SERCA1b, a neonatal form of the Ca\(^2+\)-ATPase in mammals, is expressed in adult extraocular muscles of fish and birds but not in extraocular muscles of adult mammals. Thus distinct expression patterns in the SR proteins of lower vertebrates may be a common finding once investigated in further detail.

The anatomic arrangement of fish muscles provides the tool for separating the pure slow-twitch from fast-twitch muscle, making the expression and binding studies possible. To determine whether the expression of unique RyR1 isoforms in fast- and slow-twitch muscles of fish results in functional differences, we assayed SR fractions from the different muscle fiber types for their affinity for \([\text{H}]\)ryanodine. Ryanodine binding in skeletal muscle SR preparations is typically activated by submicromolar Ca\(^2+\) concentrations and inhibited by millimolar Ca\(^2+\) concentrations. \([\text{H}]\)ryanodine binding to both fast- and slow-twitch SR preparations from fish exhibited the classic bell-shaped dependence on Ca\(^2+\) concentration that is characteristic of skeletal RyR isoforms. The pCa for peak binding was, however, different for the two muscle fiber types. The fast-twitch muscle showed peak binding at pCa 4, whereas the slow-twitch muscle fibers exhibited peak binding at pCa 5 (Figs. 8 and 9). This indicates that the RyR isoforms of slow-twitch muscle fibers have a significantly lower threshold for Ca\(^2+\) activation.

Nonmammalian skeletal muscles typically coexpress both the RyR1 and RyR3 isoforms (25). Immunoblot analysis of SR preparations using an antisera that recognizes an epitope common to all RyR isoforms revealed that although two isoforms can be detected in marlin fast-twitch muscle (RyR1 and RyR3), the slow-twitch muscle SR preparation only expresses a single RyR isoform (Fig. 9). The mobility of the single band on the protein gels and recognition by the C010 antibody along with the RNase hybridization results with the probe generated from the full-length cDNA for RyR1 slow isoform indicate this is the RyR1 slow protein. In addition, we have generated an RyR1-specific antibody that recognizes only the RyR1 protein in fast- and slow-twitch muscles of marlin (17). Because of this result, the differences observed between the fast-twitch and slow-twitch SR preparations for ryanodine binding could be ascribed to the influence of the coexpression of the RyR1 and RyR3 isoforms. However, the toadfish swim bladder muscle is known to be composed of a homogeneous fiber type that only expresses the RyR1 isoform (25). A SR preparation derived from toadfish swim bladder muscle also exhibits the bell-shaped dependency on Ca\(^2+\) concentration with a peak binding at pCa 4, similar to the fast-twitch white muscle preparations of marlin and tuna (Fig. 8E). Therefore, the shift in peak binding for ryanodine binding for the
slow-twitch muscle preparation cannot be attributed to the absence of the RyR3 isoform in the preparation. These results were demonstrated for two fish species from which significant quantities of slow-twitch muscle can be isolated (tuna and marlin). Toadfish have extremely small amounts of slow-twitch muscle fibers, which limits the ability to do protein analyses.

The absence of the RyR3 protein as revealed by Western blots raises interesting questions. It may be due to a reduced need to amplify the RyR1 signal as has been proposed in the two-component model for Ca^{2+} release (27) or a property of the RyR1 slow isoform that necessitates building triads with only this protein. It is possible that the slow-twitch muscle fibers in fish operate in vivo at lower thresholds of Ca^{2+} than fast-twitch fibers, possibly necessitating the construction of the triad with RyR isoforms that share similar properties (low threshold for activation via Ca^{2+}). If triads were built with a mixture of RyR isoforms that had different sensitivities to Ca^{2+} for opening and closing, Ca^{2+} release may not be as efficient.

Previous studies have described physiological differences in the mechanism of EC coupling between fast- and slow-twitch muscles. Salviati and Volpe (36) compared the kinetics of Ca^{2+} release from rabbit skinned fast and slow-twitch fibers, determining that the Ca^{2+} release channels of both tissue types respond to known modulatory agents but show different sensitivities. The SR preparations of slow-twitch muscle fibers had a lower threshold for caffeine, whereas the fast-twitch SR preparations were found to be more sensitive to ryanodine. Lee et al. (24) further showed with planar lipid bilayer recordings that rat fast- and slow-twitch muscle Ca^{2+} release channels have different rates of initial Ca^{2+} release and mean channel closed times. The Ca^{2+} released from the slow-twitch vesicles was 28% less than from fast-twitch SR vesicles. Recently, Delbono and Meissner (11) compared the intracellular transients in rat fast- and slow-twitch fibers using the low-affinity Ca^{2+} indicator Mag-fura 2 and demonstrated that rat fast-twitch muscles released myoplasmic Ca^{2+} faster than slow-twitch muscle. They also determined with binding experiments that slow-twitch muscles have a lower ratio of dihydropyridine receptors to RyRs, concluding that a lower number of the RyRs in slow-twitch muscle are directly controlled by the dihydropyridine receptor. Fish muscle fibers also demonstrate markedly different Ca^{2+} transients (34). In fish, the observed differences in Ca^{2+} transients and force generation by specific muscle fibers are most likely because of several molecular and biochemical modifications of SR and myofibrillar proteins, including the expression of fiber type-specific skeletal RyR isoforms.

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