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The Fastest Contracting Muscles of Nonmammalian Vertebrates Express Only One Isoform of the Ryanodine Receptor

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ABSTRACT The skeletal muscles of chickens, frogs, and fish have been reported to express two isoforms (α and β) of the sarcoplasmic reticulum calcium release channel (ryanodine receptor or RYR), while mammals express only one. We have studied patterns of RYR isoform expression in skeletal muscles from a variety of fish, reptiles, and birds with immunological techniques. Immunoblot analysis with a monoclonal antibody that recognizes both nonmammalian RYR isoforms and a polyclonal antibody specific to the α isoform show two key results: (*a*) two reptilian orders share with mammals the pattern of expressing only the α (skeletal) RYR isoform in skeletal muscle; and (*b*) certain functionally specialized muscles of fish and birds express only the α RYR isoform. While both isoforms are expressed in the body musculature of fish and birds, the α isoform is expressed alone in extraocular muscles and swimbladder muscles. The appearance of the α RYR isoform alone in the extraocular muscles and a fast-contracting sonic muscle in fish (toadfish swimbladder muscle) provides evidence that this isoform is selectively expressed when rapid contraction is required. The functional and phylogenetic implications of expression of the α isoform alone are discussed in the context of the mechanism and evolution of excitation-contraction coupling.

INTRODUCTION

A key step in excitation-contraction (EC) coupling is the transduction of the depolarization signal from the transverse (T) tubule to the sarcoplasmic reticulum (SR), resulting in calcium release and muscle contraction (Endo, 1977). Two integral membrane proteins located in junctional T tubule and SR membranes, respectively, the dihydropyridine receptor (DHPR) and SR calcium release channel or ryanodine receptor (RYR), mediate the process of signal transduction. The coupling of membrane depolarization to muscle contraction depends on the close proximity and functional linkage of these proteins.

The mechanism of EC coupling is different in vertebrate cardiac and skeletal muscles. In cardiac cells, sarcolemmal membrane depolarization results in a calcium current through the voltage-dependent DHPR (Bean, 1989). This calcium current triggers calcium release by the RYR (Cannell et al., 1987; Näbauer et al., 1989). In vertebrate skeletal muscle, unlike cardiac muscle, EC coupling occurs in the absence of extracellular calcium. A calcium current through the DHPR is not required, and EC coupling has been proposed to occur by direct mechanical coupling between the DHPR and the RYR (Rios and Pizarro, 1991). Mammalian cardiac and skeletal muscles express different isoforms of both receptors. The role of the DHPR isoforms in the different modes of coupling in cardiac and skeletal muscle has been revealed in an elegant set of experiments. Cloned skeletal and cardiac DHPRs were expressed in dysgenic mouse myotubes, which lack endogenous DHPR and EC coupling responses (Beam et al., 1986). By directly comparing the cardiac and skeletal DHPR isoforms, along with molecular chimeras, the experiments demonstrated that a single cytoplasmic loop of the DHPR is largely responsible for the type of coupling (cardiac or skeletal) expressed in a rescued dysgenic cell (Tanabe et al., 1990; Adams et al., 1990). Despite the elucidation of how DHPR isoforms influence the mode of EC coupling, the role of RYR isoforms in the differences between these types of coupling has not been thoroughly investigated.

Three tissue-specific isoforms of the RYR have been identified in mammals. The skeletal isoform (RYR1) is restricted to skeletal muscle (Marks et al., 1989; Takeshima et al., 1989); the cardiac isoform (RYR2) is present in cardiac muscle, certain brain cells (Hakamata et al., 1992; Otsu et al., 1990), and some nonexcitable tissues (Tunwell and Lai, 1993); and the brain isoform (RYR3) is expressed in brain tissue and in smooth muscle (Hakamata et al., 1992). Sequences of the cloned mammalian isoforms show 70% homology between cardiac and brain and 67% between cardiac and skeletal (Hakamata et al., 1992; Marks et al., 1989; Nakai et al., 1990). Although the isoforms have distinct amino acid sequences, the translated proteins are similar in size and share many physiological properties (Inui et al., 1987; Lai et al., 1988; Anderson et al., 1989; Herrmann-Frank et al., 1991).

While mammalian skeletal muscles express a single isoform of the RYR, four species of nonmammalian vertebrates representing birds, fish, and amphibians have been reported to express two isoforms in skeletal muscles (Airey et al., 1990; Olivares et al., 1991). The two isoforms, called α and β, have different mobilities on polyacrylamide gels and are immunologically distinct (Airey et al., 1990; Olivares et al., 1991; Lai et al., 1992). The α and β RYRs from frogs have been shown to relate immunologically to the mammalian

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skeletal and cardiac isoforms, respectively (Lai et al., 1992), but differences between the skeletal β and cardiac isoforms in chickens have also been shown (Sutko et al., 1992). The molecular relationships of the α and β isoforms are not yet known, although some differences in the calcium sensitivity of the two frog isoforms have been reported recently (Murayama and Ogawa, 1992).

The α and β isoforms have been localized to the same muscle fibers in chicken and frog muscle (Airey et al., 1990; Lai et al., 1992). Earlier structural work (Block et al., 1988) indicating that two different types of coupling occur in skeletal muscle triad junctions has been combined with these studies to form the hypothesis that the two RYR isoforms are situated side by side in the triad junction (Airey et al., 1990; Olivares et al., 1991; Lai et al., 1992). The presence of two RYR isoforms in the triads of nonmammalian skeletal muscle has important implications for the mechanism of EC coupling in skeletal muscle. Models of EC coupling in skeletal muscle are based on physiological and anatomical data from nonmammalian muscle fibers, yet they rarely account for the potential presence of two functionally different RYR isoforms. To understand the mechanism of EC coupling in nonmammalian skeletal muscle, it is necessary to answer two major questions about RYR isoforms. First, do the α and β isoforms differ in function and how? Second, what is the identity of the β isoform and does its suggested similarity to the cardiac RYR (Lai et al., 1992) imply a cardiac-type coupling mode (i.e., calcium-induced calcium release) in skeletal muscle?

In light of these questions it is of interest to examine cases in which one or the other isoform is preferentially expressed and observe the functional consequences. Differences in developmental expression of the α and β RYR isoforms have been observed in chick embryos (Sutko et al., 1991). However, prior studies have not examined the appearance of RYR isoforms in nonmammalian adult muscles with distinct functional properties or the relationship of muscle fiber types to skeletal RYR isoform expression. In this study we report that fish, birds, and reptiles show differential expression of RYR isoforms in their skeletal muscles. We have identified distinct muscles in these animals that express a single isoform of the RYR rather than both isoforms. A relationship between muscle contraction speed and the RYR isoforms expressed is evident in these findings. This pattern of expression could prove to be a valuable tool for future studies of the function of vertebrate RYR isoforms.

MATERIALS AND METHODS

Tissue collection

Live toadfish (*Opsanus tau*) were purchased from the Marine Biological Laboratory (Woods Hole, MA) and maintained in saltwater tanks until use. Fish were anesthetized with MS222 (0.5 g/liter), and the swimming muscles and swimbladder muscle were rapidly dissected out and used immediately for SR isolation. Extraocular muscles from several toadfish were frozen in liquid nitrogen and pooled for SR isolation. Superior and medial rectus extraocular muscles and swimming muscle were obtained from freshly caught blue marlin (*Makaira nigricans*) and either used immediately for SR isolation or freeze-clamped in liquid nitrogen and stored for later use. Extraocular and swimming muscles from other fish (mako shark, *Isurus oxyrinchus*; dogfish, *Squalus acanthias*; striped bass, *Morone saxatila*; wahoo, *Acanthocybium solanderi*; yellowfin tuna, *Thunnus albacares*; swordfish, *Xiphias gladius*; and spearfish, *Tetrapturus angustirostris*) were removed from freshly caught or captive reared specimens and frozen in liquid nitrogen. Extraocular and neck muscles from chickens (*Gallus domesticus*) were collected immediately after sacrifice at a local poultry supplier and frozen in liquid nitrogen. Painted turtles (*Chrysemys scripta*) were purchased from Carolina Biological Supply. Whiptail lizards (*Cnemidophorus tigris*) and zebra-tailed lizards (*Callisaurus draconoides*) were collected from California desert populations. Garter snakes (*Thamnophis radix*) were collected in Illinois. Tail and body wall muscle from rattlesnakes (*Crotalus atrox*) was a gift of S. Lindstedt (Northern Arizona University), and limb and extraocular muscle from American alligator (*Alligator mississippiensis*) was provided by M. Fagan (Florida Alligator Trappers Association). Captive reptiles were chilled to 0°C and euthanized, and muscles were dissected immediately for SR isolation.

Isolation of SR vesicles

Muscle tissues were minced in 5 to 10 volumes of ice-cold solution A (0.1 M NaCl, 20 mM Na-piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), pH 7.3, 5 mM EGTA, 0.5 mM diisopropylfluorophosphate (DIFP), 1 mM benzamidine, 1 μM leupeptin, 1 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor) and homogenized in three 20-s bursts with a Tekmar homogenizer at half-speed. Homogenates were centrifuged for 20 min at 1900 $\times$ *g*, and the supernatants were filtered through three layers of cheesecloth. The filtered supernatants were centrifuged for 30 min at 90,000 $\times$ *g* in a Beckman Ti70 rotor. Pellets were homogenized in 1–5 ml of solution B (0.3 M sucrose, 0.6 M KCl, 10 mM K-PIPES, pH 7.0, 0.1 mM EGTA, 0.5 mM DIFP, 1 mM benzamidine, 1 μM leupeptin, 1 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor)/g starting tissue and incubated for 1 h on ice. The resulting preparations were centrifuged for 1 h at 90,000 $\times$ *g* in a Beckman Ti70 rotor. The pellets were resuspended to 10–20 mg protein/ml in 0.3 M sucrose, 5 mM K-PIPES, pH 7.0, 0.5 mM DIFP, and frozen in liquid nitrogen in small aliquots. For SR preparations that were made fresh from wild caught fish a slightly different protocol was used. Muscle tissues were homogenized with five passes with a motor driven Potter-Elvehjem homogenizer and centrifuged at 1900 $\times$ *g* as above. An additional spin at 9750 $\times$ *g* for 20 min was included to reduce contamination by mitochondrial membranes. SR vesicles in the 9750 *g* supernatant were subsequently collected by a 2-h, 20,000 $\times$ *g* spin in a Sorvall SS34 rotor. The incubation in solution B was not done in these preparations.

Isolation of the ryanodine receptor

The 30 S ryanodine receptor complex was isolated by gradient density centrifugation according to Lai et al. (1988). SR vesicles prepared as above were suspended in 5 ml of 0.6 M NaCl, 20 mM Na-PIPES, pH 7.1, 0.1 mM Na-EGTA, 1 mM benzamidine, 0.5 mM DIFP, 1 μM leupeptin, 1 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor, and centrifuged for 30 min at 90,000 $\times$ *g*. The pellets were solubilized for 2 h on ice at 1–1.5 mg protein/ml with 1.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3 mg/ml phosphatidylcholine in solution C (1 M NaCl, 20 mM Na-PIPES, pH 7.1, 0.1 mM Na-EGTA, 0.15 mM $CaCl_2$, 1 mM benzamidine, 0.5 mM DIFP, 1 μM leupeptin, 1 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor). The solubilized SR were loaded onto linear 5–20% sucrose gradients containing 1.0% CHAPS and 4 mg/ml phosphatidylcholine in solution C. Gradients were centrifuged 15 h at 4°C in a Beckman SW41 rotor at 26,000 rpm.

Gel electrophoresis and immunoblotting

SR vesicle fractions were routinely analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 3–12% or 3–15%

gradient gels. Membranes were solubilized in an SDS sample buffer containing 0.1 M Tris-Cl, pH 6.8, 0.5 mM EDTA, 2% SDS, and 10% glycerol, and loaded for Coomassie blue staining (20–60 μg/lane). Molecular masses of polypeptides in the gels were estimated using as molecular mass markers: mammalian skeletal RYR (565 kDa), myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa). A Hoefer GS 300 scanning densitometer was used for the calibrations.

Samples to be analyzed by immunoblotting were resolved on 5–12% SDS-PAGE gradient gels containing the hydrolyzable crosslinker dihydroxyethylenebisacrylamide (DHEBA) (1:27 DHEBA/acrylamide ratio)-(Airey et al., 1991). Gels were transferred to PVDF membranes (Millipore Corporation, Bedford MA) in a Novablot apparatus for 2 h at 0.8 mA/cm^2 with a transfer buffer consisting of 10 mM 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES), pH 9.6, 10% ethanol, and 0.0375% SDS. Transferred membranes were blocked with 2% nonfat dry milk in Tris-buffered saline and probed with either an anti-canine cardiac muscle RYR monoclonal antibody (RYR CO10) (Lai et al., 1992) or a purified anti-rat skeletal muscle RYR antiserum (Meissner et al., 1989) at 1:1000 dilution. The monoclonal antibody (MAb) RYR CO10 recognizes a conserved epitope and binds to both α and β isoforms in fish, amphibians, birds, and reptiles as well as both mammalian isoforms. The skeletal RYR antiserum recognizes only the skeletal isoform of mammals and crossreacts with the bird, reptile, and fish α isoform (see results). Alkaline phosphatase-linked secondary antibodies (BioRad, Hercules, CA) were used, and the bands were visualized by reaction with bromochloroindoyl phosphate and nitro blue tetrazolium. Apparent molecular masses of the transferred proteins were calibrated with prestained molecular mass markers. The prestained markers were initially calibrated to unstained markers in Coomassie blue-stained gels by scanning densitometry. The markers and their apparent molecular masses are α-2 macroglobulin (186 kDa), β-galactosidase (118 kDa), fructose-6-phosphate kinase (87 kDa), pyruvate kinase (68 kDa), and fumarase (57 kDa).

Histology

Unfixed extraocular muscle from blue marlin was freeze-clamped in liquid nitrogen and sectioned at −30°C in a cryostat. Eight-μm thick sections were mounted on gelatin-coated slides and stained for acid-stable myosin ATPase activity according to a modification of Guth and Samaha (1970). Briefly, sections were preincubated in 100 mM Na-acetate, pH 4.3, for 1 min or in an alkali buffer (50 mM sodium barbitol, 50 mM sodium acetate, 30 mM $CaCl_2$, pH 10.15). Slides were then transferred to 50 mM Na-barbital, 50 mM Na-acetate, pH 9.4, 30 mM $CaCl_2$, 2.5 mM ATP and incubated for 60 min. After rinsing in 1% $CaCl_2$, the sections were incubated in 2% $CoCl_2$ for 3 min and developed 3 min with 1% $(NH_4)_2S$. Sections were viewed and photographed with a Zeiss Axioplot microscope.

RESULTS

A single isoform of the RYR is detected in extraocular muscles of fish

The epaxial and hypaxial (swimming) muscles of toadfish were previously reported to express two isoforms of the RYR (Olivares et al., 1991). We have surveyed by SDS-PAGE and immunoblot analysis a variety of muscles in seven species of fish and find that the presence of two RYR isoforms is a

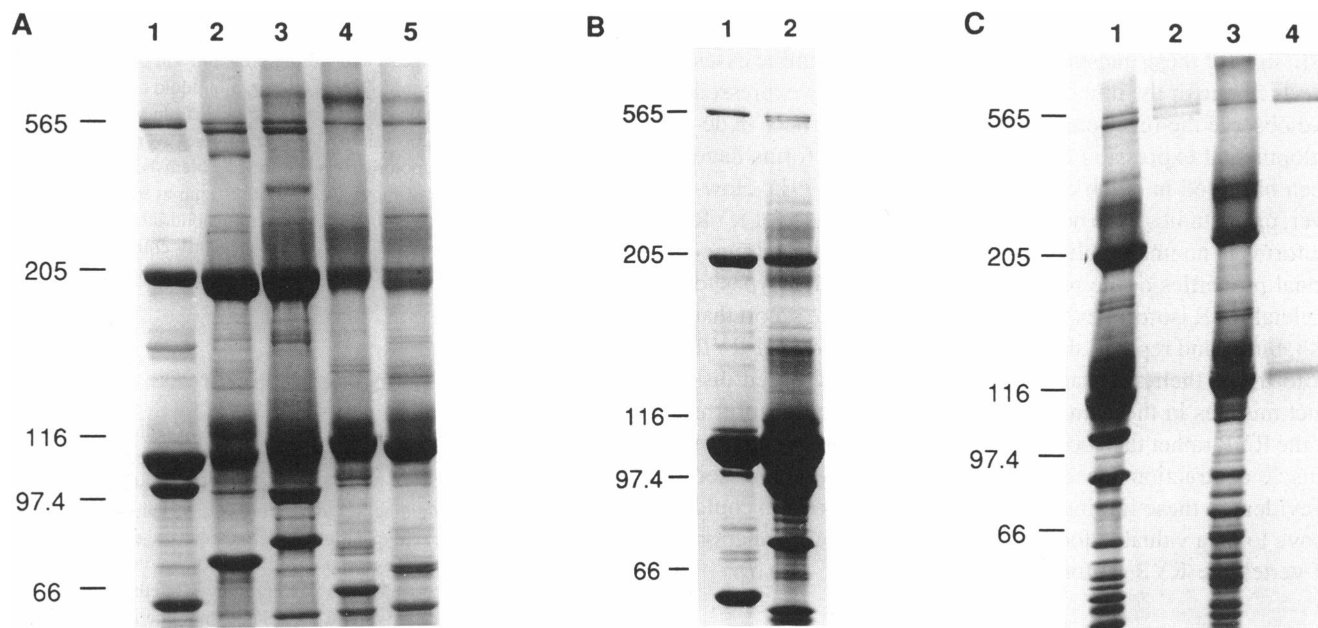

FIGURE 1 SDS-PAGE analysis demonstrates that two high molecular weight polypeptides are present in SR from fish swimming muscles while extraocular muscles have only one. (*A*) Crude SR fractions from swimming muscles and extraocular muscles were solubilized in SDS sample buffer, loaded (40 μg/lane) onto a 3–12% gradient SDS-PAGE gel, and stained with Coomassie brilliant blue. Samples loaded are: lane 1, rabbit skeletal muscle; lane 2, striped bass swimming muscle; lane 3, toadfish swimming muscle; lane 4, blue marlin superior rectus; lane 5, blue marlin medial rectus. (*B*) Crude SR fractions from swimming muscle of mako shark show two high molecular weight polypeptides as in the teleost fishes in *A*. The two polypeptides in shark muscle (*lane 2*) migrate slightly differently than do the teleost polypeptides and show less separation. Rabbit skeletal muscle SR is shown for comparison (*lane 1*). Gel conditions as in *A*. (*C*) Sucrose gradient purification of the ryanodine receptor from fish swimming muscle yields two high molecular weight polypeptides while a single high molecular weight polypeptide is purified from fish extraocular muscle. Samples were resolved on a 3–15% gradient SDS-PAGE gel and stained with Coomassie brilliant blue. Lanes 1 and 2, crude SR (20 μg) and purified ryanodine receptor (0.12 μg) from striped bass swimming muscle. Lanes 3 and 4, crude SR and purified ryanodine receptor from blue marlin medial rectus. A small amount of calcium ATPase (molecular mass 100 kDa) remains in the purified preparation from medial rectus (*lane 4*).

general feature of fish swimming musculature. SDS-PAGE analysis of crude SR fractions (Fig. 1, *A* and *B*) reveals two high molecular weight polypeptides with mobilities similar to the mammalian RYR (M_r 565,000) in swimming muscles of several teleost fish and two sharks. In contrast, only a single high molecular weight polypeptide band was seen in extraocular muscles. The mobility of the extraocular muscle polypeptide is similar to that of the 565-kDa mammalian skeletal muscle RYR and to the less mobile of the two high molecular weight polypeptides of fish skeletal muscle. In partial purification of the 30 S ryanodine receptor complex from fish swimming muscles and extraocular muscle, two high molecular weight proteins co-purify from swimming muscle while extraocular muscle yields a single high molecular weight protein (Fig. 1 *C*). These results suggest that extraocular muscles express only one of the two RYR isoforms detected in fish swimming muscle.

Immunological identification of the ryanodine receptor isoforms in fish swimming and extraocular muscles

Immunoblots of crude SR preparations from swimming muscle of striped bass and toadfish show that both α and β isoforms of the RYR are recognized by MAb RYR CO10 (Fig. 2 *A*, *lanes 2* and *3*). In contrast, only one immunoreactive protein band is detected in extraocular muscles from blue marlin, wahoo, and yellowfin tuna (*lanes 4* and *5* of Fig. 2 *A*, *lanes 2–4* of Fig. 2 *B*). All of the fish extraocular muscle preparations studied to date show a single RYR isoform. These include preparations from individual superior and medial rectus muscles from marlin, wahoo, and tuna as well as pooled preparations from toadfish and tuna that include all of the extraocular muscles. The protein band identified by MAb RYR CO10 has the same mobility as the upper band of the two present in swimming muscle. This band corresponds to the α (Airey et al., 1990; Olivares et al., 1991) or skeletal-like (Lai et al., 1992) RYR isoform.

In further experiments we have characterized the isoform of the RYR present in fish extraocular muscles by using an antiserum specific for the rat skeletal muscle RYR. Immunoblots of crude SR demonstrate that this antiserum cross-reacts with the upper band (α or skeletal-like isoform) of fish swimming muscles and recognizes the single extraocular muscle isoform (Fig. 3). No cross-reactivity with the lower band isoform in swimming muscle from fish is observed. These data support the hypothesis that the extraocular muscle RYR is related to the α isoform (upper band) from swimming muscle.

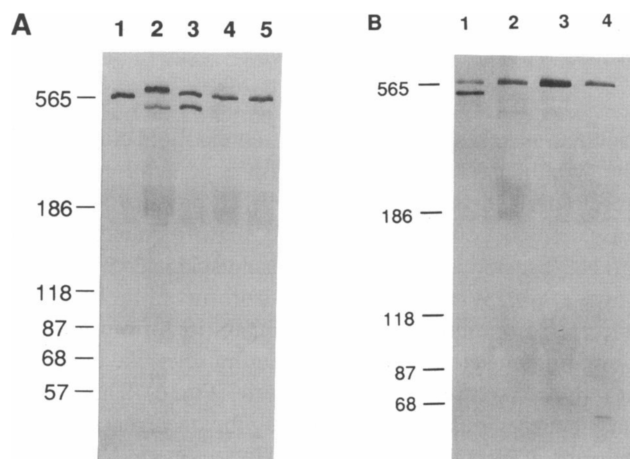

FIGURE 2 Immunoblots with antibody RYR CO10 demonstrate the presence of two isoforms of the RYR in swimming muscles of fish but a single isoform in extraocular muscles. (*A*) Crude SR fractions were separated on 5–12% DHEBA-PAGE gels and transferred to PVDF (see "Methods"). Lane 1, rabbit skeletal muscle (30 μg); lane 2, striped bass swimming muscle (75 μg); lane 3, toadfish swimming muscle (70 μg); lane 4, blue marlin superior rectus (100 μg); lane 5, blue marlin medial rectus (70 μg). (*B*) Crude SR fractions from extraocular muscles from wahoo (*lane 2*), blue marlin medial rectus (*lane 3*), and yellowfin tuna (*lane 4*) are compared to toadfish swimming muscle SR (*lane 1*).

Extraocular muscles of birds also express a single RYR isoform

The discovery that all species of fish examined express only the α RYR isoform in extraocular muscles prompted us to examine the pattern of isoform expression in nonfish species. Chicken neck muscles (Fig. 4) have two RYR isoforms as reported earlier for pectoral and thigh muscles (Airey et al., 1990; Olivares et al., 1991). In contrast, SR preparations made from chicken extraocular muscles have only the α isoform. Thus, the expression of a single RYR isoform in extraocular muscles is not a peculiarity of fish, but is a pattern seen at least in birds as well.

Expression of the α RYR isoform does not correlate with muscle fiber type

Previous authors have reported that both α and β RYR isoforms are present in the same muscle fibers in chicken and frog muscle (Airey et al., 1990; Lai et al., 1992); however the

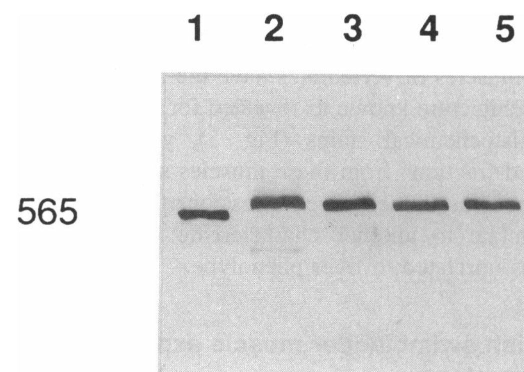

FIGURE 3 Rat skeletal muscle RYR antiserum recognizes the extraocular muscle isoform and only the upper band of swimming muscle in fish. Crude SR were separated as in the legend to Fig. 2. Lane 1, rabbit skeletal muscle (15 μg); lane 2, striped bass swimming muscle (80 μg); lane 3, toadfish swimming muscle (80 μg); lane 4, blue marlin superior rectus (100 μg); lane 5, blue marlin medial rectus (100 μg). Compare to Fig. 2 *A*.

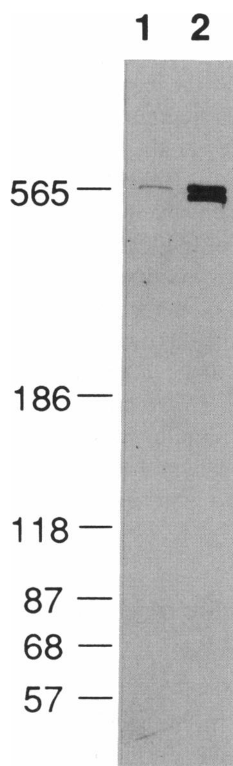

FIGURE 4 Avian extraocular muscles also show differential expression of the RYR isoforms. The α isoform is expressed alone in extraocular muscles while neck muscles express both isoforms. Shown above is an immunoblot with MAb RYR CO10, which recognizes both isoforms. Lane 1, crude SR from extraocular muscles (120 μg); lane 2, SR from neck muscles (90 μg).

relation of RYR isoform expression to muscle fiber type was not examined. The immunofluorescent co-localization studies with isoform specific antibodies were done on chicken pectoralis (Airey et al., 1990) and frog gastrocnemius (Lai et al., 1992) muscles. In both of these muscles, a single fast-twitch fiber type predominates (Putnam and Bennett, 1983; Rosser and George, 1986), so it is unclear whether isoform expression related to fiber phenotype would have been uncovered. The extraocular muscles provide a strong argument that isoform expression is not related to fiber type. Extraocular muscles of vertebrates have the most complex fiber type architecture known as revealed for fish superior rectus with histochemical stains (Fig. 5), yet SR membrane-enriched fractions from these muscles show a single RYR isoform. Thus, the selective expression of the α RYR isoform must relate to another characteristic of the extraocular muscles unrelated to fiber phenotype.

Toadfish swimbladder muscle expresses the α isoform alone

Extraocular muscles are characterized by their high contraction speeds across many fiber types (Bach-y-Rita and Ito, 1966; Lennerstrand and Baker, 1987). Indeed, the twitch fibers in extraocular muscles are the fastest in the vertebrate body. To test a possible correlation between contraction speed and RYR isoform expression, we examined a nonextraocular muscle specialized for extremely rapid contraction. The swimbladder muscle of toadfish is a sound-generating muscle derived from the body wall. To produce sound the muscle fibers contract at extremely high frequencies up to 150 Hz. Tension is developed in this muscle in 3–5 ms (Skoglund, 1961). While both α and β isoforms of the RYR are detected in immunoblots of SR preparations from toadfish swimming muscles, the swimbladder muscle, like extraocular muscles, shows only the α isoform (Fig. 6). This is contrary to a previous report mentioning the occurrence of both isoforms in swimbladder muscle (Olivares et al., 1991). The results of this study suggest that expression of the α isoform alone is not an unusual characteristic of extraocular muscles but is more likely a functional specialization related to rapid contraction.

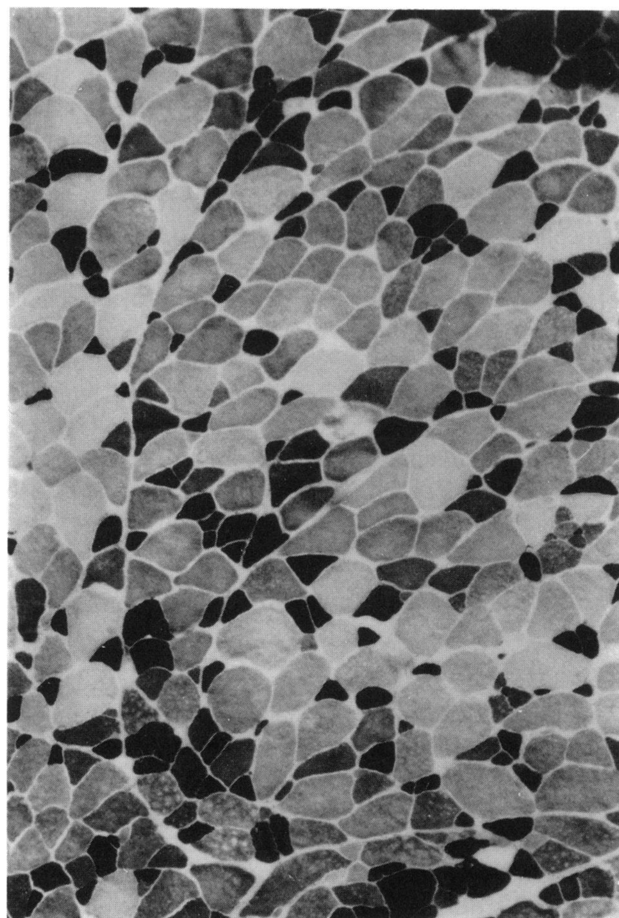

FIGURE 5 Histochemical staining reveals the complex fiber architecture typical in a vertebrate extraocular muscle (at least five fiber populations). This cross section is of the superior rectus extraocular muscle from blue marlin. The section was incubated at pH 10.15 to reveal alkali stable myosin ATPase. The smallest dark staining fibers are histologically similar to fast-twitch oxidative glycolytic fibers. The large, pale-staining fibers most closely resemble fast twitch glycolytic fibers.

Several reptiles express the α RYR isoform alone throughout the body

Both birds and mammals arose from reptilian ancestors, and a careful study of reptiles may clarify the evolutionary events

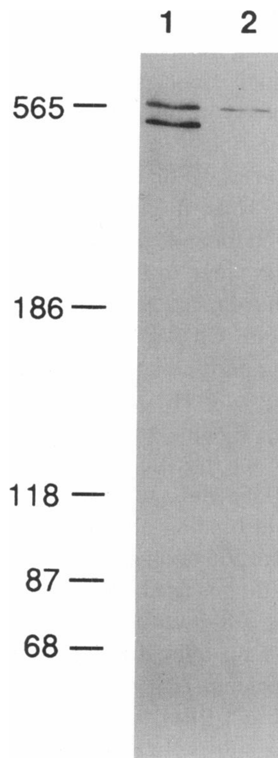

FIGURE 6 Toadfish swimbladder muscle, a specialized high frequency muscle, expresses only the α RYR isoform. An immunoblot with MAb RYR CO10 reveals two isoforms in toadfish swimming muscle (90 μg, *lane 1*) and only the α isoform in swimbladder (100 μg, *lane 2*).

that led to the loss of expression of the β RYR isoform in mammals but retention of both isoforms in birds. We have examined the patterns of RYR isoform expression in skeletal muscles from all of the major reptilian lineages (turtles, crocodiles, snakes, and lizards). Included in the survey was the rattlesnake tail-shaker muscle, which vibrates the rattle at approximately 90 Hz, and has many of the morphological modifications seen in swimbladder (hypertrophy of the SR volume and reduction of the myofibrillar volume (Schaeffer and Lindstedt, 1992)). Fig. 7 shows SDS gels and an immunoblot of SR fractions from garter snake, rattlesnake, zebra-tailed lizard, and turtle. A striking result is found among the reptiles. Like birds, turtles and alligators (alligator not shown) have two RYR isoforms in their skeletal muscles, while lizards and snakes only express the α isoform, as seen in mammals. The fast-contracting tail-shaker muscle of rattlesnakes also has only the α RYR isoform. This observation is not due to the lack of recognition of the reptilian β isoform by MAb RYR CO10, as both the turtle skeletal muscle β (*lane 5* of Fig. 7 *B*) and cardiac muscle (*lane 4* of Fig. 7 *C*) isoforms are recognized. While our survey is far from comprehensive, it demonstrates that some reptiles express only the α RYR isoform in their skeletal muscles, a condition previously thought to be restricted to mammals.

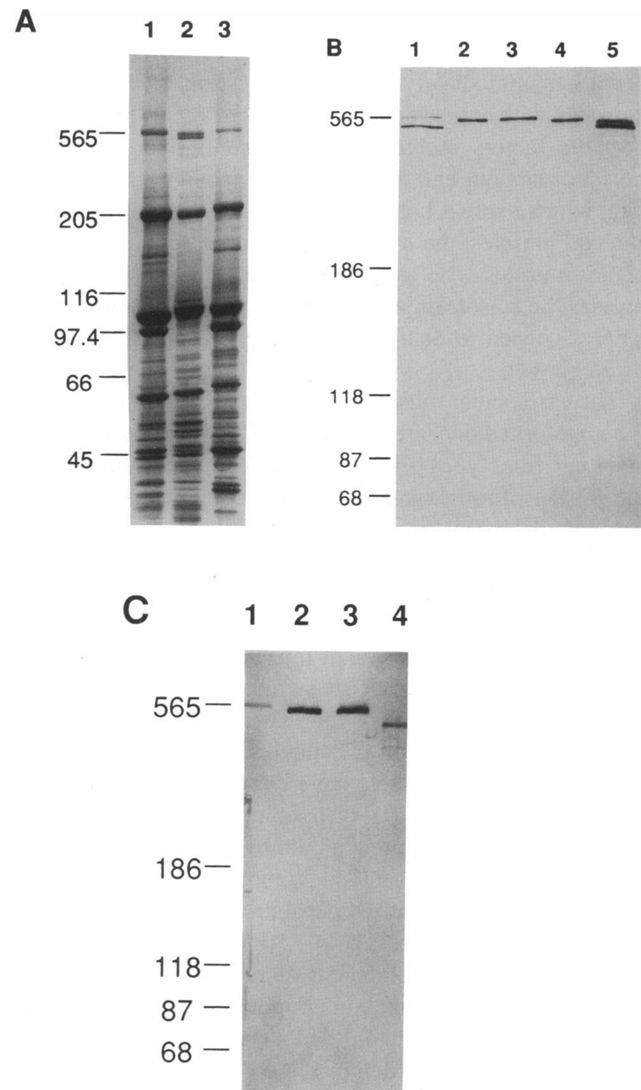

FIGURE 7 Reptiles exhibit both patterns of RYR expression in body muscles. (*A*) A 3–15% SDS-PAGE gel demonstrates two high molecular weight polypeptides in heavy SR from turtle (*lane 2*), while crude SR from whiptail lizard (*lane 3*) shows a single polypeptide that runs similarly to that in rabbit skeletal muscle (*lane 1*). (*B*) An immunoblot of crude SR preparations with MAb RYR CO10 shows a single RYR isoform in lizard skeletal muscle (*lane 2*), garter snake body (*lane 3*), and rattlesnake rattle muscles (*lane 4*), while turtle skeletal muscle has two (*lane 5*). Toadfish swimming muscle is shown for comparison (*lane 1*). (*C*) The mobility of the rattlesnake body and rattle muscle RYR (*lanes 2* and *3*) are compared here to the fish α isoform (toadfish swimbladder, *lane 1*) and turtle cardiac muscle RYR (*lane 4*) in an immunoblot with MAb RYR CO10. The antibody cross-reacts with all reptile cardiac tissues examined. The single RYR isoform expressed in snake co-migrates with the fish α isoform.

DISCUSSION

Our investigation of vertebrate skeletal muscle RYR isoforms has led to two new findings that have important implications for excitation-contraction coupling: (*a*) Within the lower vertebrates, certain skeletal muscles express the α RYR isoform alone, while both α and β are expressed in the majority of muscles. (*b*) Among the reptiles, the primitive vertebrate condition of expression of two RYR isoforms in skeletal muscles is present in crocodiles and turtles, while the snakes and lizards have the mammal-like condition of expressing only the α isoform. The significance of differential expression of skeletal RYR isoforms is discussed below from both functional and phylogenetic perspectives.

Our results indicate that differential expression of RYR isoforms is a common phenomenon in vertebrates. This observation immediately raises the question of what factors correlate with expression of one isoform versus two. Several possible factors including development, fiber phenotype, muscle function, and phylogeny are apparent. In birds and fish, which express both the α and β RYR isoforms in most skeletal muscles, the α isoform is expressed alone in extraocular muscles. The toadfish swimbladder muscle also expresses the α isoform alone. These muscles provide the examples needed to distinguish among several of the factors listed above.

The extraocular muscles of fish and birds, which express the α isoform alone, are composed of a variety of fast-twitch and tonic fiber types (Fig. 5). Thus, fiber type cannot explain the RYR isoform expression pattern in eye muscles. A developmental correlation is also unfeasible because of the different origin of the extraocular muscles and the swimbladder muscle. One common feature of these muscles, however, is their specialization for high contraction speed. Table 1 shows contraction speeds of skeletal muscles including extraocular muscles and the toadfish swimbladder muscle. Extraocular muscles are among the fastest contracting muscles in vertebrates. Even the presumed "slow" fibers in mammalian extraocular muscle have twitch times of 20–27 ms (Bach-y-Rita and Ito, 1966), comparable to fast-twitch skeletal fibers of mammalian limb muscles (Gordon and Phillips, 1953). The toadfish swimbladder muscle is even more extraordinary and is capable of producing unfused twitches at frequencies up to 150 Hz (Skoglund, 1961). This requires the development of peak tension in 3–5 ms and equally rapid relaxation. Importantly, high frequency operation demands not only rapid twitch onset, but also rapid inactivation of calcium release and recovery of contractile activity. Numerous morphological features such as hypertrophy of the sarcoplasmic reticulum and high mitochondrial content are associated with the specialization of the muscles for high frequency contraction. These structures are all involved in the release and reuptake of calcium in the muscle fiber.

Activation and inactivation of calcium release necessarily involve the RYR. Thus, it is reasonable to examine these parameters to search for an explanation for the occurrence of the α RYR isoform alone in fast-contracting muscles. Biochemical studies have recently sought to analyze the differences between the α and β RYR isoforms. The frog RYR isoforms have been purified and demonstrate a difference in calcium sensitivity of [^{3}H]ryanodine binding (Murayama and Ogawa, 1992). Ryanodine preferentially interacts with the open state of the release channel (Chu et al., 1990; Fleischer et al., 1985; Meissner, 1986; Pessah et al., 1987), so the difference in binding observed may represent a difference in the proportion of open channels at the calcium and ryanodine concentrations used. Indeed, in single channel recordings with frog SR, two populations of channels have been observed to have very different open probabilities at low levels of free calcium ($P_{o1} = 0.18 \pm 0.02$ and $P_{o2} = 0.71 \pm 0.05$ at 3 μM $CaCl_2$; Bull and Marengo, 1993). If these do represent the α and β RYR isoforms, then the calcium release properties of the two should be sufficiently different to explain some differential expression of the isoforms.

As discussed further below, the presence of two RYR isoforms in skeletal muscles represents the primitive or generalized condition for vertebrates. The α and β isoforms have been maintained stably throughout vertebrate evolution, some 400 million years, so there must be strong physiological selection for both to be present. The presence of two isoforms may allow for flexibility or increased modulation in the control of calcium release in muscle cells. Not only do unique calcium release properties of the two channels permit

TABLE 1 Contraction speeds of selected skeletal muscles

Species	Muscle type	Time to peak tension (ms)	Predominant RYR isoform expressed
Toadfish*	Swimbladder muscle	3–8 (25°C)	Skeletal (α)
Catfish‡	Extraocular muscles		
	Lateral rectus	12.1 ± 1.26 (23°C)	Skeletal (α)
Cat§	Extraocular muscle		
	East twitch	5.0–7.0 (37°C)	Skeletal
	Slow twitch	20–27 (37°C)	Skeletal
Cat¶	Extensor digitorum		
	longus (fast)	19 (37°C)	Skeletal
	Soleus (slow)	70 (37°C)	Skeletal
Snake‖	Costocutaneous	37–54 (23°C)	Skeletal (α)
Turtle**	Neck retractor muscle	400 (23°C)	Skeletal (α)/cardiac (β)
Frog‡‡	Iliofibularis		
	Fast fibvers	29.3 ± 2.3 (23°C)	Skeletal (α)/cardiac (β)
	Slow fibers	53.8 ± 4.5 (23°C)	Skeletal (α)/cardiac (β)

* Skoglund (1961).
‡ Lennerstrand and Baker (1987).
§ Bach-y-Rita and Ito (1966).
¶ Gordon and Phillips (1953).
‖ Ridge (1971).
** Henneman and Olsen (1965).
‡‡ Lännergren and Smith (1966).

this flexibility, but the different isoforms also have the potential to be regulated differently. At least two factors can be identified that may influence regulation of calcium release. The first is the mode of EC coupling by each of the RYR isoforms. In vertebrate skeletal muscle, in which direct mechanical coupling between the DHPR and the RYR is believed to control calcium release (Rios and Pizarro, 1991), structural evidence suggests that not all RYRs in the triad junction are able to make direct mechanical connections (Block et al., 1988). The RYRs are arranged in an alternating pattern with the DHPRs of the T-tubule membrane so that only half of the RYRs can make direct contacts. Physiological evidence suggests that in frog skeletal muscle some RYRs are controlled by calcium-induced calcium release, responding secondarily to the calcium released by channels that are presumably directly linked to DHPRs (Jacquemond et al., 1991). While the frog α and β RYR isoforms have been related immunologically to the mammalian skeletal and cardiac isoforms respectively (Lai et al., 1992), it is not known if this similarity extends to the coupling mode employed by each isoform. Thus, we cannot predict which RYRs are directly coupled and which are not, but the presence of two isoforms clearly allows for greater flexibility in the calcium release response. It is important to note that the structural model of the triad junction was based on the toadfish swimbladder muscle (Block et al., 1988). This muscle expresses only the α RYR isoform, so it is apparent that the α isoform, at least in toadfish swimbladder muscle, is capable of coupling in both ways (Block et al., manuscript in preparation).

The second aspect of regulation of calcium release is the control by ligands including intracellular calcium, magnesium, adenine nucleotides, and other ligands. The presence of two RYR isoforms with potentially different sensitivities to calcium and other ligands also contributes to the flexibility of the calcium release response in muscles with both RYR isoforms. In the fastest contracting muscles, this flexibility is compromised, presumably in favor of the RYR isoform better suited for rapid contraction. How would such flexibility be useful and potentially selected for millions of years? One hypothesis is that ectothermic vertebrates have to recruit their muscle fibers over a much wider range of temperatures and hence physiological conditions than do endotherms. A frog that has to jump early in the morning at 15°C to escape a predator may heat up later in the day to 30°C while basking in the sun. Two isoforms may be more capable of meeting the physiological demands of muscles than one. Mammals recruit their muscles at relatively high contraction speeds and under homeostatic temperature conditions all of the time. This may be the reason why the β isoform has been lost. Examination of more primitive mammalian groups may in fact provide evidence for such a hypothesis.

Phylogenetic considerations

One of the unexpected outcomes of this study was the finding that several major groups of reptiles, in addition to the mammals, express only the α RYR isoform throughout the skeletal muscles. The physiological basis for this pattern of expression is less clear than it is in the specialized muscles in fish and birds and an understanding of the expression pattern requires an evolutionary perspective. We have examined the evolutionary history of the expression of the two RYR isoforms in vertebrates so that physiological adaptations of individual muscles could be distinguished from evolutionary constraints. A powerful tool for analyzing isoform evolution is to place what is known about RYR isoforms in a phylogenetic context (Harvey and Pagel, 1991). In Fig. 8, the pattern of RYR isoform expression in skeletal muscle so far known is mapped on a well corroborated phylogeny of vertebrates. Several distantly related invertebrate taxa in which the RYR has been studied, a nematode and two arthropods, have been mapped onto the phylogeny for comparison with the vertebrates. The invertebrate RYR has not been sequenced nor has it been directly linked to one of the mammalian isoforms (Formelová et al., 1990; Kim et al., 1992; Seok et al., 1992). Hence, the relationship of the vertebrate skeletal RYR isoforms to these invertebrate RYRs is unclear.

The earliest vertebrate nodes all lead to lineages that express both α and β isoforms while more advanced taxa express only the α isoform (Fig. 8). To determine what is the primitive condition of RYR isoform expression (one or two skeletal isoforms) in vertebrates, parsimony analysis is used to classify the character state (primitive or advanced) by a well accepted set of rules (Stewart, 1993). One uses outgroup analysis to polarize the character, that is, to decide what is the basal or primitive characteristic of a trait. In the case of RYR isoform distribution, we would like to address whether α alone, or α and β together, is the primitive trait. This analysis can be applied at any level of resolution of the tree, as long as an outgroup comparison is made. One could polarize the isoform distribution among fish only, among higher vertebrates, or among all vertebrates; however each of these analyses requires different comparisons at the onset. Among vertebrates, one can use fishes and amphibians to analyze RYR isoform distribution in higher vertebrate taxa (reptiles, birds, and mammals). By doing this we learn the primitive condition in vertebrates is the expression of two isoforms (as in most fish, bird, and amphibian muscle) while the advanced condition, as represented in snakes, lizards, and mammals, is the expression of only one isoform of the RYR in skeletal muscles. One additional analysis yields a similar conclusion. Among the fish we can examine the isoform distribution in the skeletal muscles, extraocular muscles, or sonic muscles (toadfish is only one example of a fish that uses muscle to make sound; there are many more). The presence of two isoforms is the primitive condition for fish, while expression of only one isoform in the sound-producing muscle and extraocular muscles is the advanced condition, in this case linked to the specialization for high contraction speed. Thus, two phylogenetic analyses indicate that the two-isoform condition is primitive, while the α only condition is more advanced (recently evolved).

From the information available, it is not possible to decide whether the α or β isoform is the more primitive of the two.

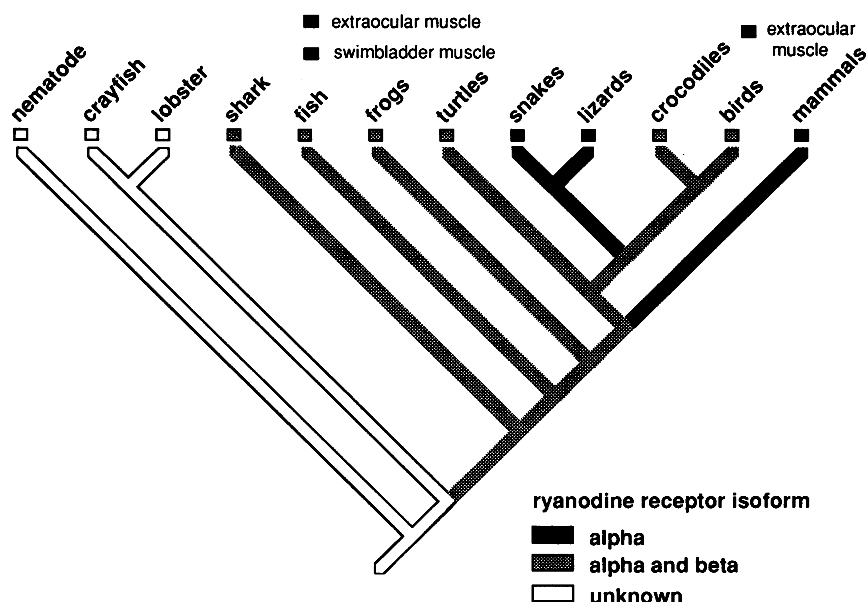

FIGURE 8 Phylogenetic distribution of skeletal muscle RYR expression patterns in vertebrates. The different patterns of skeletal muscle RYR expression are treated as character states (see key) and are mapped on a phylogeny of vertebrates using MacClade (Maddison and Maddison, 1992). Invertebrate taxa whose RYRs have been studied are included for comparison. The expression of both RYR isoforms (*shaded branches*) is the primitive condition in vertebrates. Expression of the α isoform alone (*filled branches*) has arisen in two separate lineages and in specialized muscles (*filled boxes*) in at least two other lineages.

A more complete evolutionary understanding of the relationship between α and β in vertebrates and the single polypeptide isolated from invertebrate muscles awaits further resolution of the physiology and molecular structure of the invertebrate muscle RYR isoforms. Too little is known about invertebrate RYR isoforms to use them properly as an outgroup to all vertebrates. However, from what we do know, an intriguing story in RYR isoform evolution is emerging. Thus far, only one RYR isoform has been isolated from the invertebrate muscles that have been examined (Formelová et al., 1990; Kim et al., 1992; Seok et al., 1992). In crayfish, physiological studies of muscle indicate that the RYR isoform functions by calcium-induced calcium release as does mammalian cardiac muscle (Gyorke and Palade, 1992). It is possible that the cardiac RYR isoform (RYR2) or the smooth muscle isoform (RYR3) is most closely related to the RYR expressed in invertebrate muscle tissues. Elucidation of the exact nature of the RYR in a deuterostome invertebrate (e.g., an acorn worm) would aid in construction of the evolutionary relationships between the vertebrate skeletal muscle isoforms and help in understanding the evolution of the tissue distribution for the entire RYR isoform gene family.

Only vertebrates are currently known to express two skeletal muscle RYR isoforms, but the event that gave rise to the α and β isoforms must have occurred early on in vertebrate history. Once this event occurred (as indicated on Fig. 8), this condition was retained for millions of years of vertebrate evolution. We hypothesize that the presence of two RYR isoforms together in skeletal muscle provides greater flexibility in signal transduction. This may have to do with activation of the muscle over a wide temperature range, or perhaps modulation of the amount of calcium released and degree of fiber activation. In either case, this flexibility may be compromised in the specialized fast-contracting muscles with only the α isoform in favor of an RYR complement better suited for rapid contraction. It should be noted that, since the two RYR isoform condition is far more widespread among vertebrates than is the single isoform state, it remains possible that certain mammals may also retain the ability to express the β isoform. This possibility cannot be ruled out until a more extensive survey of mammals is completed.

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