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Characterization of RyR1-slow, a ryanodine receptor specific to slow-twitch skeletal muscle

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Morrissette, Jeffery, Le Xu, Alexandra Nelson, Gerhard Meissner, and Barbara A. Block. Characterization of RyR1-slow, a ryanodine receptor specific to slow-twitch skeletal muscle. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1889–R1898, 2000.—Two distinct skeletal muscle ryanodine receptors (RyR1s) are expressed in a fiber type–specific manner in fish skeletal muscle (11). In this study, we compare [^{3}H]ryanodine binding and single channel activity of RyR1-slow from fish slow-twitch skeletal muscle with RyR1-fast and RyR3 isolated from fast-twitch skeletal muscle. Scatchard plots indicate that RyR1-slow has a lower affinity for [^{3}H]ryanodine when compared with RyR1-fast. In single channel recordings, RyR1-slow and RyR1-fast had similar slope conductances. However, the maximum open probability (P_o) of RyR1-slow was threefold less than the maximum P_o of RyR1-fast. Single channel studies also revealed the presence of two populations of RyRs in tuna fast-twitch muscle (RyR1-fast and RyR3). RyR3 had the highest P_o of all the RyR channels and displayed less inhibition at millimolar Ca^{2+}. The addition of 5 mM Mg-ATP or 2.5 mM β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) to the channels increased the P_o and [^{3}H]ryanodine binding of both RyR1s but also caused a shift in the Ca^{2+} dependency curve of RyR1-slow such that Ca^{2+}-dependent inactivation was attenuated. [^{3}H]ryanodine binding data also showed that Mg^{2+}-dependent inhibition of RyR1-slow was reduced in the presence of AMP-PCP. These results indicate differences in the physiological properties of RyRs in fish slow- and fast-twitch skeletal muscle, which may contribute to differences in the way intracellular Ca^{2+} is regulated in these muscle types.

sarcoplasmic reticulum; excitation-contraction coupling; calcium; muscle fiber types

IN SKELETAL AND CARDIAC MUSCLE, depolarization of the external membrane triggers a rapid release of stored Ca^{2+} from the sarcoplasmic reticulum (SR), which generates muscle contraction. This release is mediated by ryanodine receptors (RyRs), large protein channels made up of four identical polypeptide subunits of ~565 kDa (6, 12). In mammals, three RyR isoforms, encoded by three distinct genes, have been cloned and sequenced: RyR1 from skeletal muscle (38, 41), RyR2 from cardiac muscle (26), and RyR3 from brain (13).

Mammalian skeletal muscle primarily expresses RyR1 in mature animals, although a low level of RyR3 expression has also been reported in developing mammalian skeletal muscle and adult soleus and diaphragm muscles (5). This low expression of RyR3 may be a result of phylogenetic history, because nonmammalian skeletal muscle expresses two RyR isoforms in high abundance. The RyR isoforms in nonmammals were originally named α and β because of unknown homologies to the mammalian RyRs (1). Cloning and sequencing of α and β from bullfrog skeletal muscle revealed that these isoforms were homologous to mammalian RyR1 and RyR3, respectively (27, 28).

Although RyR isoforms share many properties, such as high-affinity [^{3}H]ryanodine binding, large single channel conductance, and Ca^{2+}-dependent channel gating, there are physiological characteristics that distinguish the three proteins. Ca^{2+} release, [^{3}H]ryanodine binding, and single channel studies have shown that RyR2 is more sensitive to activation by micromolar Ca^{2+} and less sensitive to inhibition by millimolar Ca^{2+} than RyR1 (23, 30, 34). RyR2 was also found to be less sensitive to adenine nucleotide stimulation and Mg^{2+} inhibition (20–22). Recently, RyR3 was characterized and found to be less sensitive to both Ca^{2+} activation and Ca^{2+} inhibition (15, 24, 25). Furthermore, RyR3 was shown to be more resistant to inhibition by Mg^{2+} than RyR1 (15, 24).

Studies of mammalian and nonmammalian muscle have revealed physiological differences between fast-twitch and slow-twitch fibers. Slow-twitch muscle fibers have a longer time to peak contraction and a longer time to one-half relaxation than fast-twitch muscle fibers (33, 40). Likewise, the duration of the myoplasmic Ca^{2+} transient in slow-twitch muscle is longer than that of fast-twitch fibers (9, 33). Both qualitative and quantitative modifications of several muscle proteins seem to underlie these physiological differences (32). Slow-twitch fibers compared with fast-twitch fibers contain slower myosin isoforms, which

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have a reduced maximal velocity of shortening (31), a decreased content of SR (2), with a slower isoform of the Ca^{2+} pump (SERCA2 in slow-twitch vs. SERCA1 in fast-twitch) (19, 39), and a lower concentration of the cytoplasmic Ca^{2+} buffering protein parvalbumin (14). Calsequestrin, a Ca^{2+} binding protein located in the lumen of the SR, also has a unique isoform expression pattern in slow-twitch and fast-twitch muscles of rabbit and rat (7).

Investigations into the physiological differences between slow- and fast-twitch muscle fibers can be greatly enhanced with studies using fish as model systems. Fish, especially the tunas (of the family Scombridae), are ideal organisms in which to study fiber type-specific proteins because of their anatomic separation of pure slow-twitch and fast-twitch muscle and their overall abundance of slow-twitch fibers within their body plan. Studies using antibodies raised to the fiber-type specific SERCAs have determined that the tuna's deep red muscle, which is used in continuous swimming, is nearly 100% slow oxidative (type I) fibers. The fast-twitch white muscle, used in burst swimming, is made up of over 95% fast glycolytic (type II) fibers (39).

A novel RyR isoform has recently been sequenced from fish skeletal muscle and was shown to be expressed in a fiber type-specific manner (11). Immunological and phylogenetic analyses indicated it was homologous to the RyR1 ("skeletal") family, but strikingly, ribonuclease protection assays (RPAs) showed that the mRNA for this isoform was present in slow-twitch skeletal muscle but excluded from fast-twitch skeletal muscle. We called the novel slow twitch-specific isoform RyR1-slow because of its phylogenetic homology to the skeletal muscle RyR1 family, yet distinct from the RyR expressed in fast-twitch skeletal muscle, RyR1-fast. An antibody that recognizes all RyR isoforms indicated that RyR1-slow is the only RyR expressed in slow-twitch muscle of many fish species, and thus slow-twitch fibers of fish are an exception to the two-isoform (RyR1 and RyR3) expression pattern found in most nonmammalian skeletal muscle (11). Other exceptions to the two-isoform expression pattern exist in some specialized muscle of fish, such as the super-fast contracting swimbladder muscle of toadfish (*Opsanus tau*), which expresses only RyR1-fast (11, 25). In this study, we take advantage of these naturally occurring expression patterns in fish to compare the physiological properties of RyR1-slow, RyR1-fast, and RyR3 in yellowfin tuna (*Thunnus albacares*) and toadfish. Given the molecular evidence indicating the expression of two distinct RyR1 isoforms in fish slow- and fast-twitch skeletal muscle, we hypothesized that these RyRs may also differ in their physiological properties.

MATERIALS AND METHODS

Heavy SR vesicle preparation. Approximately 30.0 g of freshly dissected tuna fast-twitch swimming muscle, tuna slow-twitch swimming muscle, or toadfish fast-twitch swimbladder muscle was homogenized using a Waring blender in 10 volumes of ice-cold buffer containing 300.0 mM sucrose, 5.0 mM EGTA, 10.0 mM Na_2EDTA, 20.0 mM K-PIPES, pH 7.3, 1.1 μM diisopropylfluorophosphate, and a protease inhibitor cocktail containing 1.0 μM pepstatin A, 1.0 mM iodoacetamide, 0.1 mM phenylmethylsulfonylfluoride, 1.0 μM leupeptin, 1.0 mM benzamidine, 0.1 μM aprotinin, and 6.0 mg/ml trypsin inhibitor. The protease inhibitors were readded to each buffer at every step of the procedure. After homogenization, the slurry was centrifuged for 20 min at 2,000 *g* in a Sorval SS34 rotor. The supernatant was passed through two layers of cheesecloth, and the crude microsomes were pelleted by centrifugation at 100,000 *g* for 50 min in a Beckman Ti50.2 rotor. The pellets were resuspended in 300.0 mM sucrose and 5.0 mM K-PIPES, pH 7.0. This material was layered on top of discontinuous sucrose gradients (2.0 ml 45%, 3.0 ml 36%, 3.0 ml 30%, 3.0 ml 25%) containing 0.4 M KCl, 0.1 mM Na_2EGTA, 0.1 mM $CaCl_2$, and 5.0 mM K-PIPES, pH 6.8, and centrifuged 16 h at 23,000 rpm in a Beckman SW41 rotor. Heavy SR (HSR) membrane vesicles were recovered from the 36–45% interface and pelleted at 100,000 *g*. Vesicles were resuspended in 300.0 mM sucrose and 5.0 mM K-PIPES, pH 7.0, frozen in liquid N_2, and stored at −80°C until use.

[^{3}H]ryanodine binding assay. Triplicate samples of HSR vesicles at a concentration of 0.5–3.0 mg/ml were incubated for 2 h at 30°C in buffer containing 0.2 M KCl, 1.0 mM Na_2EGTA, 20.0 mM K-PIPES, pH 7.1, and [^{3}H]ryanodine at the concentration indicated in Figs. 1, 2, 7. The free Ca^{2+} of the binding medium was adjusted to 0.1–10,000 μM with the addition of $CaCl_2$ according to the affinity constants of Fabiato (10). After incubation, the samples were filtered onto Whatman GF/B glass fiber filters, preincubated for 30 min in 5% polyethylinemine, washed three times with ice-cold distilled water, and counted by scintillation. Nonspecific binding was determined in the presence of a 1,000-fold excess unlabeled ryanodine and was subtracted from each sample.

Single channel measurements. RyRs were purified from 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) solubilized tuna slow-twitch and fast-twitch HSR vesicles by sucrose gradient centrifugation as previously described (17). The 30S Ca^{2+} release channels were reconstituted into proteoliposomes, which were fused to lipid bilayers consisting of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine at a ratio of 5:3:2 and a final lipid concentration of 50.0 mg/ml decane. Single channel activity was recorded in a symmetric KCl buffer (0.25 M KCl, 20.0 mM K-HEPES, pH 7.4). Signals were filtered at 2 kHz through a low-pass Bessel filter, digitized at 10 kHz, and analyzed with pClamp 6.0.3 software (Axon Instruments, Burlingame, CA). The single channel data were fitted and analyzed according to the Hill equation as described in Fig. 4.

Miscellaneous. Protein concentrations were measured in duplicate by the method of Bradford (4). All values are reported as a means ± SE. Statistical significance was determined by using the Student's *t*-test. All chemicals were of analytic grade and purchased from Sigma (St. Louis, MO). [^{3}H]ryanodine (68.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Phospholipids used in the bilayer studies were purchased from Avanti Polar Lipids (Alabaster, AL).

RESULTS

RyR1-slow has a lower affinity for [^{3}H]ryanodine. HSR vesicle preparations from tuna slow- and fast-twitch muscle and toadfish swimbladder muscle all exhibited saturation kinetics on increasing [^{3}H]ryano-

dine concentration from 0.5 to 150.0 nM (Fig. 1*A*). Scatchard analysis indicated the presence of a single class of high-affinity [^{3}H]ryanodine binding sites in each preparation with dissociation constants (K_d) of 92.3 ± 8.7, 17.9 ± 2.1, and 9.5 ± 2.1 nM for tuna slow-twitch, tuna fast-twitch, and toadfish swimbladder muscle, respectively (Fig. 1*B*). Tuna fast-twitch muscles express both RyR1-fast and RyR3 in relatively equal proportions (25), thus the K_d measured is a mixture of the affinities of the two different isoforms. However, the K_d values for RyR1 and RyR3 have been reported to be nearly identical in rabbit diaphragm muscle (24). It is likely that the two isoforms also share similar affinities for ryanodine in fish muscle, because the Scatchard plot for tuna fast-twitch muscle lacks any curvature indicative of two distinct K_d values.

The maximum binding (B_{max}) values obtained from the Scatchard analysis were 1.09 ± 0.35, 2.08 ± 0.88, and 2.75 ± 0.15 pmol/mg protein for tuna slow-twitch, tuna fast-twitch, and toadfish swimbladder muscle, respectively (Fig. 1*B*). The B_{max} values for tuna slow-twitch muscle and tuna fast-twitch muscle were not significantly different; however, the B_{max} of tuna slow-twitch muscle was significantly lower than that of the toadfish swimbladder muscle ($P < 0.05$).

Adenine nucleotides attenuate the Ca^{2+} dependent inhibition of [^{3}H]ryanodine binding to RyR1-slow. Figure 2 displays the Ca^{2+} dependence of [^{3}H]ryanodine binding to tuna slow- and fast-twitch muscle HSR and toadfish fast-twitch swimbladder HSR. With Ca^{2+} as the only activator, all [^{3}H]ryanodine binding curves were bell shaped. Peak binding was 0.31, 0.66, and 0.42 pmol/mg for tuna slow- and fast-twitch muscle HSR and toadfish swimbladder HSR, respectively. The addition of the adenine nucleotide β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), a nonhydrolyzable analog of ATP, increased [^{3}H]ryanodine binding to tuna slow- and fast-twitch muscle HSR and toadfish swimbladder HSR at all Ca^{2+} concentrations. Peak binding in the presence of 2.5 mM AMP-PCP was 0.89, 1.23, and 1.52 pmol/mg for tuna slow- and fast-twitch muscle HSR and toadfish swimbladder HSR, respectively. The overall shape of the normalized Ca^{2+} activation/inactivation curve for toadfish swimbladder muscle (RyR1-fast only) or tuna fast-twitch muscle (RyR1-fast and RyR3) was unchanged in the presence of AMP-PCP (Fig. 2, *B* and *C*). However, the fast-twitch muscle preparation displayed significantly less inhibition at 1.0 mM Ca^{2+} in the presence of AMP-PCP, when compared with the toadfish swimbladder curve ($P < 0.05$). This is likely due to the expression of RyR3, which has been shown to be less sensitive to Ca^{2+}-dependent inhibition (15, 24, 25). In contrast to toadfish swimbladder and tuna fast-twitch muscle HSR, the Ca^{2+} dependency of tuna slow-twitch HSR was highly modified by AMP-PCP. In the presence of the adenine nucleotide, the shape of the curve for slow-twitch muscle (RyR1-slow only) displayed a broad peak of B_{max} between 10 μM and 1.0 mM [Ca^{2+}] (Fig. 2*A*). This resulted in the reduced inhibition of [^{3}H]ryanodine binding at millimolar Ca^{2+}. At 1.0 mM Ca^{2+}, [^{3}H]ryanodine binding to RyR1-slow was still ~90% of maximum in the presence of AMP-PCP but only ~30% of maximum without AMP-PCP.

Single channel recordings of RyRs from fish skeletal muscle. To further characterize the properties of RyR1-slow and RyR1-fast and to exclude the possibility that other proteins in the HSR preparations may have been responsible for the observed differences in the [^{3}H]ryanodine binding data above, CHAPS solubilized RyRs

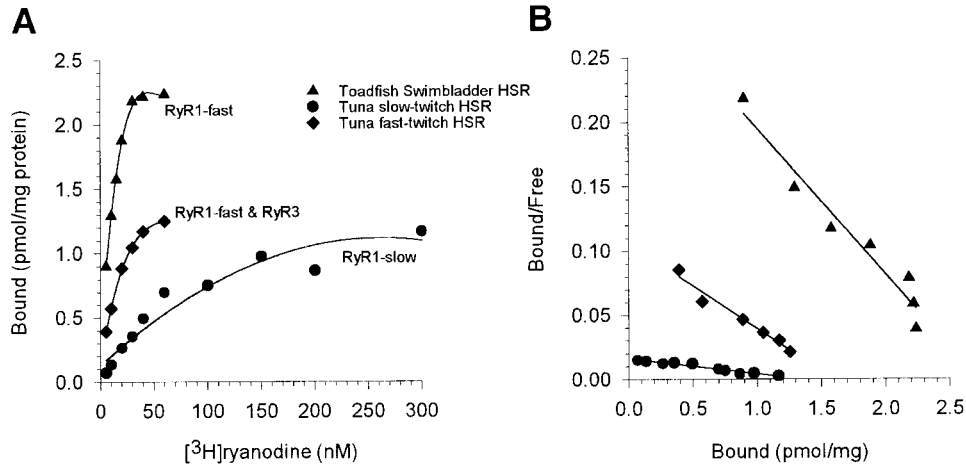

Fig. 1. Scatchard analysis of [^{3}H]ryanodine binding to tuna slow- and fast-twitch muscle and toadfish swimbladder muscle. One representative experiment of four is shown. Binding was conducted in the presence of 2.5 mM β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) and the Ca^{2+} concentration that elicited maximal binding (B_{max}; i.e., 10 μM for tuna slow-twitch and 100 μM for tuna fast-twitch and toadfish swimbladder muscle). *A*: equilibrium binding of [^{3}H]ryanodine to tuna slow-twitch muscle heavy sarcoplasmic reticulum (HSR; ●), tuna fast-twitch muscle HSR (◆), and toadfish swimbladder muscle HSR (▲). *B*: Scatchard representation of the binding data. Fitted lines from the 4 experiments yielded B_{max} and dissociation constant (K_d) values of 1.09 ± 0.35 pmol/mg protein, 92.3 ± 8.7 nM for tuna slow-twitch muscle; 2.08 ± 0.88 pmol/mg protein, 17.9 ± 2.1 nM for tuna fast-twitch muscle; and 2.75 ± 0.15 pmol/mg protein, 9.5 ± 2.1 nM for toadfish swimbladder muscle. RyR, ryanodine receptor.

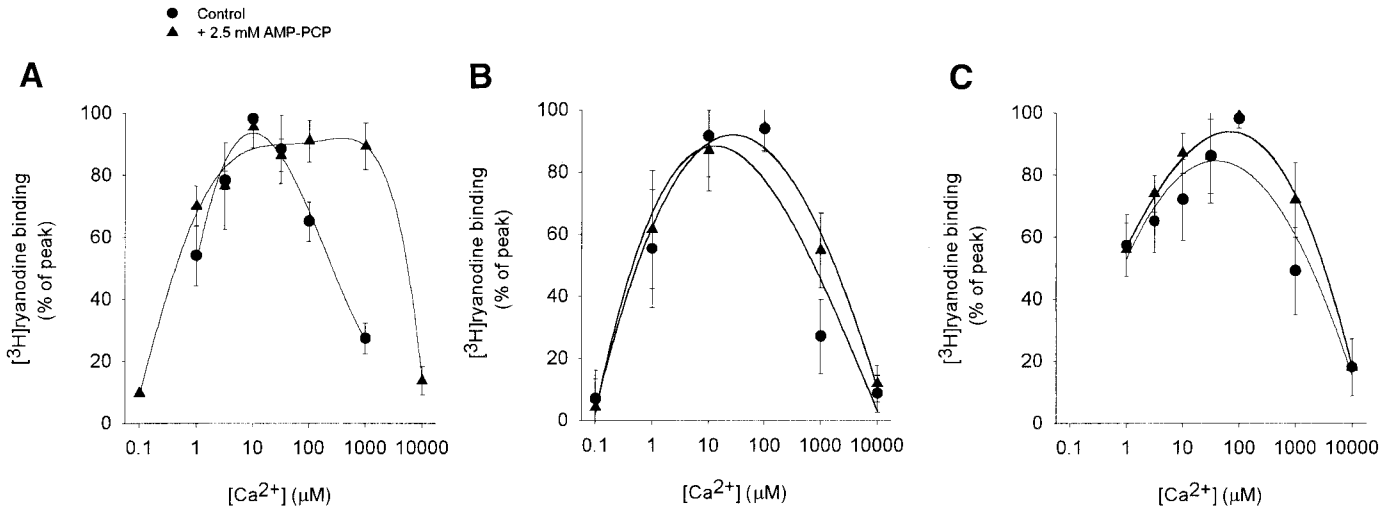

Fig. 2. Ca^{2+} dependence of [^{3}H]ryanodine binding to tuna slow- and fast-twitch and toadfish swimbladder muscle in the presence or absence of AMP-PCP. Triplicate samples of tuna slow-twitch HSR vesicles (*A*; $n = 7$), toadfish swimbladder muscle HSR vesicles (*B*; $n = 4$), or tuna fast-twitch HSR vesicles (*C*; $n = 3$) were incubated at 30°C for 2 h with 90 nM [^{3}H]ryanodine for slow-twitch muscle or 20 nM [^{3}H]ryanodine for fast-twitch muscle and toadfish swimbladder muscle. The incubation buffer contained 1.0 mM EGTA and the necessary Ca^{2+} to reach the specified free Ca^{2+} concentrations. Each data point was normalized and expressed as % of maximal or peak binding for that experiment. The smooth curves represent third-order polynomial fits to the data with r^2 values ≥0.90, except the curve for slow-twitch muscle in the presence of AMP-PCP, which was best fit with a fifth-order polynomial ($r^2 = 0.988$). ●, Control values (no AMP-PCP); ▲, presence of 2.5 mM AMP-PCP. B_{max} in the absence and presence of AMP-PCP, respectively, was 0.31 ± 0.05 and 0.89 ± 0.08 pmol/mg for tuna slow-twitch muscle; 0.42 ± 0.24 and 1.52 ± 0.55 pmol/mg for toadfish swimbladder muscle, and 0.66 ± 0.11 and 1.23 ± 0.08 pmol/mg for tuna fast-twitch muscle.

were purified by sucrose density centrifugation from tuna slow- and fast-twitch muscle and toadfish fast-twitch swimbladder muscle. The purified RyRs were reconstituted into proteoliposomes and incorporated into planar lipid bilayers to record channel currents. Single channel analysis indicated that all RyRs formed large conductance Ca^{2+} channels that were modulated in typical fashion by Ca^{2+}, adenine nucleotides, and caffeine. Tuna fast-twitch white muscle expresses both RyR1-fast and RyR3, and two populations of channels were apparent in the recordings of RyRs purified from fast-twitch muscle. Because RyR3 has been shown to be less sensitive to Ca^{2+}-dependent inhibition compared with RyR1, we chose the P_o at 1.0 mM *cis*-Ca^{2+} (Ca^{2+} at the cytoplasmic face of the channel) as the criteria for separating two populations of channels from tuna fast-twitch muscle. At 1 mM *cis*-Ca^{2+}, the P_o of all recordings for *group 1* were <0.02 and for *group 2* were >0.22. *Group 1* channels, represented by the traces shown in Fig. 3*A*, *left*, and Fig. 4, displayed lower channel activity (P_o) at all [Ca^{2+}] and nearly complete channel inhibition at millimolar [Ca^{2+}] when compared with the *group 2* channels represented in Figs. 3*A*, *right*, and 4. This second class of channels displayed higher channel P_o at all [Ca^{2+}] and a decreased level of inhibition at 1.0 mM Ca^{2+}, two characteristics previously described for fish RyR3 channels (25). The single channel data were fit to the Hill equation to quantify the differences in Ca^{2+}-dependent activation/inhibition between the two channel populations. This analysis revealed that both channel populations from tuna fast-twitch muscle displayed similar activation constants [(K_a) − [Ca^{2+}], which elicited half-maximal channel activity], but distinct inhibition constants [(K_i) − [Ca^{2+}], which inhibited one-half of the maximal channel activity] (Table 1). The K_i was over 2.5-fold higher for *group 2* than for *group 1*. This analysis also unveiled similar cooperativity in the Ca^{2+}-dependent activation or inhibition of *group 1* or *group 2* channels as evident by their similar Hill coefficients (Table 1). Thus, two populations of RyRs, RyR1-fast channels (*group 1*), with a lower P_o and nearly complete Ca^{2+}-dependent inhibition, and RyR3 channels (*group 2*), with a higher P_o and incomplete channel inhibition at 1.0 mM *cis*-Ca^{2+}, can be discerned in RyR single channel recordings from tuna fast-twitch muscle RyRs.

Representative single channel recordings of RyR1-fast and RyR1-slow purified from toadfish fast-twitch swimbladder muscle and tuna slow-twitch red muscle, respectively, are shown in Fig. 3*B*. Both channels displayed a low P_o and the nearly complete channel inhibition at millimolar Ca^{2+} that was characteristic of RyR1-fast from tuna fast-twitch muscle (Fig. 4). RyR1-slow displayed the lowest channel activity, reaching a maximum P_o of only 0.12. RyR1-fast, whether purified from toadfish swimbladder muscle or from tuna fast-twitch muscle, had a maximum P_o ~0.30. Hill analysis yielded similar activation constants of 7.7 ± 1.4 μM for RyR1-slow and 3.6 ± 0.4 μM for RyR1-fast, values similar to the K_a measured for RyR1-fast from tuna fast-twitch muscle. However, RyR1-slow from tuna slow-twitch muscle displayed a lower Ca^{2+} K_i compared with RyR1-fast from toadfish swimbladder mus-

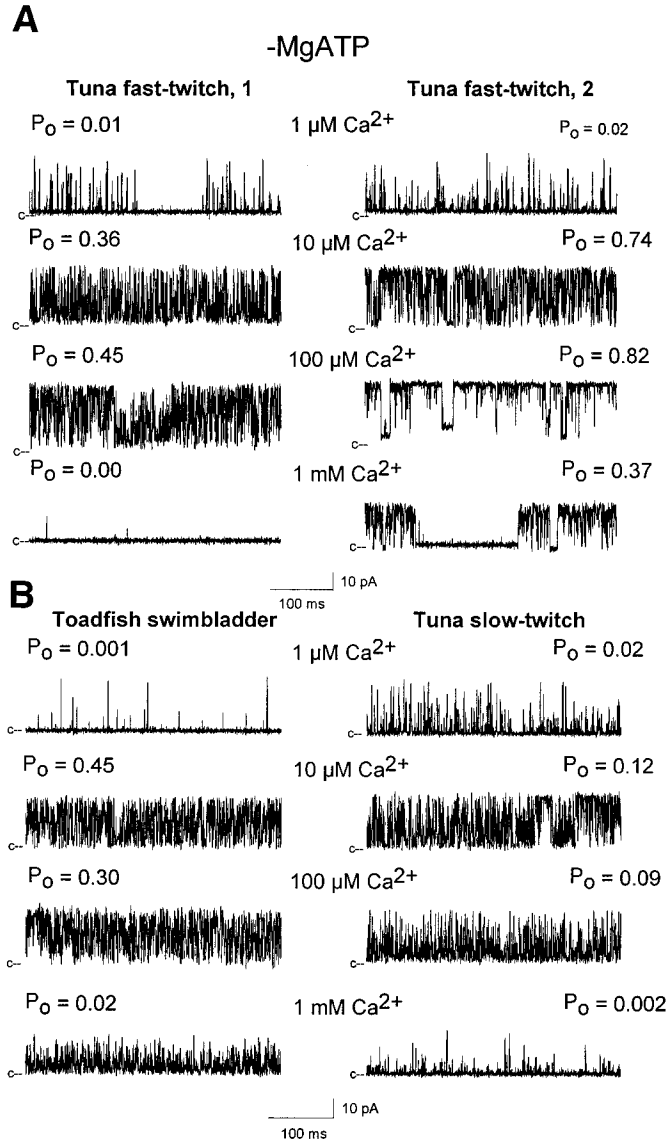

Fig. 3. Ca^{2+} dependence of the single channel activity of tuna slow- and fast-twitch and toadfish swimbladder RyRs. Purified RyRs from tuna slow- and fast-twitch and toadfish swimbladder muscle were reconstituted into proteoliposomes and fused to lipid bilayers. Single channel currents were recorded in symmetrical 0.25 M KCl, 20.0 mM K-HEPES solutions, pH 7.4, at a holding potential of +35 mV. *A*: representative traces of the single channel activity of 2 distinct populations of RyRs from tuna fast-twitch muscle. *B*: representative traces of the single channel activity of RyR1-fast from toadfish swimbladder muscle (*left*) or RyR1-slow from tuna slow-twitch muscle (*right*) under the indicated *cis* (cytoplasmic) $[Ca^{2+}]$. Channel openings are upward deflections from the closed state indicated by the c at the *left* of each trace. P_o, open probability.

cle. K_i was 59 ± 20 μM for RyR1-slow and 251 ± 48 μM for RyR1-fast (Table 1). Thus lower maximal P_o and complete channel inhibition at millimolar Ca^{2+} are properties that distinguish the RyR1s from RyR3. Furthermore, RyR1-slow can be distinguished from RyR1-fast by its significantly lower maximal P_o.

As shown in the current-voltage plots in Fig. 5, all fish skeletal muscle RyRs form high conductance channels in symmetrical 0.25 M KCl solutions. Amplitudes of the single channels were measured at fixed voltages, and the slope conductances were obtained by linear regression. RyR1-slow purified from tuna slow-twitch muscle had a slope conductance of 786 ± 7 pS (n = 6), whereas RyR1-fast purified from toadfish fast-twitch swimbladder muscle had a slope conductance of 766 ± 8 pS (n = 4). These values were not found to be significantly different. All channels recorded from tuna fast-twitch muscle displayed similar conductances with a mean value of 820 ± 6 pS (n = 6). The measured slope conductances of fish RyRs are typical of other skeletal muscle RyRs recorded under symmetrical 0.25 M KCl solutions (29, 36).

The attenuation of Ca^{2+}-dependent inactivation of RyR1-slow by adenine nucleotides that was observed in the [^{3}H]ryanodine binding studies was further investigated using single channel recordings. Figure 6*A* shows representative single channel traces of RyR1-fast from swimbladder muscle and RyR1-slow from tuna slow-twitch muscle in the presence of 5.0 mM *cis*-Mg-ATP at several *cis*-Ca^{2+} concentrations. The P_o of both RyR1-slow and RyR1-fast was increased on the addition of 5.0 mM Mg-ATP (Fig. 6*A*). Furthermore,

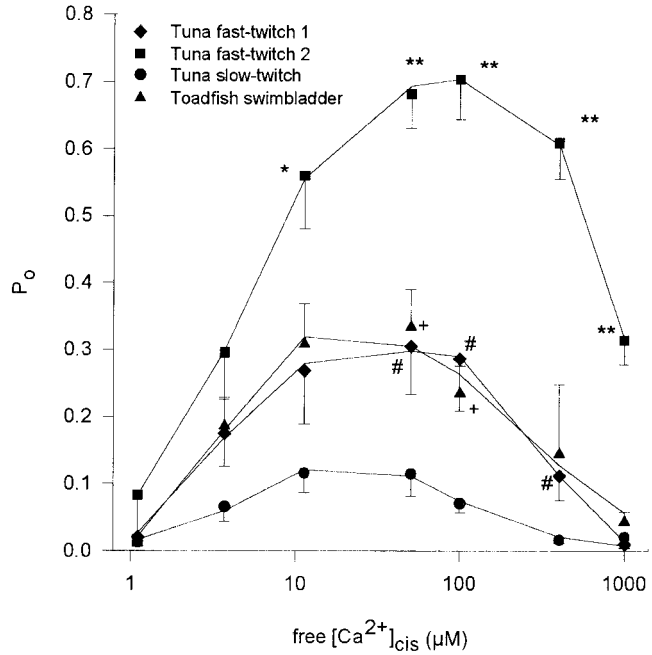

Fig. 4. Ca^{2+} dependence of single channel activities of tuna slow- and fast-twitch and toadfish swimbladder muscle RyRs. Plot of the P_o of RyR1-slow from tuna slow-twitch muscle (●, n = 5) and RyR1-fast from toadfish swimbladder muscle (▲, n = 12) as a function of the $[Ca^{2+}]$ at the cytoplasmic face of the channel. Two distinct populations of channels recorded from tuna fast-twitch muscle are also plotted: *group 1* (RyR1-fast) (◆, n = 6) and *group 2* (RyR3) (■, n = 5). * And ** indicate that differences between *groups 1* and *2* at corresponding $[Ca^{2+}]$ are significant at $P < 0.05$ and $P < 0.001$, respectively. + And # indicate that difference between toadfish swimbladder and slow-twitch and between *group 1* and slow-twitch at corresponding $[Ca^{2+}]$ are significant at $P < 0.05$, respectively. The difference between toadfish swimbladder and tuna fast-twitch 1 at all $[Ca^{2+}]$ are not significant. Solid lines are fitted results according to $P_o = P_{o\,max}\{1/[1+(K_a/[Ca])^{n_a}]\} \times \{1 - [1/(1+[K_i/[Ca]]^{n_i})]\}$ with the parameters shown in Table 1.

Table 1. *Ca^{2+} activation and inhibition constants for tuna slow-twitch, toadfish fast-twitch swimbladder, and two distinct populations of RyRs from tuna fast-twitch muscle incorporated into lipid bilayers*

	Tuna Fast-Twitch *Group 1* (RyR1-Fast)	Tuna Fast-Twitch *Group 2* (RyR3)	Toadfish Swimbladder (RyR1 Fast)		Tuna Slow-Twitch (RyR1-Slow)	
	−Mg-ATP	−Mg-ATP	−Mg-ATP	+Mg-ATP	−Mg-ATP	+Mg-ATP
K_a (μM)	3.3 ± 0.2	4.7 ± 0.1	3.6 ± 0.4	7.2 ± 0.8	7.7 ± 1.4	40 ± 7
n_a	2.1 ± 0.3	1.4 ± 0.4	2.3 ± 0.9	3.0 ± 0.8	1.3 ± 0.3	1.6 ± 0.4
K_i (μM)	332 ± 20	888 ± 15	251 ± 48	2,515 ± 491	59 ± 20	1,800 ± 300
n_i	2.8 ± 0.5	2.1 ± 0.9	1.2 ± 0.2	1.2 ± 0.3	1.2 ± 0.2	2.3 ± 0.5

Values are means ± SE. Ca^{2+} dependency data of ryanodine receptor 1 (RyR1) fast, RyR1-slow, and RyR3 in the presence and absence of MgATP. Data in Figs. 4 and 6 were fitted to the Hill equation to yield K_a, the [Ca^{2+}] that produced one-half maximal channel activity and K_i, the [Ca^{2+}] that inhibited one-half of the maximal channel activity. The Hill coefficients, n_a and n_i, indicate the degree of cooperativity of Ca^{2+}-dependent activation and inhibition, respectively.

the addition of Mg-ATP increased the Ca^{2+} K_a for RyR1-slow approximately fivefold, but had less of an effect on the K_a for RyR1-fast, increasing it only twofold (Table 1). Similar to the effect AMP-PCP had on [^{3}H]ryanodine binding to RyR1-slow, Mg-ATP attenuated the Ca^{2+}-dependent inhibition of RyR1-slow when incorporated into lipid bilayers (Fig. 6*C*). The addition of Mg-ATP to RyR1-slow resulted in a 30-fold increase in Ca^{2+} K_i and a concomitant increase in the apparent cooperativity of Ca^{2+}-dependent inhibition indicated by the increase in the Hill coefficients for Ca^{2+}-dependent inactivation (Table 1). The addition of Mg-ATP to RyR1-fast channels also reduced the Ca^{2+}-dependent inhibition, but not nearly to the extent seen with the RyR1-slow channels (Fig. 6*B*). There was an ~10-fold increase in the K_i as a result of Mg-ATP addition to RyR1-fast (Table 1). The attenuation of Ca^{2+}-dependent inhibition of toadfish swimbladder RyR1-fast channels by adenine nucleotides was something only minimally observed in the [^{3}H]ryanodine binding experiments (Fig. 2*B*). This discrepancy may have resulted from the use of different adenine nucleotides in the binding and single channel studies or from the fact that the RyRs were purified for the single channel studies, whereas the [^{3}H]ryanodine binding was conducted on HSR.

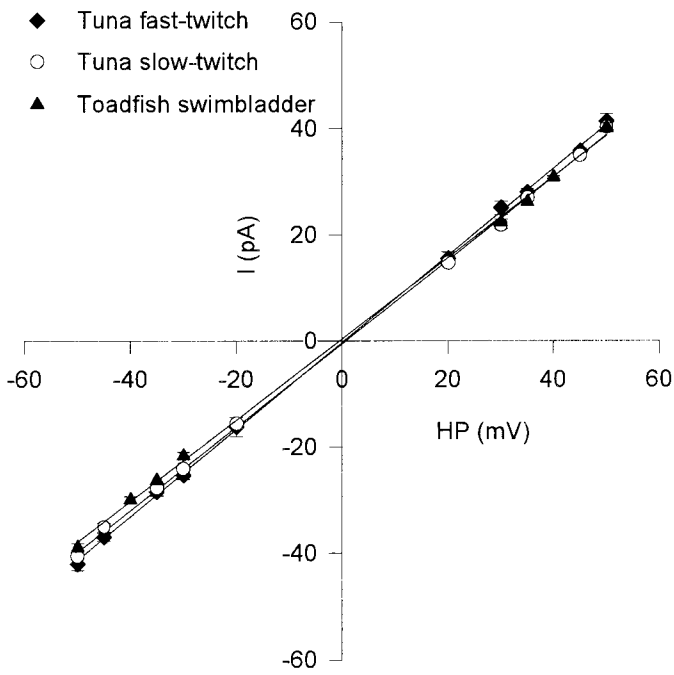

Fig. 5. Current-voltage relationship of K^+ current through tuna slow- and fast-twitch and toadfish swimbladder muscle RyRs. Single channel currents (I) of RyRs purified from tuna slow- or fast-twitch or toadfish swimbladder muscle were recorded in symmetrical 0.25 M KCl, 20 mM K-HEPES solutions, pH 7.4, at several holding potentials (HP). Slope conductances were 786 pS (r^2 = 0.9993) for RyR1-slow from tuna slow-twitch muscle (○, n = 6), 820 pS (r^2 = 0.9996) for tuna fast-twitch muscle RyRs (◆, n = 6), and 766 pS (r^2 = 0.9991) for RyR1-fast from toadfish swimbladder muscle (▲, n = 4).

Mg^{2+} inhibition of RyR1-slow, RyR1-fast, and RyR3. One theory on the mechanism by which RyRs are inhibited by millimolar concentrations of Ca^{2+} and or Mg^{2+} is that the divalent cations close the channel by binding to low-affinity sites on the protein (16). A shared inhibitory binding site for Ca^{2+} and Mg^{2+} suggests adenine nucleotides may reduce Mg^{2+}-dependent inhibition of RyR1-slow in a fashion similar to the way Ca^{2+}-dependent inhibition was reduced. Figure 7 shows the effect of 0–10 mM $MgCl_2$ on [^{3}H]ryanodine binding in tuna slow- and fast-twitch muscle and toadfish fast-twitch swimbladder muscle HSR. For each HSR preparation, the binding was conducted at the optimal [Ca^{2+}] that yielded B_{max}. In the presence of 2.5 mM AMP-PCP, Mg^{2+}-dependent inhibition of RyR1-slow is reduced compared with RyR1-fast. Similar to the effect seen at 1.0 mM Ca^{2+}, [^{3}H]ryanodine binding to slow-twitch muscle at 1.0 mM Mg^{2+} in the presence of 2.5 mM AMP-PCP was still ~85% of control (no Mg^{2+}) (Fig. 7), but only ~35% of control in the absence of AMP-PCP (Fig. 7, *inset*). In contrast, 1.0 mM Mg^{2+} inhibited binding to RyR1-fast with equal potency in the absence or presence of AMP-PCP. The distinct effect of adenine nucleotides on RyR1-slow was not due to the difference in activating [Ca^{2+}], because the same results were obtained when binding to tuna slow-twitch HSR was performed at 100 μM Ca^{2+} (data not shown). A comparison of the binding data for the toadfish fast-twitch swimbladder HSR (RyR1-fast) and tuna fast-twitch HSR (RyR1-fast and RyR3) reveals the decreased sensitivity of RyR3 to Mg^{2+} inhibition that has been described in other studies (15, 24). In the presence (Fig. 7) or absence (Fig. 7, *inset*) of AMP-PCP, the addition of 1.0 mM $MgCl_2$ to tuna fast-twitch mus-

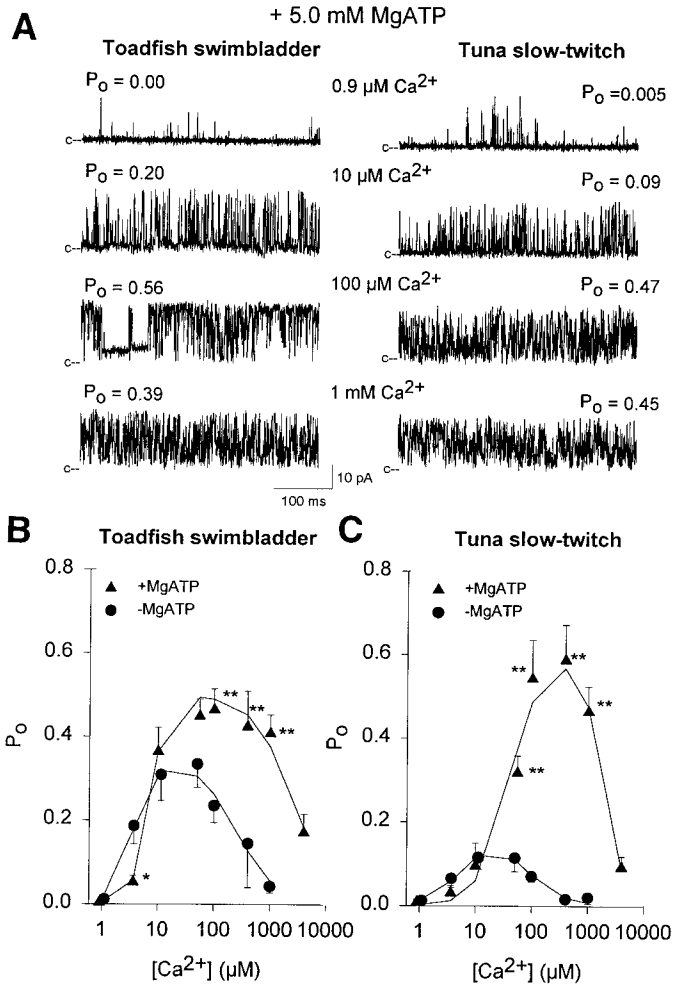

Fig. 6. Ca^{2+} dependence of single channel activity of RyR1-slow and RyR1-fast in the presence of Mg-ATP. K^+ currents through RyR1-fast (*left*) or RyR1-slow (*right*) were recorded in symmetrical 0.25 M KCl, 5.0 mM Mg-ATP, 20 mM K-HEPES solutions, pH 7.4, at an HP of +35 mV. *A*: representative traces of the single channel activity of RyR1-fast (*left*) or RyR1-slow (*right*) under the indicated *cis*-[Ca^{2+}]. Channel openings are upward deflections from the closed state indicated by the c at the *left* of each trace. *B*: plot of the P_o of RyR1-fast from toadfish swimbladder muscle as a function of *cis*-[Ca^{2+}] in the absence (●, $n = 12$) and presence (▲, $n = 8$) of 5.0 mM Mg-ATP. *C*: plot of the P_o of RyR1-slow as a function of *cis*-[Ca^{2+}] in the absence (●, $n = 5$) and presence (▲, $n = 7$) of 5.0 mM Mg-ATP. * And ** indicate that differences are significant between −Mg-ATP and +Mg-ATP at corresponding [Ca^{2+}] ($P < 0.05$ and 0.001, respectively). Solid lines are fitted results according to equation shown in Fig. 4, with parameters shown in Table 1.

cle HSR resulted in little inhibition of [^{3}H]ryanodine binding. Binding was still ~70% of the control value (no $MgCl_2$) in both cases. In contrast, [^{3}H]ryanodine binding to toadfish swimbladder HSR, with or without AMP-PCP present, was reduced to ~40% of the control by the addition of 1 mM $MgCl_2$.

DISCUSSION

Slow- and fast-twitch skeletal muscles display distinct physiological properties that allow the muscles to be recruited for different biological activities. The extent of these activities can range from rapid movements such as those used by a tuna to capture prey to the sustained movements used to power a tuna across an ocean basin during a long migration. Although slow- and fast-twitch skeletal muscle are used to perform these very distinct functions, all vertebrate skeletal muscle has the same basic structure and uses the same basic contractile system (32). It is the specific modifications of the proteins involved in the contractile system that allow the muscles to perform these diverse tasks. Several muscle proteins, including myosin, Ca^{2+}-ATPase, troponin C, and calsequestrin, have been shown to be morphologically and physiologically distinct in slow-twitch and fast-twitch skeletal muscle. RyRs can now be considered a part of this molecular machinery that varies between fiber types. Molecular evidence indicated that distinct RyR1 proteins were expressed in tuna slow- and fast-twitch skeletal muscle (11). The objective of this study was to characterize the novel RyR1-slow from tuna slow-twitch muscle fibers and compare its [^{3}H]ryanodine binding characteristics and single channel properties to RyR1-fast from fast-twitch skeletal muscle. In the course of these experiments, properties of the fast-twitch muscle RyR3 could also be deduced. We conclude that RyR1-slow has at least three physiological characteristics that distin-

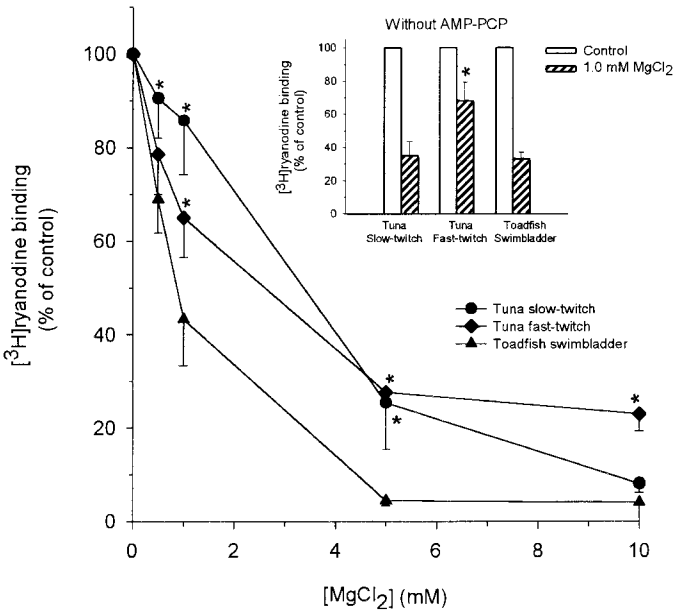

Fig. 7. Effect of Mg^{2+} on [^{3}H]ryanodine binding to tuna slow- and fast-twitch muscle and toadfish swimbladder muscle. Triplicate samples of tuna slow-twitch HSR vesicles (●, $n = 7$) or tuna fast-twitch HSR vesicles (◆, $n = 6$) or toadfish swimbladder muscle HSR (▲, $n = 4$) were incubated at 30°C for 2 h with the [^{3}H]ryanodine concentrations corresponding to the measured dissociation constant (K_d) of each tissue, i.e., 90 nM for slow-twitch muscle, 20 nM for fast-twitch muscle, and 10 nM for swimbladder muscle. Binding was conducted at the Ca^{2+} concentration that yielded B_{max} in each tissue, i.e., 10 μM for slow-twitch muscle and 100 μM for fast-twitch and swimbladder muscle. Specific binding in the presence of 2.5 mM AMP-PCP as a function of the [Mg^{2+}] is indicated as % of control (No Mg^{2+}). *Inset*: specific binding in the absence of AMP-PCP is given in the absence of Mg^{2+} (control, open bar) and in the presence of 1.0 mM Mg^{2+} (hatched bar). *Significantly different from the toadfish swimbladder data at $P \leq 0.05$.

guish it from RyR1-fast: *1*) RyR1-slow has a reduced affinity for [^{3}H]ryanodine, *2*) with Ca^{2+} as the only activator, RyR1-slow displays a lower channel activity than RyR1-fast, and *3*) adenine nucleotides cause a shift in the Ca^{2+} activation curve resulting in an increase in the activation constant for RyR1-slow and also reduce the inhibition of RyR1-slow by millimolar concentrations of Ca^{2+} and/or Mg^{2+}. These results provide evidence for physiological differences between two distinct RyR1 isoforms expressed in slow- and fast-twitch muscle of fish. This study also confirms two properties of RyR3, a high P_o and a lack of Ca^{2+}/Mg^{2+}-dependent inhibition, that were previously described for RyR3 isoforms (15, 24, 25).

Evidence that the RyR1s expressed in tuna slow- and fast-twitch skeletal muscle were physiologically distinct was first apparent in equilibrium [^{3}H]ryanodine binding experiments. With a measured K_d of ~90 nM, RyR1-slow has an affinity for [^{3}H]ryanodine that is approximately one order of magnitude less than the affinity of all the RyRs described to date. This suggests that the binding site for ryanodine may be structurally different between RyR1-slow and the other RyR isoforms. Once it is determined precisely where ryanodine binds to the receptors, a comparison of the primary sequence of RyR1-slow and RyR1-fast may yield useful insight into the specific amino acids involved in determining the affinity for ryanodine binding.

The maximal [^{3}H]ryanodine binding was 2.5-fold less in tuna slow-twitch muscle compared with toadfish fast-twitch muscle. This difference in B_{max} values is in accord with the 1.5- to 4.0-fold increase in the maximal [^{3}H]ryanodine binding of fast-twitch fibers with respect to slow-twitch fibers that has been reported for mammalian skeletal muscle HSR preparations and is consistent with the increased amount of SR in fast-twitch muscle preparations (7, 8, 18).

Both the [^{3}H]ryanodine binding experiments and the single channel recordings reveal the decreased Ca^{2+}- and Mg^{2+}-dependent inhibition of RyR3 that has been previously described (15, 24, 25). Furthermore, the single channel recordings confirm a higher maximum P_o for RyR3 channels compared with RyR1 channels in fish (25). Thus lower maximal P_o and complete channel inhibition at millimolar Ca^{2+} are properties that distinguish the RyR1s from RyR3. Of the three RyR isoforms studied, RyR1-slow had the lowest maximum channel activity, a property that could be used to distinguish RyR1-slow from RyR1-fast. It is interesting to note the significant increase in the maximal channel activity of RyR3 compared with the RyR1s under conditions in which Ca^{2+} is the lone channel activator. This may reflect the in vivo mode of activation of RyR3, which is thought to function as a Ca^{2+}-induced Ca^{2+} release channel activated by Ca^{2+} ions. RyR1 channels are thought to be activated in vivo through a mechanical coupling to the dihydropyridine receptor channels in the plasma membrane.

Single channel measurements of RyR1-slow and RyR1-fast indicated both channels displayed similar slope conductances of ~775 pS, which was typical of other RyR1s recorded in symmetrical 0.25 M KCl solutions (29, 36). A higher slope conductance of 820 pS was measured for channels purified from tuna fast-twitch muscle. This may suggest that RyR3 has a slightly higher conductance than the RyR1s under these conditions. However, too few channels were recorded from tuna fast-twitch muscle to discern two groups of channels with distinct conductances. A report by O'Brien et al. (25) claimed RyR3 had a substantially lower conductance than RyR1. However, it is difficult to compare this study with that of O'Brien et al., because two different RyR preparations and two distinct current carriers were used in the two studies.

The most distinguishing characteristic of the slow-twitch Ca^{2+} release channel is its unique regulation by adenine nucleotides. In the presence of adenine nucleotides, RyR1-slow shows an increase in channel activity, but also displays a shift of the Ca^{2+} activation curve that results in the disinhibition of the channel at millimolar Ca^{2+} and/or Mg^{2+}. This suggests a model in which adenine nucleotide binding to RyR1-slow causes a conformational change in the channel that no longer allows the binding of Ca^{2+} or Mg^{2+} to the inhibitory site. The single channel experiments of Fig. 6 show the properties of RyR1-slow under conditions that most closely mimic the true intracellular environment. In these recordings, the Ca^{2+} activation constant of RyR1-slow increased nearly fivefold in the presence of Mg-ATP, but the Ca^{2+} activation constant of RyR1-fast was only slightly increased under the same conditions. The Ca^{2+} inhibition constant of RyR1-slow increased 30-fold in the presence of Mg-ATP. The Ca^{2+} K_i of RyR1-fast also increased, but only 10-fold. Thus the addition of Mg-ATP increased both the Ca^{2+} activation and inhibition constants of RyR1-slow but increased only the Ca^{2+} K_i of RyR1-fast. Interestingly, using the consensus sequence, GXGXXG, seven adenine nucleotide binding sites have been identified in the sequence of RyR1-slow. Five were conserved in all RyR1 sequences, but two located between amino acids 4303–4308 and 4310–4315, respectively, were unique to RyR1-slow (11). Whether these two additional sites contribute to the unique regulation of RyR1-slow by adenine nucleotides requires further investigation.

The nearly complete attenuation of the Ca^{2+}-dependent inhibition of RyR1-slow in the presence of adenine nucleotides may allow this channel to function over a wider range of physiological Ca^{2+} concentrations. This may have major functional implications in a slow-twitch muscle that in tuna is continuously contracting and relaxing. Tuna swim continuously to pass oxygenated water over their gills. The slow-twitch oxidative fibers of the tuna's deep red muscle powers the fish during this uninterrupted activity. In a continuously active muscle, such as the heart or the tuna's slow-twitch muscle, intracellular Ca^{2+} levels may fluctuate from beat to beat or contraction to contraction. With the reduced capability of slow-twitch fibers to handle these fluctuations, due to their lower Ca^{2+}-ATPase activity and lower concentrations of Ca^{2+} buffering proteins, an RyR protein that can function at higher

physiological Ca^{2+} concentrations may become necessary. However, the functional significance of adenine nucleotides in the release of Ca^{2+} from the SR in situ is not well understood. In theory, if the levels of adenine nucleotides are changed in the vicinity of the channel under physiological conditions, the activity of the RyR and its regulation by Ca^{2+} can be significantly altered, which may contribute to muscle function under various physiological pressures.

It remains unclear as to whether the expression of fiber type-specific RyR isoforms is solely a property of fish or a property of higher vertebrates as well. Data from two studies on mammalian muscle show a reduced affinity for ryanodine in slow-twitch fibers consistent with the results described here for RyR1-slow. Salviati and Volpe (35) showed that ryanodine was much less effective in inhibiting Ca^{2+} loading in rabbit soleus muscle (slow twitch) than it was in inhibiting loading in rabbit adductor magnus muscle (fast twitch). Similarly, Su and Chang (37) showed a two- to fourfold decrease in the sensitivity to ryanodine-induced depression of tension in the soleus compared with the adductor magnus. However, recent studies comparing the properties of RyRs in slow-twitch and fast-twitch skeletal muscle of mammals have found few differences between the two. Lee et al. (18) found that, although the initial Ca^{2+} release rates in response to caffeine were three times slower in rabbit slow-twitch muscle than in fast-twitch muscle, the properties of the RyRs in both muscle types were similar. [^{3}H]ryanodine binding studies showed no significant differences in terms of affinity for [^{3}H]ryanodine, sensitivity to Ca^{2+}, or modulation to known activators and inhibitors such as caffeine, ATP, ruthenium red, and Mg^{2+}. However, consistent with our data for RyR1-slow, Lee et al. (18) found no difference in the slow-twitch or fast-twitch channel's conductance, yet, the slow-twitch channels had a much longer mean closed time, thus a lower P_o, than fast-twitch channels. Damiani and Margreth (7) using rat and rabbit slow- and fast-twitch muscle showed similar $-\log[Ca^{2+}]$ relationships, K_d values for [^{3}H]ryanodine, and similar modulation by caffeine, doxorubicin, and ruthenium red. More recently, Bastide and Mounier (3) using monkey muscle showed the same conductance, Ca^{2+} sensitivity, and caffeine sensitivity for slow- and fast-twitch RyRs. These mammalian studies are hampered by the challenges associated with the fact that mammalian skeletal muscle is a heterogeneous mixture of fiber types. The HSR membranes isolated from the "slow" or "fast" muscle will be mixture of slow- and fast-twitch SR. Considering that fast-twitch fibers contain more SR and thus a higher density of RyRs and the data from this report indicating a lower affinity of slow-twitch RyRs for [^{3}H]ryanodine, it is quite possible that the expression of a slow twitch-specific RyR may have been masked in these mammalian studies. Further studies incorporating molecular techniques should be able to resolve the question of whether mammalian slow- and fast-twitch skeletal muscle fibers express distinct RyR1s.

In summary, fish slow- and fast-twitch skeletal muscles express functionally distinct RyR1 proteins that may help explain some of the fundamental differences between slow and fast skeletal muscle contraction. Furthermore, the distinct physiological properties of RyR1-slow and RyR1-fast may contribute to differences in the way intracellular Ca^{2+} is regulated in these muscle types.

Perspectives

In our previous study (11), it was determined that fish slow- and fast-twitch skeletal muscle express molecularly distinct ryanodine receptors. This study expands on the first study by describing functional differences between the slow- and fast-twitch RyRs. Given the critical role RyRs play in muscle contraction, these results may indicate distinct ways in which intracellular Ca^{2+} is regulated during slow- and fast-twitch muscle contraction. This may have major implications on the way in which different skeletal muscle fibers are recruited for various animal activities and behaviors.

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