Physiological Differences Between the α and β Ryanodine Receptors of Fish Skeletal Muscle

John O'Brien,* Hector H. Valdivia,‡ and Barbara A. Block*
*Department of Organismal Biology and Anatomy, University of Chicago, Chicago, Illinois 60637, and ‡Department of Physiology, University of Maryland, Baltimore, Maryland 21201 USA

ABSTRACT Two isoforms of the sarcoplasmic reticulum Ca^{2+} release channel (ryanodine receptor or RYR) are expressed together in the skeletal muscles of most vertebrates. We have studied physiological properties of the two isoforms (α and β) by comparing SR preparations from specialized fish muscles that express the α isoform alone to preparations from muscles containing both α and β. Regulation of channel activity was assessed through [^{3}H]ryanodine binding and reconstitution into planar lipid bilayers. Distinct differences were observed in the calcium-activation and -inactivation properties of the two isoforms. The fish α isoform, expressed alone in extraocular muscles, closely resembled the rabbit skeletal muscle RYR. Maximum [^{3}H]-ryanodine binding and maximum open probability (P_o) of the α RYR were achieved from 1 to 10 μM free Ca^{2+}. Millimolar Ca^{2+} reduced [^{3}H]ryanodine binding and P_o close to zero. The β isoform more closely resembled the fish cardiac RYR in Ca^{2+} activation of [^{3}H]ryanodine binding. The most prominent difference of the β and cardiac isoforms from the α isoform was the lack of inactivation of [^{3}H]ryanodine binding and P_o by millimolar free Ca^{2+}. Differences in activation of [^{3}H]ryanodine binding by adenine nucleotides and inhibition by Mg^{2+} suggest that the β and cardiac RYRs are not identical, however. [^{3}H]ryanodine binding by the α RYR was selectively inhibited by 100 μM tetracaine, whereas cardiac and β RYRs were much less affected. Tetracaine can thus be used to separate the properties of the α and β RYRs in preparations in which both are present. The distinct physiological properties of the α and β RYRs that are present together in most vertebrate muscles support models of EC coupling incorporating both directly coupled and Ca^{2+}-coupled channels within a single triad junction.

INTRODUCTION

Calcium release from intracellular stores plays an important role in metabolic regulation and signal transduction in a wide variety of cell types. A family of proteins, the calcium release channels or ryanodine receptors (RYRs), are central to this process in many cells. Ryanodine receptors were first described as the calcium release channel in skeletal muscle sarcoplasmic reticulum (SR). They have been shown to correspond to the "foot proteins" that span the gap between the SR membrane and the T-tubule in the triad junction (Inui et al., 1987a; Lai et al., 1988). Biochemical purification and cloning have revealed at least three different tissue-specific isoforms of the RYR in mammalian tissues. The RYR in skeletal muscle is the type 1 RYR (RYR1), which is expressed primarily in skeletal muscle (Marks et al., 1989), although a shortened transcript of RYR1 has been found in the brain (Takeshima et al., 1993) and an immunologically similar RYR is abundant in cerebellar Purkinje cells (Ouyang et al., 1993). The cardiac RYR (RYR2) was initially purified (Inui et al., 1987b; Anderson et al., 1989) and cloned (Nakai et al., 1990; Otsu et al., 1990) from heart, but is also expressed throughout the nervous system (Kuwajima et al., 1992). A third isoform (RYR3) has been cloned from brain (Hakamata et al., 1992). It is expressed primarily in brain, but is also present in smooth muscle and some endothelial cells (Hakamata et al., 1992).

All three RYR isoforms have structural and functional features in common. The functional channel consists of a homotetramer of an approximately 565 kDa polypeptide whose cytoplasmic domains form the feet structures. The channel is formed by the transmembrane domains of all four subunits (Lai et al., 1989). Each of the isoforms is activated by micromolar calcium, adenine nucleotides, caffeine, and nanomolar ryanodine and inhibited by magnesium, ruthenium red, and micromolar ryanodine (Meissner, 1994). A number of differences, however, attest to the fact that the RYR isoforms are not functionally equivalent. Although all three RYR isoforms are approximately the same size, there is only about 67% amino acid sequence identity between them (66% skeletal and cardiac; 67% cardiac and brain; 67% skeletal and brain; Nakai et al., 1990; Hakamata et al., 1992). Comparisons between the cardiac and skeletal muscle isoforms show that the cardiac muscle RYR is more sensitive to activation by micromolar free calcium and is less sensitive to inhibition by millimolar calcium (Pessah et al., 1985; Michalak et al., 1988; Chu et al., 1993). The cardiac isoform is also more readily phosphorylated by calmodulin-dependent protein kinase (CaM-Kinase) and cyclic AMP-dependent protein kinase than is the skeletal isoform (Witcher et al., 1991; Strand et al., 1993), and shows altered conductance properties when phosphorylated (Witcher et al., 1991).

The functional differences in RYRs may, in part, underlie the differences in the way each RYR channel is involved in the calcium release signal transduction pathway. Calcium release through the cardiac muscle RYR is triggered by an

Received for publication 15 April 1994 and in final form 7 September 1994.
Address reprint requests to Barbara Block, Stanford University, Department of Biological Sciences, Hopkins Marine Station, Pacific Grove, CA 93950. Tel.: 408-655-6236; Fax: 408-375-0793.

0006-3495/95/02/471/12 $2.00

influx of external calcium through the sarcolemmal voltage-dependent calcium channel (dihydropyridine receptor or DHPR) in a calcium-induced calcium release process (Stern, 1992). The skeletal muscle RYR, on the other hand, is proposed to be linked mechanically to the T-tubule DHPR (Schneider and Chandler, 1973; Rios et al., 1993) and does not require a current of external calcium for activation. Calcium release is instead proposed to be triggered by a conformational change in the DHPR that is directly coupled to the skeletal RYR. The region(s) of the skeletal RYR responsible for this form of coupling have not yet been identified.

In nonmammalian vertebrates, the presence of a single, distinct isoform of each channel in cardiac and skeletal muscle is not the rule. Most skeletal muscles of birds, fish, amphibians, and certain reptiles express two distinct RYR isoforms together (Airey et al., 1990; Olivares et al., 1991; Lai et al., 1992; O'Brien et al., 1993). The isoforms, termed α and β, were identified by their different mobilities on SDS gels and differences in their immunological reactivity and peptide maps (Airey et al., 1990; Olivares et al., 1991). The two isoforms are detected together in the same muscle fibers and are thought to be present in the same triad junctions (Airey et al., 1990; Olivares et al., 1991; Lai et al., 1992). Immunological results suggest that the α isoform in frogs, chicken, fish, and reptiles has similarity to the mammalian skeletal muscle RYR (Lai et al., 1992; O'Brien et al., 1993), whereas the frog β isoform has similarity to the mammalian cardiac muscle RYR (Lai et al., 1992; Airey et al., 1993). Careful peptide mapping, phosphorylation, and immunological studies in chicken have revealed that both of the RYR isoforms in skeletal muscle are distinct from the cardiac muscle isoform, although β more closely resembles the cardiac isoform (Airey et al., 1993).

Currently, the functional significance of the coexpression of two RYR isoforms in nonmammalian skeletal muscle remains unknown. The α and β RYR isoforms have been present together since the beginnings of vertebrate evolution (O'Brien et al., 1993). A central question concerning their presence over this 400 million year time span is this: Does the presence of two isoforms confer physiological advantages to the muscle, or is it merely an evolutionary step in the progression to the single isoform condition seen in mammals and some reptiles? To understand the functional roles of the α and β RYR isoforms, it is necessary to learn how they differ physiologically. Recently, we have studied the distribution of RYR isoforms in skeletal muscles of a variety of vertebrates and have discovered that the two isoforms are differentially expressed in muscles with different functional specializations (O'Brien et al., 1993). The α isoform is expressed alone in fish and bird extraocular muscles and the toadfish swimbladder muscle. These muscles are unique in that they are among the fastest-contracting muscles in vertebrates. This pattern of expression has important implications for the physiological roles of the α and β RYR isoforms in skeletal muscle. It suggests that the functional properties of the α isoform are better suited for triggering high frequency muscle contractions.

In the present study, we have taken advantage of the differential expression of skeletal muscle RYR isoforms in fish muscles to examine the physiological properties of the α and β isoforms. We find that there are differences in the calcium activation and inactivation properties of the two isoforms. These physiological differences have important implications for the properties of Ca^{2+} release at skeletal muscle triad junctions. The distinct properties of calcium release may likewise provide the basis on which the isoforms are selected for use in muscles with different functional demands.

MATERIALS AND METHODS

SR isolation

Crude SR vesicles were prepared from a variety of muscle tissues from blue marlin (*Makaira nigricans*) and toadfish (*Opsanus tau*) as previously described (O'Brien et al., 1993). A series of SR preparations was prepared from blue marlin muscles to allow comparison of the [^{3}H]ryanodine binding properties of SR from tissues with different complements of RYR isoforms. For each blue marlin, equal quantities (5 g) of heart, superior rectus extraocular muscle (containing only the α RYR isoform), and white epaxial (swimming) muscle (containing both α and β isoforms) were used to prepare SR fractions under identical conditions. Crude SR fractions from four individual fish were prepared this way.

Heavy SR preparations more enriched in the RYR were made from toadfish white swimming muscle (α and β isoforms). Crude SR, prepared as described above, were suspended in 0.3 M sucrose, 0.6 M KCl, 10 mM K-piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), pH 7.0, 100 μM EGTA, 1 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 1 mM benzamidine, and 1.2 mM diisopropylfluorophosphate (DIFP) and layered over discontinuous sucrose gradients. The gradients consisted of 5 ml of 45% sucrose, 8 ml of 36% sucrose, 9 ml of 30% sucrose, and 9 ml of 20% sucrose in 0.4 M KCl, 5 mM K-PIPES, pH 6.8, 100 μM EGTA, 100 μM $CaCl_2$, plus the protease inhibitors listed above. Gradients were centrifuged for 16 h at 22,000 rpm (64,000 $\times$ g_{av}) in a Beckman SW28 rotor, and the heavy SR fraction collected from the 36–45% sucrose interface. Heavy SR preparations for comparison with fish SR were made from frozen rabbit skeletal muscle obtained from Pel-Freeze Biological Supply (Rogers, AR).

[^{3}H]Ryanodine binding

[^{3}H]Ryanodine binding to SR vesicles was used as a probe for the open state of the RYR calcium channel (Meissner, 1986; Pessah et al., 1987; Chu et al., 1990). SR vesicles (0.2–1.0 mg protein/ml) were incubated for 6 h at 30°C in a medium consisting of (unless otherwise stated in the figure legends) 0.2 M KCl, 20 mM K-PIPES, pH 7.1, 1 mM EGTA, 2 mM nitrilotriacetic acid (NTA), 0.5 mg/ml serum albumin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.6 mM DIFP, 6.4 nM [^{3}H]ryanodine, and $CaCl_2$ to set the free calcium concentration from 1 nM to 3.3 mM (pCa 9.0–2.5). Aliquots of the vesicle suspension were filtered through Whatman GF/B filters presoaked in 5% (w/v) polyethyleneimine and rinsed with 15 ml of ice-cold water. Radioactivity remaining bound to the filters was measured by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM unlabeled ryanodine and was subtracted from the data to yield specifically bound [^{3}H]ryanodine. Data for each assay represent means for two filtered aliquots. At least two replicate assays were performed per preparation. The calcium concentration at the [^{3}H]ryanodine binding peak was determined for each preparation by analysis of the intersection of regression lines fit to log-log plots of the binding data according to Nickerson et al. (1989). For comparisons between tissue types, the [^{3}H]ryanodine binding data for each

preparation were normalized to the maximum binding measured in that preparation. Comparisons were made at each experimental [Ca^{2+}] using a *t*-test.

Measurements of [^{3}H]ryanodine binding affinity were performed as described above except that the buffer contained 0.1 mg/ml BSA, 1 mM β-γ-methyleneadenosine 5′-triphosphate (AMP-PCP), 100 μM free calcium, and 1 to 100 nM [^{3}H]ryanodine. For ryanodine concentrations higher than 10 nM, [^{3}H]ryanodine was mixed with unlabeled ryanodine. Samples were incubated 16 h at 30°C, and aliquots filtered through GF/B filters and treated as above. Nonspecific binding was measured in the presence of 100 μM unlabeled ryanodine.

Free calcium concentrations were calculated with a computer program using stability constants for EGTA and NTA from Fabiato and Fabiato (1979). The concentrations of EGTA solutions were measured by titration with a Radiometer calcium standard using a Radiometer F2112Ca calcium electrode. Working $CaCl_2$ solutions were calibrated by titration with a calibrated EGTA solution in the same way. Protein was assayed by the Amido Black procedure of Kaplan and Pedersen (1985).

Planar bilayer recordings

Phospholipid bilayers composed of phosphatidylethanolamine and phosphatidylserine (1:1 dissolved in decane to 20 mg/ml) were "painted" with a teflon rod across an aperture of ≈300 μm diameter in a delrin cup. The *cis* chamber was the voltage control side connected to the head stage of a 200A Axopatch amplifier, whereas the *trans* side was held at virtual ground. Agar/KCl bridges were used to connect the chambers to Ag/AgCl electrodes immersed in 0.2 M KCl. The *cis* (600 μl) and *trans* (900 μl) chambers were initially filled with 50 mM Cs-methanesulfonate and 10 mM Tris-HEPES, pH 7.2. After bilayer formation, an asymmetric Cs-methanesulfonate gradient (300 mM *cis*/50 mM *trans*) was established. Cs^+ instead of Ca^{2+}, was chosen as the charge carrier to precisely control Ca^{2+} around the channel, to increase the conductance of the channel ($g_{Cs^+}/g_{Ca^{2+}} = 2$; Smith et al., 1988), and to avoid interference from K^+ channels present in the SR membrane. Cl^- channels were blocked by replacement of Cl^- with the impermeant anion methanesulfonate.

Incorporation of channels was achieved as previously described (Valdivia et al., 1992). Heavy SR from toadfish white swimming muscle (α and β isoforms) were added to the *cis* chamber, which corresponds to the cytoplasmic side of the SR, whereas the *trans* side corresponded to the lumenal side. Contaminant Ca^{2+} (≈4–8 μM) served to activate channel openings. After visualization of the channel, the Cs-methanesulfonate concentration in the *trans* chamber was increased to 300 mM. This maneuver greatly improved bilayer stability and prevented further vesicle insertion, allowing recordings to be made from the same channel for 30–60 min. Routinely, a current-voltage relationship was constructed to determine unitary channel conductance. A Ca^{2+}-EGTA mixture was added to the *cis* chamber to a final concentration of 0.059:1.00 mM, respectively. The calculated free Ca^{2+} concentration was 10 nM, which was verified with a calcium electrode using a Molecular Probes (Eugene, OR) Ca^{2+} calibration kit (range 1 nM to 1 mM). Aliquots of $CaCl_2$ and NaOH were then added to the *cis* chamber to yield the specified free [Ca^{2+}] without pH deviation. For each [Ca^{2+}], single-channel data were collected at steady voltages (+40 and −40 mV) for 2–5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10 kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5–2 kHz. Data acquisition and analysis were done with Axon Instruments (Burlingame, CA) software and hardware (pClamp v5.7, Digidata 200 AD/DA interface).

Materials

[^{3}H]ryanodine was purchased from NEN (Boston, MA), and unlabeled high-purity ryanodine (99.9%) was from Calbiochem (La Jolla, CA). Other chemicals were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Comparison of α and β RYR isoforms to cardiac muscle RYR by [^{3}H]ryanodine binding

The α isoform is expressed alone in fish extraocular muscles (O'Brien et al., 1993). By comparing the characteristics of the α RYR from these muscles to those of cardiac muscle RYR and white swimming muscle (which contains both α and β RYR isoforms), we observed distinct differences in the [^{3}H]ryanodine binding properties of the isoforms. In these experiments, we used a series of SR preparations from marlin superior rectus extraocular muscle (α isoform only), white muscle (α and β isoforms), and heart. All of the preparations displayed high-affinity [^{3}H]ryanodine binding (Table 1) with K_d values ranging from 3.9 to 12 nM. None of the K_d values were significantly different from each other, although the *p* value for the difference between superior rectus muscle (α only) and white swimming muscle (α and β) was 0.062 (*t*-test). [^{3}H]ryanodine binding activity in all tissues was strongly dependent on the presence of channel activators. To study the role of Ca^{2+} in channel activation, we first compared the dependency of [^{3}H]ryanodine binding on [Ca^{2+}] under conditions in which Ca^{2+} is the sole activator of the channel. The Ca^{2+} activation curves of [^{3}H]ryanodine binding for the fish muscle SR preparations are shown in Fig. 1 *A–C*. There were distinct differences in the Ca^{2+} activation and inactivation properties of [^{3}H]ryanodine binding in the preparations. [^{3}H]Ryanodine binding to marlin superior rectus muscle SR (α isoform only) displayed a bell-shaped dependence on free Ca^{2+} (Fig. 1 *A*). Ryanodine binding was activated with a threshold of approximately 0.1 μM Ca^{2+} and was maximal between 10 and 100 μM free Ca^{2+} for most preparations. The mean [^{3}H]ryanodine binding peak was at pCa 4.96 (5 experiments with 4 preparations). Higher concentrations of Ca^{2+} inhibited binding with 74% inactivation at 3.3 mM (see normalized curve, Fig. 1 *D*). A different Ca^{2+} activation and inactivation profile was observed with marlin cardiac muscle SR (Fig. 1 *B*). The threshold of activation was approximately 0.1 μM Ca^{2+}, but maximum ryanodine binding was reached at pCa 4.30 (4 preparations). Furthermore, ryanodine binding was only inactivated 18% by free Ca^{2+} as high as 3.3 mM. Marlin white muscle SR (Fig. 1 *C*), which contain both α and β RYR isoforms, had [^{3}H]ryanodine binding characteristics intermediate between those of the cardiac muscle and superior rectus preparations. The peak of [^{3}H]-ryanodine binding was at pCa 4.28, similar to the cardiac muscle preparations, but there was more inactivation (41%) by 3.3 mM free Ca^{2+}. The difference in [^{3}H]ryanodine binding peak between the superior rectus and heart preparations

TABLE 1 [^{3}H] Ryanodine binding properties of marlin muscle SR preparations

Muscle (number of preparations)	K_d (nM)	B_{max} (pmol/mg)
White muscle (α and β isoforms) (2)	3.87 ± 0.04	3.71 ± 0.23
Superior rectus (α isoforms) (3)	9.84 ± 2.75	2.18 ± 0.79
Heart (3)	12.06 ± 5.37	1.57 ± 0.63

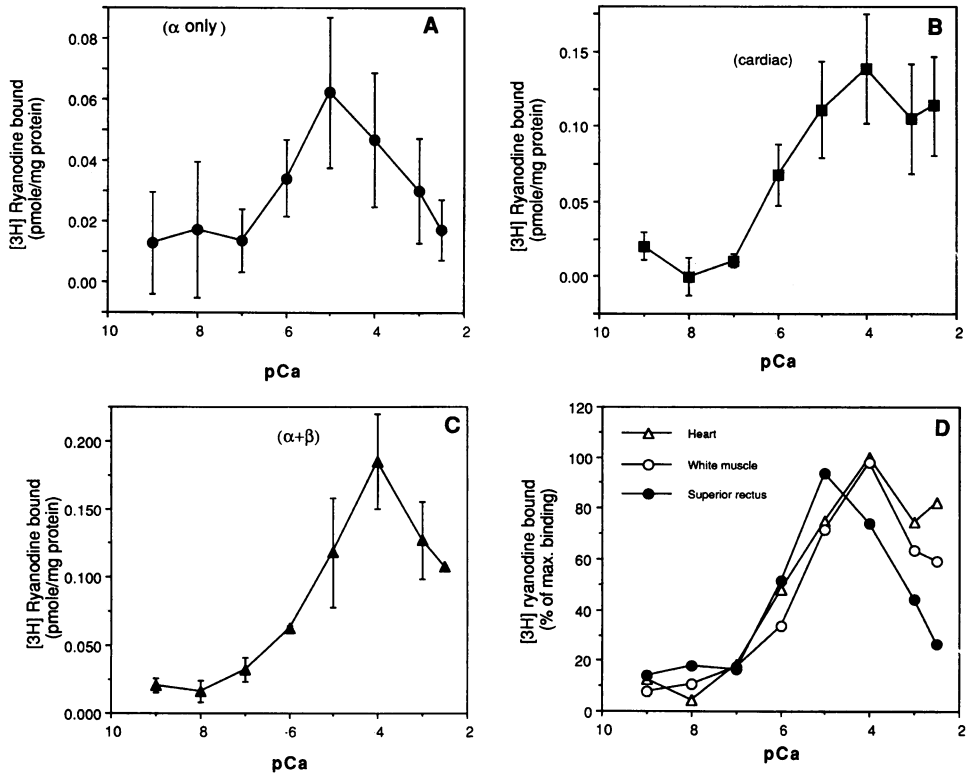

FIGURE 1 Calcium dependence of [^{3}H]ryanodine binding to SR vesicles from marlin muscles. SR vesicles were incubated with 6.4 nM [^{3}H]ryanodine under conditions in which Ca^{2+} is the sole channel activator (see Materials and Methods). (*A*) Superior rectus muscle SR, containing the α isoform only, shows a strongly biphasic response to Ca^{2+}. [^{3}H]Ryanodine binding is nearly completely blocked at pCa 2.5. (*B*) Cardiac muscle SR has a [^{3}H]ryanodine binding curve that is shifted to the right and displays little inhibition of binding at pCa 2.5. (*C*) White swimming muscle SR, containing both α and β isoforms, has a [^{3}H]-ryanodine binding curve similar to that of cardiac muscle SR. (*D*) Normalized curves for the three tissues reveal the differences in position of the ryanodine binding peak and degree of inactivation at millimolar Ca^{2+} between superior rectus (α only; ●), heart (cardiac; △), and white swimming muscle (α and β; ○) SR preparations. Data are means ± SE for four preparations. Error bars have been omitted from *D* for clarity.

was statistically significant ($p = 0.034$), whereas a significant difference was not established between superior rectus and white muscle ($p = 0.061$). The p-value for the similarity of [^{3}H]ryanodine binding peak between white muscle and heart preparations was 0.91. The degree of inactivation of [^{3}H]ryanodine binding at 3.3 mM free Ca^{2+} (pCa 2.5) was significantly different between all three preparations. [^{3}H]-ryanodine bound at pCa 2.5 was significantly lower in white muscle SR than in heart ($p = 0.043$), and [^{3}H]ryanodine bound to superior rectus SR was significantly lower than to white muscle ($p = 0.024$). Comparison of superior rectus and heart yielded a p of 0.001. The similarity of the white muscle [^{3}H]ryanodine binding peak to that of heart as well as the reduced inactivation at high [Ca^{2+}] compared with superior rectus SR (with only the α isoform present) suggests that a portion of the [^{3}H]ryanodine binding activity in white muscle SR has properties similar to those of the fish cardiac RYR.

Tetracaine has differential effects on [^{3}H]ryanodine binding by the RYR isoforms

To discern the [^{3}H]ryanodine binding contributions of the α and β isoforms in white skeletal muscle preparations that contain both isoforms, it is necessary to selectively activate or inhibit the isoforms. The [^{3}H]ryanodine binding characteristics of the different types of SR preparations were modified by application of the local anesthetic tetracaine. Dose-response curves of the effect of tetracaine on each of the marlin muscle SR types at pCa 4.0 in the presence of 2.5 mM AMP-PCP and 2.5 mM Mg^{2+} (0.4 mM free Mg^{2+}) are shown in Fig. 2 *A*. The K_i values from these curves are 66 μM for superior rectus (α only), 89 μM for white muscle (α and β), and 360 μM for heart. The heart K_i for tetracaine was significantly higher than either superior rectus or white muscle K_i values ($p < 0.05$ for both comparisons), but no statistical difference was found between superior rectus and white muscle K_i values. Hill plots of these data (Fig. 2 *B–D*) are linear at high tetracaine concentration for all tissues, but curved below a threshold level. The slopes of the linear portion are different for each tissue type, with a slope of -1.01 ± 0.05 (regression coefficient ± SE of determination) for superior rectus (α isoform), -1.35 ± 0.10 for heart, and -1.29 ± 0.08 for white muscle (α and β). The slope of the superior rectus Hill plot was significantly different than that of either the heart or white muscle preparations ($p < 0.05$ for both comparisons), but there was no significant difference between heart and white muscle preparations. These data reveal differential inhibition of the RYR isoforms by tetracaine. The α isoform, expressed alone in superior rectus muscle, is more sensitive to tetracaine inhibition than is the cardiac isoform. Furthermore, it appears that the β isoform in white muscle may lend some degree of insensitivity to tetracaine, increasing the K_i and increasing the slope of the Hill plot.

The differential inhibition of the RYR isoforms can be seen further in experiments in which the effect of a single tetracaine concentration was tested over the range of [Ca^{2+}] (Fig. 3). These experiments, like those shown in Fig. 2, were performed in the presence of 2.5 mM AMP-PCP and 2.5 mM Mg^{2+} (free Mg^{2+} 0.2–0.5 mM) to activate [^{3}H]ryanodine binding. At 100 μM, tetracaine showed differential blocking

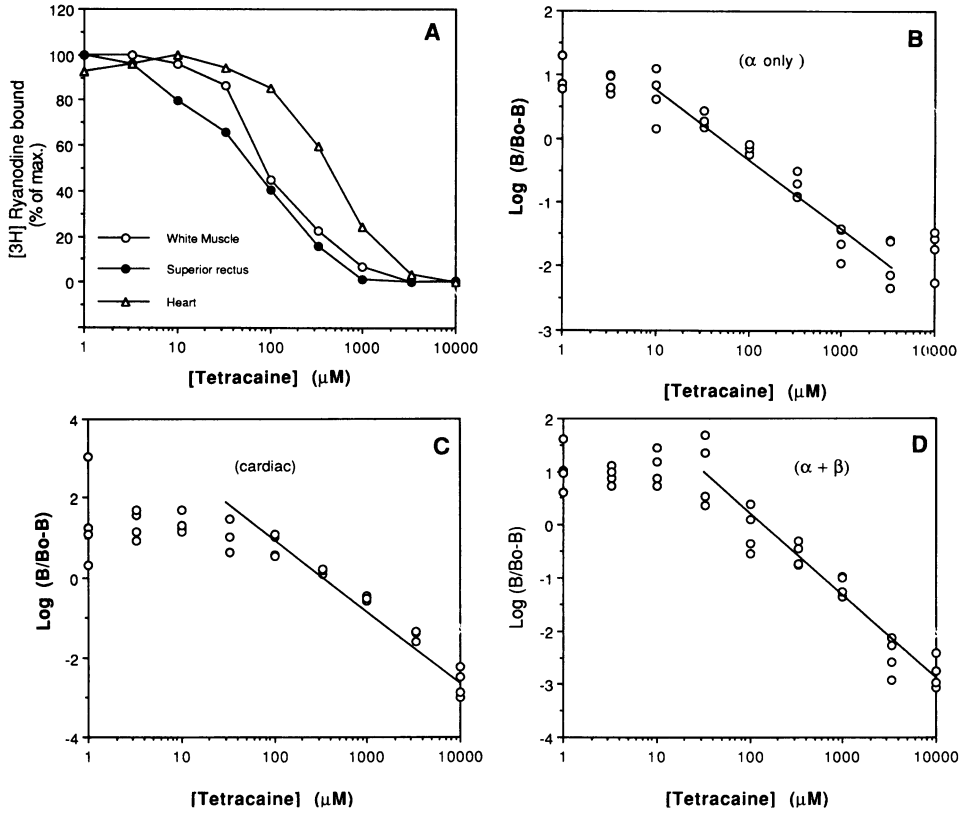

FIGURE 2 Effects of tetracaine on [^{3}H]ryanodine binding to marlin muscle SR. SR vesicles from superior rectus (α only; ●), cardiac (△), and white swimming muscle (α and β; ○) were incubated with varying concentrations of tetracaine in a medium containing 2.5 mM AMP-PCP, 2.5 mM Mg^{2+} (0.4 mM free Mg^{2+}) and 100 μM Ca^{2+}. (*A*) Dose response curves of the three SR preparations. (*B–D*) Hill plots ($\log\{bound_{tet}/(bound_{control} - bound_{tet})\}$ vs. log(tetracaine concentration)) of the same data for superior rectus, cardiac, and white muscle SR preparations.

activity on the different muscle SR preparations, and thus can be a useful tool to distinguish between the α and β RYR isoforms. [^{3}H]ryanodine binding by the α isoform of superior rectus muscle SR preparations was inhibited more than 70% by 100 μM tetracaine (Fig. 3 *A*), whereas the shape of the Ca^{2+} activation curve was largely unaffected. The cardiac

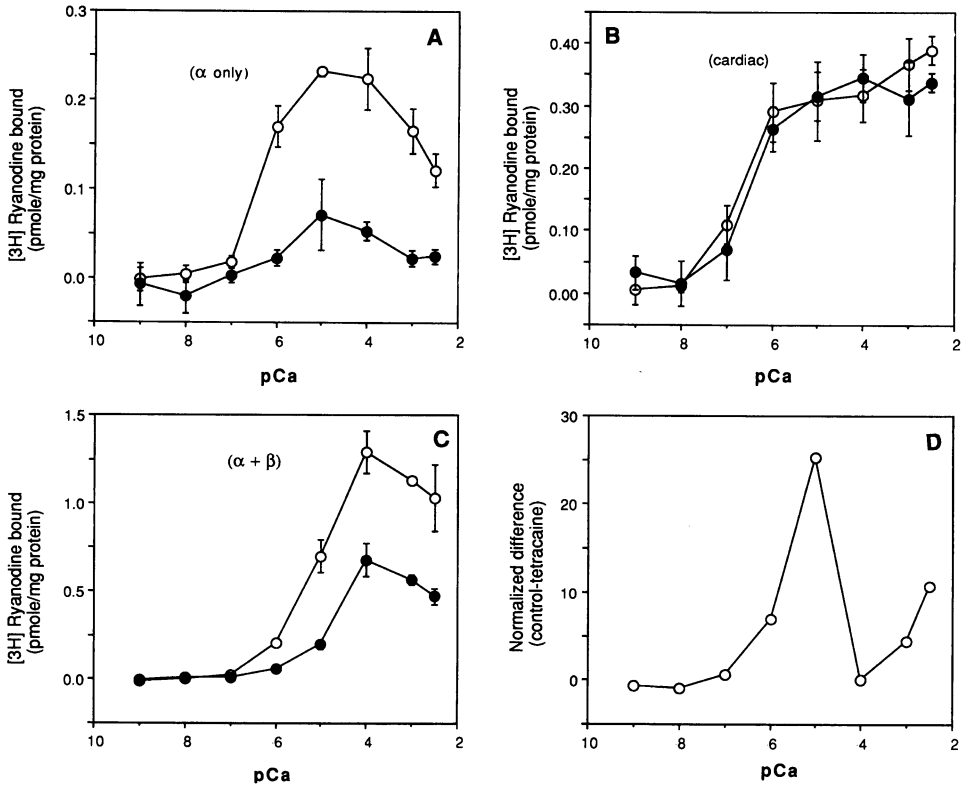

FIGURE 3 Effects of 100 μM tetracaine on the Ca^{2+} dependence of [^{3}H]ryanodine binding. Conditions were similar to those in Fig. 1 except that 2.5 mM AMP-PCP and 2.5 mM Mg^{2+} were included. Specific [^{3}H]ryanodine binding is increased three- to fivefold compared with Fig. 1. Control curves (*open symbols*) show [^{3}H]ryanodine binding in the absence of tetracaine. [^{3}H]Ryanodine binding in the presence of 100 μM tetracaine is shown with solid symbols. (*A*) Marlin superior rectus muscle SR (α isoform only). (*B*) Marlin cardiac muscle SR. (*C*) Marlin white swimming muscle SR (α and β isoforms). Data in *A–C* are means ± SDs for three determinations from single preparations. (*D*) Normalized control minus tetracaine-inhibited difference curve for white swimming muscle SR (α and β) shown in *C*. The difference curve reveals the Ca^{2+} activation profile of the form predominantly inhibited by tetracaine. Data in *D* are means ± SDs of the difference between paired controls and tetracaine-inhibited preparations normalized to the peak [^{3}H]ryanodine binding value.

RYR isoform, on the other hand, was inhibited only 5% by 100 μM tetracaine (Fig. 3 *B*) and showed no calcium inactivation under these conditions of adenylate and Mg^{2+} modulation. In the white muscle preparations, containing both α and β RYR isoforms (Fig. 3 *C*), [^{3}H]ryanodine binding was reduced by 50% at the binding peak (100 μM Ca^{2+}) and approximately 70% at 10 μM Ca^{2+}. An analysis of the difference between the control and tetracaine inhibited curves for white muscle (both curves normalized to the peak of [^{3}H]ryanodine binding) is shown in Fig. 3 *D*. The difference curve reveals a peak of tetracaine sensitivity at pCa 5, consistent with the Ca^{2+} activation profile of the α isoform. No such consistent pattern was seen with the same treatment of the tetracaine inhibition data of the other preparations (not shown). These data suggest that at 100 μM tetracaine the α isoform contribution to the [^{3}H]ryanodine binding curve of white muscle is substantially reduced. Thus, the significant remaining [^{3}H]ryanodine binding should reflect largely the β isoform contribution. The shape of the white muscle SR [^{3}H]ryanodine binding curve inhibited by tetracaine is very similar to that of the uninhibited curve, supporting the conclusion that this shape represents primarily the β isoform contribution. Comparison of this curve with the cardiac SR [^{3}H]ryanodine binding curve under the same conditions of Mg^{2+} and adenylate demonstrates that the cardiac isoform and the β isoform do not behave identically. The cardiac isoform is maximally activated at much lower free [Ca^{2+}] (about 10 μM) than is β under these conditions, and shows no evidence of inactivation by millimolar Ca^{2+}. On the other hand, the tetracaine-inhibited white muscle SR preparation still shows partial inactivation by millimolar Ca^{2+} that is at least partially attributable to the β isoform.

Comparison with [^{3}H]ryanodine binding properties of other skeletal muscle SR

Preparations from other species show characteristics of [^{3}H]-ryanodine binding that match those of the marlin muscle preparations. Heavy SR preparations from toadfish white muscle (α and β isoforms; Fig. 4 *A*) show a Ca^{2+} activation profile remarkably similar to that of marlin white muscle SR. The threshold of activation is approximately 0.1 μM Ca^{2+}, with maximum ryanodine binding at 100 μM Ca^{2+}. The inactivation at millimolar calcium is about 30%. The Ca^{2+} activation profile of [^{3}H]ryanodine binding by rabbit skeletal muscle SR (Fig. 4 *B*) follows that of the marlin superior rectus SR (α isoform alone). The Ca^{2+} activation and inactivation curves of rabbit are shifted approximately 1 pCa unit lower, but the bell-shaped profile is preserved, with complete inactivation of [^{3}H]ryanodine binding at millimolar Ca^{2+}. This Ca^{2+} activation profile was also seen in toadfish swimbladder muscle, in which the α isoform is present alone (Fig. 4 *C*). It is evident that the Ca^{2+} activation profiles of [^{3}H]-ryanodine binding are consistent between species and fall into two groupings. The fish α only preparations and mammalian skeletal muscle preparations form a group with a characteristically large degree of inactivation by millimolar Ca^{2+}. The fish white muscle preparations (α and β isoforms) and cardiac muscle preparations form a second group. They differ from the α only preparations primarily in the higher Ca^{2+} activation peak (100 μM) and in the much smaller degree of inactivation by millimolar Ca^{2+}. Thus, the majority of the ryanodine binding activity by the white muscle SR has a Ca^{2+} activation and inactivation profile similar to that of cardiac SR. From the results of preparations with the α isoform alone, it appears that the cardiac-like characteristics of the white muscle [^{3}H]ryanodine binding activity are contributed by the β isoform.

Functional differences of RYR isoforms

To examine further the functional differences of the toadfish skeletal muscle RYR isoforms, we studied the single-channel conductance and Ca^{2+} dependence of these channels incorporated into planar lipid bilayers. Incorporation of heavy SR from toadfish white muscle (α and β isoforms) into lipid bilayers revealed two different high-conductance channels with distinct properties (Fig. 5). The following controls (a)

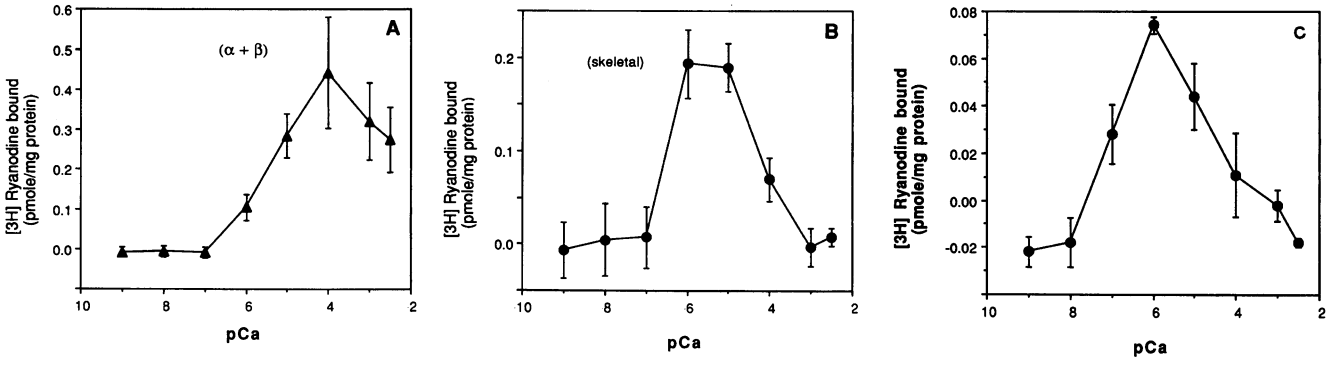

FIGURE 4 Calcium dependence of [^{3}H]ryanodine binding to heavy SR preparations from other species closely resembles that of marlin SR [^{3}H]ryanodine binding. Heavy SR preparations were incubated under conditions identical to those in Fig. 1. (*A*) Toadfish white swimming muscle heavy SR, which contain both α and β RYR isoforms, have a [^{3}H]ryanodine binding curve nearly identical to that of marlin white swimming muscle (Fig. 1 *C*). (*B*) Rabbit skeletal muscle heavy SR have [^{3}H]ryanodine binding characteristics very similar to those of marlin superior rectus SR (α isoform only; Fig. 1 *A*). The inactivation of [^{3}H]ryanodine binding by millimolar Ca^{2+} is essentially complete as it was in superior rectus SR. (*C*) Toadfish swimbladder muscle SR, α RYR only.

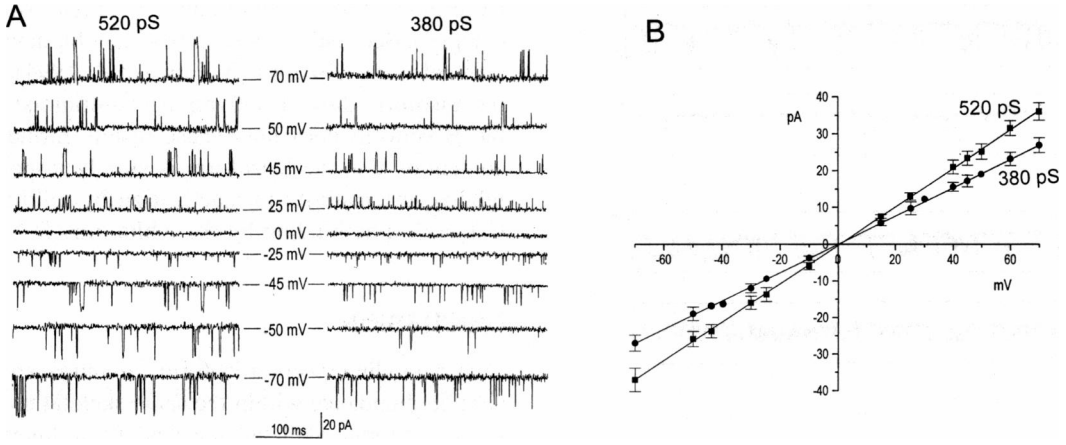

FIGURE 5 RYR isoforms of toadfish fast-twitch skeletal muscles. (*A*) Planar bilayer recordings of a 520 pS channel and a 380 pS channel from SR vesicles of toadfish white skeletal muscle. Traces were obtained at specified holding potential in symmetrical 300 mM cesium methanesulfonate, 10 mM Tris/HEPES, pH 7.2. Contaminant Ca^{2+} (4–8 μM) served to activate openings. Recordings were low pass-filtered at 1.5 kHz and digitized at 3 kHz. (*B*) Current voltage relationship of RYR isoforms. Data were collected from four 520 pS channels and three 380 pS channels. The solid lines are linear regressions to the data points with correlation coefficient = 0.99 for both channels.

to (d) (not shown) ensured that the recorded channel activity corresponded to RYRs (Valdivia et al., 1992). (a) Channel activity increased three to sixfold when 3 mM Na_2ATP was added to the *cis* side. (b) By contrast, similar addition of EGTA, which decreased free Ca^{2+} to less than 1 nM, totally abolished channel activity. (c) Ryanodine (200 nM) irreversibly modified the kinetics and conductance of the channel, inducing the appearance of a conductance state that represented approximately 50% of the full conductance state. And (d) this ryanodine-modified state was completely blocked by the addition of 5 μM ruthenium red to the *cis* chamber. Each set of traces in Fig. 5 were taken from experiments in which a single channel was observed. Although insertion of multiple channels occurred in about 30% of our recordings, we analyzed only those in which a single channel was active during the entire course of the experiment. In some instances, we ruled out the presence of multiple channels by adding *cis* 5 mM ATP under optimal free [Ca^{2+}] (10–100 μM, see below). Under these conditions, open probability (P_o) was >0.3 for any given channel, and the likelihood of encountering multiple opening levels was high. Thus, by limiting the analysis to single-channel experiments only, we could unambiguously attribute changes of activity in response to modulators to a specific RYR isoform.

Two different RYRs were detected in toadfish white muscle based on their single-channel conductance (Fig. 5 *A*). One of the two channels had a slope conductance of 520 ± 36 pS (mean ± SD, n = 6; Fig. 5 *B*) and was very similar in conductance to the RYR from rabbit skeletal muscles (460–600 pS in monovalent solutions; Smith et al., 1988; Xu et al., 1993). The second type of channel displayed a 380 ± 32 pS conductance and was similar in properties to the bovine cardiac RYR (Valdivia et al., 1994). The 520 and 380 pS channels were equally abundant in two independent preparations of SR, as judged by their frequency of presentation in bilayer experiments (n = 14 and 11 for the 520 and 380 pS channels, respectively, from a total of 29 recordings). Both channels displayed long, well resolved openings intermixed with brief, more abundant openings. Also, the presence of ultrafast components was highly likely (Tinker et al., 1992), but the temporal resolution of our recording system limited the detection of open events to those duration 0.50 ms and longer. For this reason, it was difficult to discern whether truncated openings corresponded to unresolved events or to genuine subconductance states (Liu et al., 1989). Although all events were considered for calculation of the channel open probability (P_o) by placing the open discriminator 1 SD above the mean baseline current, we constructed the current-voltage (*I-V*) relationship for both channels (Fig. 5 *B*) with data obtained with well resolved openings only. The linear regression for data points thus obtained had correlation coefficients ≥0.98.

Effects of Ca^{2+} on single-channel activity of RYR isoforms

Although the 520 and 380 pS channels displayed qualitatively similar response to modulators, each channel displayed a distinct and significantly different response to Ca^{2+}. Representative traces of channel activity of the 520 pS channel at 100 μM and 1 mM *cis* (cytoplasmic) Ca^{2+} are shown in Fig. 6 *A*, traces 1 and 2 (respectively). The 380 pS channel at 100 μM and 1 mM Ca^{2+} is shown in Fig. 6 *B*, traces 1 and 2. The effects of increasing *cis* [Ca^{2+}] on the P_o of the two channels is shown in Fig. 6 *C*. The P_o of both channels was increased by 0.1–1 μM *cis* free Ca^{2+} and achieved maximum value between 10 and 100 μM free Ca^{2+}. However, the two channels displayed different responses to high [Ca^{2+}]. The 520 pS channel reached a maximum P_o of 0.31 and was inactivated by 1 mM Ca^{2+} (Fig. 6 *A*, trace 2). The 380 pS channel was activated to nearly twice the P_o, 0.57, and was not inactivated by millimolar Ca^{2+} (Fig. 6 *B*, trace 2). In both

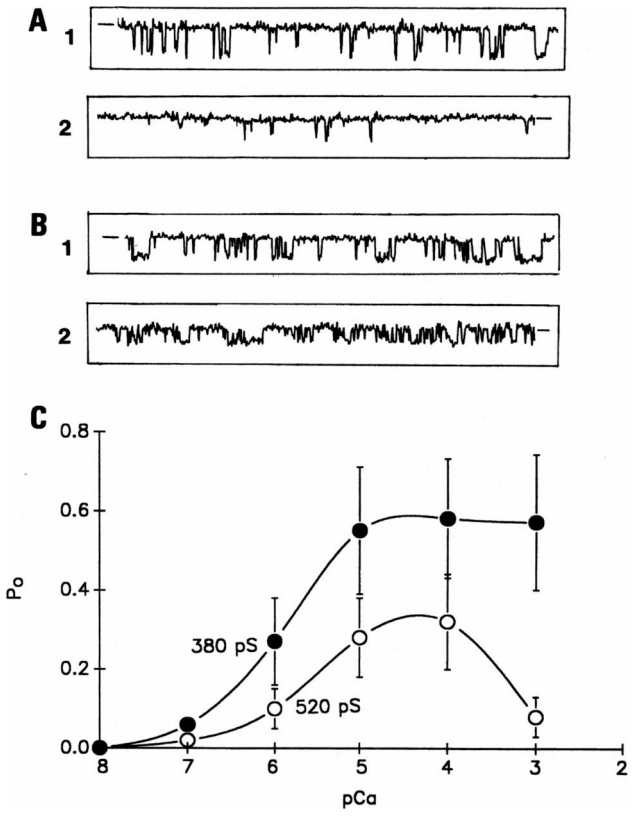

FIGURE 6 Ca^{2+} dependence of open probability of RYR channels from toadfish white swimming muscle heavy SR incorporated into planar lipid bilayers. Two types of channels were identified in this SR preparation, which contains both α and β RYR isoforms. Representative traces of the higher conductance (520 pS) channel are shown in *A*. Trace 1 shows channel activity in the presence of 10 μM *cis* (cytoplasmic) Ca^{2+}; P_o = 0.22. Trace 2 shows the channel in the presence of 1 mM *cis* Ca^{2+}; P_o = 0.04. The lower conductance channel (380 pS) is shown in *B*. Trace 1 is in the presence of 10 μM *cis* Ca^{2+}, and trace 2 in the presence of 1 mM *cis* Ca^{2+}. P_o is 0.56 in trace 1 and 0.60 in trace 2. The Ca^{2+} dependence of open probability of the two types of channels is shown in *C*. Data are means ± SD from a total of 14 channels.

channels, the addition of 1 mM Ca^{2+} caused a significant decrease in permeability to Cs^+ that was reflected in the lower current amplitude at a given holding potential (compare, for example, traces 1 and 2 in Fig. 6, *A* and *B*). This effect has been attributed to the higher selectivity of the RYR for Ca^{2+} over monovalent cations (Smith et al., 1988).

In a complementary study, Valdivia et al. (1994) have shown that the 520 pS channel was selectively activated by Imperatoxin A, a scorpion toxin that acts selectively upon the skeletal muscle isoform of the RYR (Valdivia et al., 1992). The 380 pS channel was not affected. Imperatoxin A also enhanced [^{3}H]ryanodine binding by fish SR preparations containing only the α isoform, whereas it increased [^{3}H]-ryanodine binding to fish white muscle SR preparations (α and β) only half as much (Valdivia et al., 1994). These data demonstrate that the 520 pS channel activated by Imperatoxin A is the α RYR isoform.

The Ca^{2+} activation and inactivation of P_o closely mimic the influences of Ca^{2+} on [^{3}H]ryanodine binding by the α isoform (520 pS channel) and the cardiac isoform (380 pS channel). Because we can identify unambiguously the 520 pS channel as the α isoform (Valdivia et al., 1994), we know that the α isoform shows inactivation by millimolar Ca^{2+} whereas the β isoform does not. These results further support the contention that the β isoform is largely responsible for the relative insensitivity to inactivation by millimolar Ca^{2+} of [^{3}H]ryanodine binding by the white muscle SR.

DISCUSSION

Two ryanodine receptor isoforms, α and β, are expressed in similar abundance within the same skeletal muscle fibers in most vertebrate skeletal muscles. Thus, knowledge of the physiological properties of the α and β RYRs in nonmammalian skeletal muscle is critical for understanding the mechanism of EC coupling. We have utilized a fish model system that facilitates the elucidation of the [^{3}H]ryanodine binding properties of the two distinct nonmammalian RYR isoforms. We have then complemented the binding studies with observations on single-channel conductances of the two different channels in fish swimming muscle. The expression of the α isoform alone in fish extraocular muscles has allowed us to study the α isoform in its native SR membrane independently of the β isoform. Comparisons to the α + β population of channels in fish swimming muscles and to the cardiac muscle RYR have then allowed us to infer [^{3}H]-ryanodine binding properties of the β isoform and to identify unambiguously both isoforms in single-channel studies. This approach has bypassed the need to solubilize and separately purify the different isoforms, which may have detrimental effects on the isolated channels' properties.

The α isoform of nonmammals is functionally equivalent to the mammalian skeletal RYR

The α isoform, expressed alone in extraocular muscles, has distinct and readily resolved properties of Ca^{2+} activation and inactivation. Both [^{3}H]ryanodine binding and single-channel conductance studies of the α isoform reveal its biphasic response to free Ca^{2+}. The channel is activated at micromolar Ca^{2+}, with peak activation in the 1–10 μM range, and is subsequently inhibited by millimolar Ca^{2+}. The similarity of the α RYR isoform of fish muscle to the mammalian skeletal muscle RYR is striking. The α isoform from fish extraocular and epaxial muscles displays similar properties of Ca^{2+} activation and inactivation of [^{3}H]ryanodine binding, nearly identical single-channel conductance, and similar Ca^{2+} activation and inactivation of channel P_o to the rabbit skeletal muscle RYR (mammalian data from Pessah et al., 1985; Smith et al., 1986; Chu et al., 1993; fish α from this study). These similarities of the fish α isoform to mammalian skeletal muscle RYR were also observed in toadfish swimbladder muscle, a separately derived muscle that expresses the α isoform alone. In addition to these physiological similarities, the α isoform of fish and other vertebrates is the specific target of Imperatoxin A, a scorpion toxin that se-

lectively activates [^{3}H]ryanodine binding and channel activity of the mammalian skeletal muscle RYR isoform (Valdivia et al., 1994). Finally, mammalian skeletal muscle RYR-specific antisera recognize the fish α RYR isoform but not the β isoform (O'Brien et al., 1993). These numerous similarities indicate that the fish α RYR isoform is functionally similar to the mammalian skeletal muscle RYR. Indeed, partial sequencing of the fish α RYR has demonstrated that its closest homology is to the mammalian skeletal muscle RYR (Franck et al., 1994).

The β isoform is physiologically distinct from the α RYR isoform

There are also similarities between the fish skeletal muscle β RYR isoform and the cardiac muscle isoform from both mammals and fish. Mammalian cardiac muscle RYRs are relatively insensitive to inactivation by millimolar Ca^{2+} when studied either with [^{3}H]ryanodine binding (Pessah et al., 1985; Michalak et al., 1988; Chu et al., 1993) or single-channel recording (Rousseau et al., 1986; Chu et al., 1993), as is the fish β isoform. The single-channel conductance of the toadfish β RYR isoform is also similar to that of the bovine cardiac RYR (Valdivia et al., 1994). Furthermore, both the β isoform and the mammalian cardiac RYR are insensitive to Imperatoxin A (Valdivia et al., 1994). However, there are certain differences that suggest that the cardiac muscle and β isoforms are not homologous. In the presence of AMP-PCP and Mg^{2+}, the cardiac RYR is much more sensitive to activation by Ca^{2+} than is the β isoform (pCa for half-maximal activation is 6.5 for cardiac, 4.7 for β; Fig. 3, tetracaine-modified curves). So although some properties, especially the insensitivity to inactivation by millimolar Ca^{2+}, are similar between the cardiac and skeletal muscle β RYR isoforms, the two are not functionally identical. This result is in agreement with recent biochemical studies that distinguish between these two isoforms in chickens (Airey et al., 1993).

Previous studies on the biochemical and physiological properties of the skeletal muscle RYR isoforms in amphibian muscle have also shown differences between the α and β isoforms in the Ca^{2+}-dependency of channel activation. Murayama and Ogawa (1992) purified the two isoforms from frog muscle and found differences in their [^{3}H]ryanodine binding properties. The β isoform showed a 20-fold higher sensitivity to Ca^{2+} than did α, with pCa values required for half-maximal activation of 7.1 and 5.8 for the β and α RYRs, respectively. These experiments were performed in a medium containing 1 M NaCl and 10 mM caffeine, conditions that potently activate the RYR, and cannot be directly compared with our data. Recent molecular studies from the same laboratories, however, confirm the physiological data linking α functionally with mammalian type 1 RYRs. The cDNAs for the two RYR isoforms have been cloned and sequenced in frog muscle (Oyamada et al., 1994) and have demonstrated homology between the nonmammalian RYR α isoform and the mammalian type 1 RYR (skeletal), whereas the β isoform is homologous with the mammalian type 3 RYR.

A recent study of the single-channel conductance properties of the RYR channels from frog skeletal muscle (Bull and Marengo, 1993) has revealed two distinct types of channels with very similar physiological properties to the fish α and β RYR channels. One was stimulated by 10–100 μM Ca^{2+} and blocked at 500 μM Ca^{2+}, whereas the other was stimulated by 1–10 μM Ca^{2+} but could not be blocked by high [Ca^{2+}]. The blockable channels displayed about 60% lower maximum P_o than did the nonblockable channels, but had very similar unitary conductance. The authors tentatively identified the blockable channels as the α isoform and the nonblockable channels as the β isoform based on their differences in sensitivity to activation by micromolar Ca^{2+} and the relative sensitivities to Ca^{2+} of the purified α and β isoforms reported by Murayama and Ogawa (1992). Our results with single channels from fish muscle SR agree with these assignments. Thus, the physiological similarity between the fish and frog α and β RYR isoforms demonstrates that the fundamental properties of the two isoforms are likely conserved among the vertebrates that express both. The major difference between the two types of nonmammalian RYRs is in the nature of their inactivation by calcium.

Tetracaine selectively distinguishes between the α and β RYR isoforms

Tetracaine has been an important tool for examining the mechanism of EC coupling in amphibian and mammalian muscles. Further insight into the mechanisms of Ca^{2+} release in skeletal muscle may be gained from analysis of the effects of tetracaine on the α and β RYR isoforms. In this study, we have found distinct differences in the sensitivity of the fish RYR isoforms to tetracaine. The α isoform has a ninefold lower K_i than the cardiac isoform and a nearly twofold lower K_i than the mixed α and β pool of RYRs in fish white swimming muscle. The analysis suggests that the β isoform is less sensitive to tetracaine inhibition than is the α isoform and, indeed, at 100 μm, tetracaine selectively inhibited [^{3}H]ryanodine binding in the α RYR isoform whereas the cardiac and β isoforms were less affected. The results are consistent with our identification of the α isoform as a functional homolog of mammalian skeletal muscle RYR that is also affected by tetracaine at similar concentrations (Xu et al., 1993). We used this differential tetracaine sensitivity of the two isoforms to provide a clearer view of the properties of the β isoform in tissues that express both α and β together. By preferentially blocking the α isoform, the β isoform [^{3}H]ryanodine binding characteristics were more easily delineated.

The selective inhibitory effect of tetracaine on [^{3}H]ryanodine binding by the α RYR isoform may also be relevant to the influence of tetracaine on Ca^{2+} release in skeletal muscle fibers. Many studies have shown inhibitory effects of tetracaine on SR Ca^{2+} release (e.g., Almers and Best, 1976; Csernoch et al., 1988; Pizarro et al., 1992). Pizarro et al.

(1992) studied the effects of low concentrations of tetracaine on the Ca^{2+} release flux in cut frog skeletal muscle fibers. In this study, two distinct kinetic components of the SR Ca^{2+} release flux with differing tetracaine sensitivities were identified. The two components of Ca^{2+} release flux, a fast activating and inactivating component of large amplitude and a smaller steady release component, have been described earlier in the literature (Simon and Schneider, 1988; Schneider and Simon, 1988). Tetracaine (20 μM) selectively inhibited the fast activating and inactivating component of Ca^{2+} release flux, leaving the steady component unaffected (Pizarro et al., 1992). The results indicate pharmacological differences between the noninactivating and inactivating components of the SR Ca^{2+} release flux, which has been interpreted to be a result of dual SR Ca^{2+} release pathways. These results, when combined with our data indicating a selective inhibition of [^{3}H]ryanodine binding to the α isoform by tetracaine, imply that the α isoform is at least partially responsible for the inactivating component of calcium release.

A two-component model for calcium release in nonmammals

The presence of two RYR isoforms (α and β) with different physiologies in most nonmammalian vertebrate skeletal muscles has important implications for the mechanism of Ca^{2+} release. Current models of skeletal muscle EC coupling invoke a direct mechanical coupling between the voltage-dependent, T-tubule DHPR or voltage sensor and the RYR (type 1) as the mechanism for triggering SR Ca^{2+} release (Rios et al., 1991, 1993). However, the structural model of the triad junction, generated in toadfish swimbladder muscle (Block et al., 1988), implies that only half of the RYRs in the triad may be mechanically coupled to DHPRs. Importantly, in the toadfish swimbladder muscle all RYRs are of one type, α, which implies homology with the mammalian skeletal muscle junction. In toadfish swimbladder muscle, RYRs that are not coupled directly to a D4PR are presumed to couple cooperatively with the mechanically linked RYR. A model in which a portion of the RYRs are mechanically coupled while others are activated secondarily by Ca^{2+} released by the directly coupled channels fits logically with the physiological properties of the two RYR isoforms we have identified in fish skeletal muscle. In Fig. 7 we illustrate schematically a two-component model for Ca^{2+} release in nonmammals based upon the results presented here as well as the structural model of Block et al. (1988), the physiological models of Rios (Rios et al., 1991, 1993), and the results of Pizarro et al. (1992). The two component model for Ca^{2+} release accommodates data that suggest that there are two functionally separate SR Ca^{2+} release channels in nonmammalian vertebrates. Voltage-sensitive channels are gated by voltage across the T-tubular membrane, presumably via the DHPR, whereas the other SR Ca^{2+} release channels are presumed to operate by Ca^{2+}-induced Ca^{2+} release. Activation of one channel by voltage would result in the release of Ca^{2+} and thereby activate a neighboring "slave" channel. The

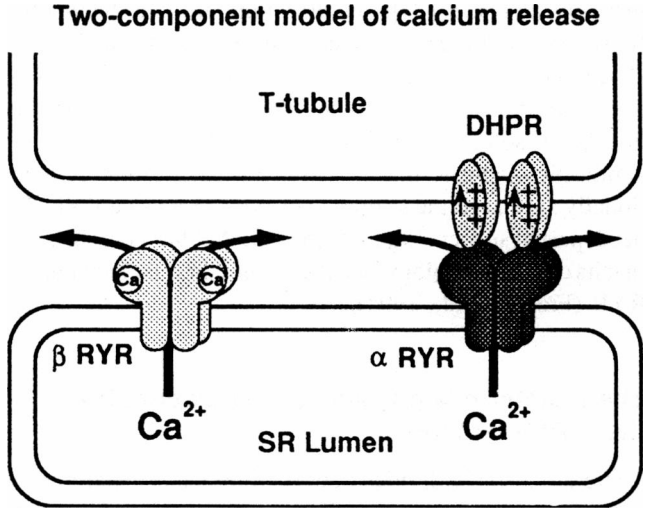

FIGURE 7 A two component model for the calcium release process in nonmammalian vertebrate skeletal muscle. The presence of two physiologically distinct Ca^{2+} release channels in skeletal muscle of nonmammalian vertebrates suggests a model in which two different forms of EC coupling are present. Direct, mechanically coupled channels are depicted making contact with the overlying T-tubule DHP receptor, which acts as a voltage sensor. Ca^{2+} release in a depolarization-induced coupling event triggers Ca^{2+} release from the close neighbor or "slave" RYR channel. The skeletal-like α RYR isoform occupies the directly coupled position (see discussion), and the β RYR isoform the Ca^{2+}-coupled position. The physical dimensions of the RYR array in toadfish triad junctions are such that adjacent RYRs may actually touch one another (Block et al., 1988) and deliver localized Ca^{2+} currents or transmit conformational changes.

extremely small distance between neighboring RYRs in a triad (~30 nm between the centers of adjacent RYRs; Block et al., 1988) would allow for tight physiological coupling of the two types of channels.

Our results indicate that in a two-component model the α RYR isoform is likely to be the RYR isoform directly coupled to the DHPR. This assignment is based on two results. The α isoform is present alone in toadfish swimbladder muscle (O'Brien et al., 1993), in which the structural evidence for direct coupling was obtained (Block et al., 1988), and it is physiologically homologous to the mammalian skeletal muscle RYR (Fig. 4, *B* and *C*), which is proposed to be directly coupled. The β isoform is a logical candidate to be the calcium-coupled RYR isoform because it has functional similarity to the cardiac RYR, which operates by Ca^{2+}-induced Ca^{2+} release.

The dual nature of Ca^{2+} release suggested by the biochemical, immunological, and structural properties of fish triad junctions has numerous parallels in physiological studies of Ca^{2+} release in voltage-clamped single cut frog muscle fibers (Schneider and Simon, 1988; Simon and Schneider, 1988; Jacquemond et al., 1991; Csernoch et al., 1993; Jong et al., 1993). These studies report the existence of two components of SR Ca^{2+} release, a fast activating and inactivating component of large amplitude, and a smaller steady release component. The interpretation of the mechanism underlying these two components is controversial. Some authors report that the inactivating component is selectively eliminated by

fast Ca^{2+} buffers (BAPTA and Fura 2) and interpret it as representing a CICR component that is rapidly Ca^{2+}-inactivated (Jacquemond et al., 1991; Csernoch et al., 1993). The remaining steady release is believed to represent a directly coupled component of Ca^{2+} release. A number of other studies have reported contradictory results. Baylor and Hollingworth (1988), Hollingworth et al. (1992), Pape et al. (1993), and Jong et al. (1993) report an enhancement of the Ca^{2+} release rate by Ca^{2+} buffers, suggesting a role for Ca^{2+} *inactivation* of Ca^{2+} release, but not supporting the evidence for Ca^{2+}-*induced* Ca^{2+} release. Significant differences in the rates of Ca^{2+} release reported and the methods used make it difficult to reconcile the conflicting reports.

One property of the Ca^{2+} release flux experiments is agreed upon: the fast inactivating component is inactivated by Ca^{2+}. The tetracaine sensitivity of the inactivating component observed by Pizarro et al. (1992) in whole fiber studies and the tetracaine sensitivity of the α RYR isoform in isolated SR vesicles shown in this study suggest that the fast inactivating component is at least partially attributable to the α RYR isoform. This is corroborated by the inhibition of [^{3}H]-ryanodine binding and reduction in channel P_o of the α isoform by high concentrations of free Ca^{2+}. The observed properties allow for a Ca^{2+} release channel that can inactivate when released Ca^{2+} reaches millimolar levels in the restricted space of the triad junction. Given the earlier conclusion that the α isoform is the directly coupled RYR, our model suggests that the directly coupled isoform may also be the Ca^{2+}-inactivating isoform.

It is clearly difficult to equate the two components of Ca^{2+} release observed in whole muscle fiber experiments with the two different channel isoforms observed in isolated vesicles. However, an attempt to synthesize the results may be a step forward in resolving Ca^{2+} release events in nonmammalian muscles. Our model of SR Ca^{2+} release places the α isoform in a directly coupled position. No direct evidence exists to exclude other models invoking, e.g., a cooperative physical interaction between adjacent RYRs, and one cannot exclude direct coupling of both α and β RYRs to the DHPRs. The physiological data from whole fibers suggest that the RYR isoforms are tightly linked. Elucidation of the mode of coupling by either RYR isoform to the DHPR and of the nature of the cooperative interactions between RYRs remains an important problem in EC coupling.

If the RYRs in all vertebrate triad junctions are cooperatively coupled to each other, the question arises: Why have two different RYR isoforms? The answer to this question is not clear, but there is evidence of evolutionary selection in the expression patterns of nonmammalian skeletal muscles for α and β isoforms together versus α alone. In an earlier study, we found that the fish and bird muscles that expressed the α isoform alone were specialized for high frequency contraction (O'Brien et al., 1993). The most prominent difference that we have observed in this study between the α and β RYR isoforms is the sensitivity of the α isoform to inactivation by high [Ca^{2+}]. Calcium inactivation of all of the RYRs in a triad may produce a Ca^{2+} release event of shorter duration compared to that produced by a triad with some inactivating and some noninactivating channels. Such a short Ca^{2+} release spike may enable the fiber to activate more quickly to a subsequent stimulation.

Superb technical assistance was provided by Mark Beamsley and Dr. Richard Londraville. The authors gratefully acknowledge the critical review of this manuscript by Dr. John Keen.

This work is supported by National Institutes of Health grant AR40246 and National Science Foundation grant IBN8958225 to B. A. Block. J. O'Brien is supported by postdoctoral fellowship AR08254 from National Institutes of Health. H. H. Valdivia is the recipient of a Minority Scientist Research Award from the American Heart Association. Specimen collection was supported, in part, by the Pacific Ocean Research Foundation.

REFERENCES

Airey, J. A., C. F. Beck, K. Murakami, S. J. Tanksley, T. J. Deerinck, M. H. Ellisman, and J. L. Sutko 1990. Identification and localization of two triad junctional foot protein isoforms in mature avian fast twitch skeletal muscle. *J. Biol. Chem.* 265:14187–14194.

Airey, J. A., M. M. Grinsell, L. R. Jones, J. L. Sutko, and D. Witcher. 1993. Three ryanodine receptor isoforms exist in avian striated muscles. *Biochemistry.* 32:5739–5745.

Almers, W., and P. M. Best. 1976. Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *J. Physiol.* 262:583–611.

Anderson, K., F. A. Lai, Q. Y. Liu, E. Rousseau, H. P. Erickson, and G. Meissner. 1989. Structural and functional characterization of the purified cardiac ryanodine receptor-Ca^{2+} release channel complex. *J. Biol. Chem.* 264:1329–1335.

Baylor, S. M., and S. Hollingworth. 1988. Fura-2 calcium transients in frog skeletal muscle fibres. *J. Physiol.* 403:151–192.

Block, B. A., T. Imagawa, K. P. Campbell, and C. Franzini-Armstrong. 1988. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.* 107:2587–2600.

Bull, R., and J. J. Marengo. 1993. Sarcoplasmic reticulum release channels from frog skeletal muscle display two types of calcium dependence. *FEBS Lett.* 331:223–227.

Chu, A., M. Diaz-Munoz, M. J. Hawkes, K. Brush, and S. L. Hamilton. 1990. Ryanodine as a probe of the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. *Mol. Pharmacol.* 337:735–741.

Chu, A., M. Fill, E. Stefani, and M. L. Entman. 1993. Cytoplasmic Ca^{2+} does not inhibit the cardiac muscle sarcoplasmic reticulum ryanodine receptor Ca^{2+} channel, although Ca($^{2+}$)-induced Ca^{2+} inactivation of Ca^{2+} release is observed in native vesicles. *J. Membr. Biol.* 135:49–59.

Csernoch, L., C.-L. Huang, G. Szücs, and L. Kovacs. 1988. Differential effects of tetracaine on charge movements and Ca^{2+} signals in frog skeletal muscle. *J. Gen. Physiol.* 96:601–612.

Csernoch, L., V. Jacquemond, and M. F. Schneider. 1993. Microinjection of strong calcium buffers suppresses the peak of calcium release during depolarization in frog skeletal muscle fibers. *J. Gen. Physiol.* 101:297–333.

Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris).* 75:463–505.

Franck, J. P. C., M. S. Beamsley, J. E. Iceen, R. L. Londraville, and B. A. Block. 1994. Cloning and characterization of the ryanodine receptor α isoform from fish. *The Physiologist.* 37:83*a*. (Abstr.)

Hakamata, Y., J. Nakai, H. Takeshima, and K. Imoto. 1992. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. *FEBS Lett.* 312:229–235.

Hollingworth, S., A. B. Harkins, N. Kurebayashi, M. Konishi, and S. M. Baylor. 1992. Excitation-contraction coupling in intact frog skeletal muscle fibers injected with millimolar concentrations of fura-2. *Biophys. J.* 63:224–234.

Inui, M., A. Saito, and S. Fleischer. 1987a. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* 262:1740–1747.

Inui, M., A. Saito, and S. Fleischer. 1987b. Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. *J. Biol. Chem.* 262:15637–15642.

Jacquemond, V., L. Csernoch, M. G. Klein, and M. F. Schneider. 1991. Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophys. J.* 60:867–873.

Jong, D. S., P. C. Pape, W. K. Chandler, and S. M. Baylor. 1993. Reduction of calcium inactivation of sarcoplasmic reticulum calcium release by fura-2 in voltage-clamped cut twitch fibers from frog muscle. *J. Gen. Physiol.* 102:333–370.

Kaplan, R. S., and P. L. Pedersen. 1985. Determination of microgram quantities of protein in the presence of milligram levels of lipid with Amido Black 10B. *Anal. Biochem.* 150:97–104.

Kuwajima, G., A. Futatsugi, M. Niinobe, S. Nakanishi, and K. Mikoshiba. 1992. Two types of ryanodine receptors in mouse brain: skeletal muscle type exclusively in Purkinje cells and cardiac muscle type in various neurons. *Neuron.* 9:1133–1142.

Lai, F. A., H. P. Erickson, E. Rousseau, Q. Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature.* 331:315–319.

Lai, F. A., Q. Liu, L. Xu, A. El-Hashem, N. R. Kramarcy, R. Sealock, and G. Meissner. 1992. Amphibian ryanodine receptor isoforms are related to those of mammalian skeletal and cardiac muscle. *Am. J. Physiol.* 263:C365–C372.

Lai, F. A., M. Misra, L. Xu, H. A. Smith, and G. Meissner. 1989. The ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. *J. Biol. Chem.* 264:16776–16785.

Liu, Q. Y., F. A. Lai, E. Rousseau, R. V., and G. Meissner. 1989. Multiple conductance states of the purified calcium release channel complex from skeletal muscle sarcoplasmic reticulum. *Biophys. J.* 55:415–424.

Marks, A. R., P. Tempst, K. S. Hwang, M. B. Taubman, M. Inui, C. Chadwick, S. Fleischer and B. Nadal-Ginard. 1989. Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 86:8683–8687.

Meissner, G. 1986. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.* 261:6300–6306.

Meissner, G. 1994. Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* 56:485–508.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid calcium release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Michalak, M., P. Dupraz, and V. Shoshan-Barmatz. 1988. Ryanodine binding to sarcoplasmic reticulum membrane: comparison between cardiac and skeletal muscle. *Biochim. Biophys. Acta.* 939:587–594.

Murayama, T., and Y. Ogawa. 1992. Purification and characterization of two ryanodine-binding protein isoforms from sarcoplasmic reticulum of bullfrog skeletal muscle. *J. Biochem.* 112:514–522.

Nakai, J., T. Imagawa, Y. Hakamata, M. Shigekawa, H. Takeshima, and S. Numa. 1990. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett.* 271:169–177.

Nickerson, D. M., D. E. Facey, and G. D. Grossman. 1989. Estimating physiological thresholds with continuous two-phase regression. *Physiol. Zool.* 62:866–887.

O'Brien, J., G. Meissner, and B. A. Block. 1993. The fastest contracting skeletal muscles of non-mammalian vertebrates express only one isoform of the ryanodine receptor. *Biophys. J.* 65:2418–2427.

Olivares, E. B., S. J. Tanksley, J. A. Airey, C. Beck, Y. Ouyang, T. J. Deerinck, M. H. Ellisman, and J. L. Sutko. 1991. Nonmammalian vertebrate skeletal muscles express two triad junctional foot protein isoforms. *Biophys. J.* 59:1153–1163.

Otsu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:13472–13483.

Ouyang, Y., T. J. Deerinck, P. D. Walton, J. A. Airey, J. L. Sutko, and M. H. Ellisman. 1993. Distribution of ryanodine receptors in the chicken central nervous system. *Brain Res.* 620:269–280.

Oyamada, H., T. Murayama, T. Takai, M. Iino, N. Iwabe, T. Miyata, Y. Ogawa, and M. Endo. 1994. Primary structure and distribution of ryanodine-binding protein isoforms of the bullfrog skeletal muscle. *J. Biol. Chem.* 269:17206–17214.

Pape, P. C., D. S. Jong, W. K. Chandler, and S. M. Baylor. 1993. Effect of fura-2 on action potential-stimulated calcium release in cut twitch fibers from frog muscle. *J. Gen. Physiol.* 102:295–332.

Pessah, I. N., R. A. Stambuk, and J. E. Casida. 1987. Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine, and adenine nucleotides. *Mol. Pharmacol.* 31:232–238.

Pessah, I. N., A. L. Waterhouse, and J. E. Casida. 1985. The calcium-ryanodine receptor complex of skeletal and cardiac muscle. *Biochem. Biophys. Res. Commun.* 128:449–456.

Pizarro, G., L. Csernoch, I. Uribe, and E. Rios. 1992. Differential effects of tetracaine on two kinetic components of calcium release in frog skeletal muscle fibres. *J. Physiol.* 457:525–538.

Rios, E., M. Karhanek, J. Ma, and A. Gonzalez. 1993. An allosteric model of the molecular interactions of excitation-contraction coupling in skeletal muscle. *J. Gen. Physiol.* 102:449–481.

Rios, E., J. Ma, and A. Gonzalez. 1991. The mechanical hypothesis of excitation-contraction (EC) coupling in skeletal muscle. *J. Muscle Res. Cell Motil.* 12:127–135.

Rousseau, E., J. S. Smith, J. S. Henderson, and G. Meissner. 1986. Single channel and $^{45}Ca^{2+}$ flux measurements of the cardiac sarcoplasmic reticulum calcium channel. *Biophys. J.* 50:1009–1014.

Schneider, M. F., and W. K. Chandler. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature.* 242:244–246.

Schneider, M. F., and B. J. Simon. 1988. Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *J. Physiol.* 405:727–745.

Simon, B. J., and M. F. Schneider. 1988. Time course of activation of calcium release from sarcoplasmic reticulum in skeletal muscle. *Biophys. J.* 54:1159–1163.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum: activation by Ca^{2+} and ATP and modulation by Mg^{2+}. *J. Gen. Physiol.* 88:573–588.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Stern, M. D. 1992. Theory of excitation-contraction coupling in cardiac muscle. *Biophys. J.* 63:497–517.

Strand, M. A., C. F. Louis, and J. R. Mickelson. 1993. Phosphorylation of the porcine skeletal and cardiac muscle sarcoplasmic reticulum ryanodine receptor. *Biochim. Biophys. Acta.* 1175:319–326.

Takeshima, H., S. Nishimura, M. Nishi, M. Ikeda, and T. Sugimoto. 1993. A brain-specific transcript from the 3′-terminal region of the skeletal muscle ryanodine receptor gene. *FEBS Lett.* 322:105–110.

Tinker, A., A. R. G. Lindsay, and A. J. Williams. 1992. A model for ionic conduction in the ryanodine receptor of sheep cardiac muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 100:495–517.

Valdivia, H. H., O. Fuentes, and B. A. Block. 1994. Imperatoxin A, a selective activator of skeletal ryanodine receptors (RYR), distinguishes between α and β RYR isoforms in non-mammalian muscle. *Biophys. J.* 66:418*a*. (Abstr.)

Valdivia, H. H., M. S. Kirby, W. J. Lederer, and R. Coronado. 1992. Scorpion toxins targeted against the sarcoplasmic reticulum $Ca^{(2+)}$-release channel of skeletal and cardiac muscle. *Proc. Natl. Acad. Sci. USA.* 89: 12185–12189.

Witcher, D. R., R. J. Kovacs, H. Schulman, D. C. Cefali, and L. R. Jones. 1991. Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J. Biol. Chem.* 266:11144–11152.

Xu, L., R. Jones, and G. Meissner. 1993. Effects of local anesthetics on single channel behavior of skeletal muscle calcium release channel. *J. Gen. Physiol.* 101:207–233.