Functional properties of the native type 3 ryanodine receptor Ca\(^{2+}\) release channel from canine diaphragm

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Abstract: mRNA and protein analyses have previously shown that the diaphragm expresses two ryanodine receptor isoforms: RyR1 and RyR3. RyR1 is the main Ca\(^{2+}\) releasing pathway in this muscle type. We now report the conducting, gating, and immunological properties of the native and purified forms of the less abundant RyR3 channel. The conductance of this native Ca\(^{2+}\) release channel was 330 pS in 50 mM/250 mM trans/cis CsCH\(_3\)SO\(_3\). It was activated by Ca\(^{2+}\) concentrations of 1–1000 \(\mu\)M, and did not inactivate at mM concentrations of Ca\(^{2+}\). Both isoforms were purified by either a sucrose density gradient or immunoprecipitation as > 450 kDa proteins on SDS-PAGE. Western blot analysis confirmed the presence of RyR1 and RyR3, which displayed conductances of 740 ± 30 and 800 ± 25 pS, respectively, in 250 mM KCl. We thus provide evidence that one form of the diaphragm SR Ca\(^{2+}\) release channels may be classified as RyR3, with gating properties different from those of the well-characterized RyR1 and RyR2 isoforms.

Key words: diaphragm, calcium channel, ryanodine receptors, skeletal muscles, excitation-contraction coupling.

Introduction

In striated muscle cells, the sarcoplasmic reticulum (SR) is responsible for the release and uptake of Ca\(^{2+}\) during excitation-contraction coupling (ECC) (Melzer et al. 1995; Schneider 1994). The release of Ca\(^{2+}\) from the SR is mediated by Ca\(^{2+}\)-release channels, also known as ryanodine receptors (RyRs), because they bind to the plant alkaloid with high affinity and specificity (Fleischer et al. 1985; Pessah and Zimanyi 1991). Three structurally related RyRs have been identified in mammalian tissues: a skeletal muscle (RyR1) isoform (Takeshima et al. 1989), a cardiac (RyR2) isoform (Imagawa et al. 1987; Lai et al. 1988a); in the case of RyR1, these subunits have been shown to correspond to the foot proteins that span the gap between the SR membrane and the T-tubule (Lai et al. 1988b). These very large homo-tetramers (~2 MDa) respond to a complex pattern of regulation by various endogenous and exogenous effector molecules (Meissner et al. 1988). The skeletal and cardiac Ca\(^{2+}\)-release channels are activated by micromolar concentrations of Ca\(^{2+}\), ATP, and caffeine, and nanomolar concentrations of ryanodine; they are inhibited by Mg\(^{2+}\), ruthenium red and micromolar concentrations of ryanodine (Murayama and Ogawa 1997). There are a number of differences between the RyR isoforms (Sutko and Airey 1996), which are encoded by three different genes and display only 67% homology (Hakamata et al. 1992; Nakai et al. 1990). For instance, the skeletal and cardiac isoforms differ in their sensitivity to cytoplasmic Ca\(^{2+}\) (Rousseau and
Meissner 1989; Rousseau et al. 1988) and to phosphorylation by calmodulin- and cyclic AMP-dependent protein kinases (Strand et al. 1993). The cardiac RyR2 is the most sensitive to these two factors, while it is less sensitive to Mg2+ inhibition (Rousseau et al. 1992). To date, two different mechanisms of in situ RyR activation have been postulated for the so-called skeletal and cardiac muscle isoforms: the RyR1 would be physically coupled to the internal loop linking domains II-III of the alpha-1 subunit of the voltage-sensitive, dihydropyridine-receptors (DHPRs) of the T-tubule membrane (Schneider 1994; Takeshima et al. 1994; Stern et al. 1997), while the RyR2 would be mainly activated by an influx of extracellular Ca2+ through DHPR-Ca2+ channels (Fabiato 1988).

Although the diaphragm has been classified as a striated skeletal muscle, this tissue shares contractile properties with both cardiac and non-respiratory skeletal muscles. Like the heart, the diaphragm contracts rhythmically for life and displays metabolic features of oxidative fibers (Coirault et al. 1994; Herve et al. 1988; Anger et al. 1995). Despite numerous studies examining the contractile properties of normal and dystrophic diaphragm fibers (Coirault et al. 1994; Herve et al. 1988; Vières et al. 1988), little is known of the electrophysiological properties of their SR membrane system. Diaphragm contraction relis on extracellular Ca2+ (Lecarpentier et al. 1993; Vières et al. 1988) and is altered by ryanodine (Herve et al. 1988). Recent studies have reported the characteristics of the SR conductances for monovalent and divalent ions (Picher et al. 1997; Sonnleitner et al. 1998). Moreover, two groups have reported that among mammalian skeletal muscles, the diaphragm expresses the highest level of RyR3, as assessed by mRNA probing and immunoblot analysis (Conti et al. 1996; Murayama and Ogawa 1997). The diaphragm SR displays a dual immunological reactivity for the RyR1 and RyR3 isoforms, but with a dominant expression for RyR1 (Giannini et al. 1995; Tarroni et al. 1997). Our previous study of the main Ca2+-releasing pathway (RyR1) revealed heterogeneity in the gating and conducting behaviors of single SR channels (Picher et al. 1997), as also recently reported by Sorrentino’s group (Sonleitner et al. 1998); it was thus of prime interest to further investigate the cause of these functional variations.

To test whether these patterns could be ascribed to the presence of different isoforms or regulatory modes (biological/physiological variability), we examined the functional properties of the diaphragm RyR3 isoform using single-channel recordings upon fusion of native SR vesicles into planar lipid bilayers. We also performed comparative biochemical and immunological analyses of purified ryanodine receptors in parallel with membrane reconstitution experiments.

**Experimental procedures**

**Preparation of sarcoplasmic reticulum vesicles**

Microsomal fractions enriched in SR vesicles were prepared from either canine or rabbit diaphragms exactly as described previously (Rousseau et al. 1992). Animal were cared for in accordance with the principles and guidelines of the CCAC (Institutional protocol: O189-97). Briefly, diaphragms were homogenized and centrifuged at 6000 x g for 20 min at 4°C in Tris-maleate buffer (pH 6.8) in the presence of protease inhibitors. The supernatant was filtered and centrifuged at 90 000 x g at 4°C for 80 min. The pellet was re-suspended in K-PIPES buffer (pH 7.0) and the membrane vesicles were separated by ultracentrifugation at 120 000 x g on a 25–45% sucrose gradient. Sucrose fractions were analyzed for their protein concentration and [3H]ryanodine binding activity. [3H]Ryanodine binding assays were performed using a procedure previously described and routinely used in our laboratory (Guiraud et al. 1997). Other types of SR microsomal fractions from canine cardiac, tracheal, and fast twitch muscles [ extensor digitorum longus (EDL)] were also prepared in our laboratory, as reported elsewhere (Rousseau and Meissner 1989; Rousseau et al. 1988). These fractions were used as control vesicle populations for either biochemical or immunological analyses.

**Purification and immuno-precipitation of ryanodine receptors**

The fraction most enriched in ryanodine receptors, recovered in 40% sucrose, was used for further purification of SR Ca2+-release channels. The microsomal fractions (25 mg of protein) were solubilized with 5 nM [3H]ryanodine and without, then solubilized in 1.2% CHAPS and 3% azolectine. The solubilized material was centrifuged in a linear 5–25% (w/v) sucrose density gradient (Lai et al. 1988a; Meissner et al. 1989). The sucrose fractions were again analyzed for their radioactivity (bound [3H]-ryanodine; Guihard et al. 1997) and protein concentration, using the Biorad DC assay kit.

Immuno-purification of ryanodine receptors was achieved following solubilization of 2.5–5 mg of SR protein (in 250 mM NaCl + 1.2% CHAPS) for 30 min at 4°C and centrifugation (10 min at 12 000 rpm; 28 000 x g). Aliquots of specific RyR-antibodies, either anti-RyR1 or anti-RyR3, were sequentially diluted (1/200) in the supernatants containing the solubilized RyRs (250–500 μL), adjusted to 1.2 μg protein/L, and incubated overnight at 4°C. The RyR-antibody complexes were then mixed with Protein A-coupled Sepharose CL-4B beads pre-equilibrated in phosphate buffer solution (PBS; 10 mg/100 μL) and incubated for 2 h at 4°C. The suspension was centrifuged at 12 000 rpm for 4 min to sediment the beads as well as the bound RyR–IgG complexes. The sedimented material was washed twice in 250 μL of PBS + 1 mM EDTA + 2.5 μL of BSA (1 mg/mL), resuspended in the loading buffer, boiled for 3 min, and either immediately submitted to SDS-PAGE or frozen and stored at ~80°C for later use.

**Western blot analysis**

Protein samples that had been separated on 6% SDS-PAGE were electrotransferred to nitrocellulose membranes at 30 V overnight, at 4°C. The nitrocellulose membranes were washed for 15 min in 200 mM Tris + 1.4 M NaCl + 0.1% Tween 20, pH 7.6 (TBS-T), blocked with 5% non-fat dry milk in TBS-T, and incubated with polyclonal antibodies raised against the ryanodine receptor isoforms. Anti-RyR1 and anti-RyR3 were kindly provided by Dr. G. Meissner, University of North Carolina (Meissner 1994). The anti-FP11-antibody, raised against a protein fragment of the RyR3, was kindly provided by Dr. S.R.W. Chen, University of Calgary (Chen et al. 1997). Control experiments involved the use of polyclonal anti-RyR2 antibodies produced in our laboratory (Guiraud et al. 1997). The membranes were incubated with donkey anti-rabbit IgG1 as secondary antibodies or protein A coupled to horseradish peroxidase (HRP) (Amersham, Baie d’Urfé, Que.).

**Bilayer formation and recording instrumentation**

The planar lipid bilayers (PLB) were formed at room temperature from a lipid mixture containing phosphatidylethanolamine,
phosphatidylserine, and phosphatidycholine in a ratio of 3:2:1 (Picher et al. 1997; Rousseau et al. 1992). The final lipid concentration was 25 mg/mL, dissolved in decane. A Telolon® stick was used to gently spread a drop of the decane-lipid mixture across an aperture to obtain an artificial membrane. Membrane thinning was assayed by applying a triangular wave test pulse. Typical capacitance values were 150–300 pF. Aliquots of SR vesicles or purified ryanodine receptor complexes (30–60 μg of protein) were added to the cis chamber in close proximity to the bilayer. For SR vesicles, the chambers contained 50/250 mM trans\textit{cis} cesium methanesulfonate (CsCH$_3$SO$_3$), plus symmetrical 10 μM free Ca$^{2+}$ (109 μM CaCl$_2$ + 100 μM Tris-EGTA) and 20 mM Tris-HEPES, pH 7.4, unless specified otherwise. The use of Tris and HEPES as large impermeant cations and anions eliminated currents through the K$^+$ and Cl$^-$ channels that are present in native SR vesicles and are fused into the PLB along with the Ca$^{2+}$-release channel (Picher et al. 1997). The fusions were either spontaneous or facilitated by applying positive potentials across the bilayer. Applied voltages were defined with respect to the trans\textit{cis} chamber, which was held at virtual ground (Picher et al. 1997). The purified ryanodine receptors were incorporated in asymmetrical 50/250 mM KCl, plus 10 μM free Ca$^{2+}$ and 20 mM Tris-HEPES, pH 7.2 and 2 mM ATP cis, unless specified otherwise. Free Ca$^{2+}$ concentrations were calculated using an apparent stability constant of 1.543 × 10$^{-7}$ (pH 7.4) for Ca$^{2+}$-EGTA buffers and computer programs published by Fabiato (1988).

Command voltages were applied to the PLB through low impedance electrodes which are very stable and have minimized junction potentials. The currents were recorded using a low noise amplifier (Dagan 3900, Minneapolis, Minn.), filtered at 5 kHz and recorded on a digital audio tape through a pulse code modulation device (DAS 75 ES-SONY, Unitrade). The currents were displayed on-line on an oscilloscope (Kikusui, 5020A, Montréal, Que.). Current recordings were played back, filtered at 500 Hz with an 8-pole Bessel filter, and sampled at 2 kHz for digital storage using a Digidata 1200 interface and the Axoscope program from Axon-Instrument Inc. (Foster City, Calif.). Signal analysis was performed using the single channel analysis (SCA) or PClamp 6.0 programs. The open probability values ($P_o$) were determined using the half-threshold discriminator method on data stored in 60- to 120-second-duration files, unless specified otherwise.

**Statistical analyses**

Average values are given as means ± standard error of the mean (SEM). Regression curves and curve-fittings were performed using Sigma Plot 2000 program from SPSS Science (Chicago, Ill.).

**Chemicals**

AMP (adenosine 5′-monophosphate), ATP (adenosine 5′-triphosphate), CaCl$_2$, cesium methanesulfonate (CsCH$_3$SO$_3$), dithiothreitol, MgSO$_4$, protease inhibitors, and Protein A-Sepharose CL-4B beads were obtained from Sigma (St. Louis, Mo.). Ruthenium red was purchased from Terochem, and Sepharose CL-4B beads were obtained from Sigma (St. Louis, Mo.). [3 H]ryanodine was purchased from New England Nuclear (NEN-Mo.). Ruthenium red was purchased from Terochem, and Sepharose CL-4B beads were obtained from Sigma (St. Louis, Mo.).

**Results**

**Calcium dependent-behavior of the Ca$^{2+}$-release channel**

Previous reports have shown that the SR Ca$^{2+}$ channels display various behaviors as a function of the free Ca$^{2+}$ concentration on their cytoplasmic side (Picher et al. 1997; Sonnleitner et al. 1998). While 1–10 mM free Ca$^{2+}$ activates the RyR3, the concentration range inactivates RyR1 (Picher et al. 1997). Hence, under similar, if not identical, experimental conditions, the SR Ca$^{2+}$ release channels display various gating and conducting properties. For instance, RyR3 has long opening times and well defined subconducting events, whereas the native RyR1 does not (except in the presence of ATP).

The effect of cytoplasmic Ca$^{2+}$ on the activity of the native SR channel was assayed after reconstitution into planar lipid bilayers in asymmetrical 50/250 mM trans\textit{cis} CsCH$_3$SO$_3$ buffer solution (Picher et al. 1997). Figure 1A shows the fluctuations of Ca$^{2+}$ current in the presence of increasing concentrations of free [Ca$^{2+}$] in the cis chamber. The open probability ($P_o$) of the channel was very low even at 1 mM Ca$^{2+}$ ($P_o = 0.005$) and characterized by very short open events. The channel was maximally activated by 1 mM [Ca$^{2+}$] ($P_o = 0.87$). Figure 1A also illustrates that the channel’s unitary amplitude was not affected by cumulative increases in [Ca$^{2+}$] on the cytoplasmic face of the channel. The unitary conductance of this channel was 335 pS in asymmetrical CsCH$_3$SO$_3$ buffer solution, which is similar to the average unitary conductance value (370 pS) reported previously for the RyR1-Ca$^{2+}$ release channel under identical conditions (Picher et al. 1997). However, the single channel recordings display specific characteristics, such as long open times and pronounced sub-conductance events, which are not typical of the reconstituted native RyR1 isofrom from SR canine diaphragm (Picher et al. 1997).

Figure 1B shows the relationship between cytoplasmic cis [Ca$^{2+}$] and the $P_o$ of this type of infrequently observed channel. The channel under investigation was activated by micromolar [Ca$^{2+}$] and did not inactivate at higher [Ca$^{2+}$].
The IC₅₀ value (20 μM) for ryanodine in diaphragm SR vesicles was close to the value determined (22 μM) for the other skeletal muscles, but quite different from the value determined (5.2 μM) in cardiac SR. Finally, [³H]ryanodine binding was less sensitive to Ca²⁺ in diaphragm and skeletal SR (EC₅₀ = 40 and 50 μM, respectively) than in cardiac SR (EC₅₀ = 2 μM; Table 1, bottom row).

Western blot analysis of various SR vesicle populations

Immunological analysis of microsomal fractions prepared from either diaphragm, tracheal, and skeletal muscles were performed using specific antibodies raised against the RyR1, RyR2, and RyR3 isoforms, respectively (see Materials and methods section). Figure 2 shows that both the RyR1 and RyR3 isoforms were present in the canine diaphragm and rabbit skeletal SR vesicle populations. However, the RyR3 immunoreactive bands were consistently much less intense (left panel) than the bands detected with the RyR1 antibody (right panel). No immunostaining was observed in the membrane-proteins derived from tracheal smooth muscle that were used as a control (Fig. 2; middle lanes). RyR2-antibodies do not react with any of the protein bands of the diaphragm SR (Giannini et al. 1995; Sonnleitner et al. 1998); similar results were obtained in our laboratory (data not illustrated). Lower molecular weight protein bands were detected by both antibodies in the lanes corresponding to SR skeletal muscle (Fig. 2). These bands probably derive from protein cleavages of the main band of high Mr (>450 kDa) at the top. These results suggest the presence of both RyR1 and RyR3 isofoms in the SR from canine diaphragm, with a lower occurrence of type 3. Similar results have been obtained using bovine (Sonnleitner et al. 1998) or rabbit (Murayama and Ogawa 1997) SR preparations (data illustrated below).

Purification and characterization of the diaphragm RyRs

The purification of the RyRs from the diaphragm SR membranes was achieved through [³H]ryanodine binding, 1% CHAPS solubilization, and ultra-centrifugation on linear 5–25% sucrose gradients, as reported elsewhere (Lai et al. 1988a; Picher et al. 1997). The fractions were pumped out of the gradient tubes, passed through a UV-cell detector, collected, and counted for their relative contents in radioactive material. The results are reported in Fig. 3A, where a small peak of protein was detected in fractions 9 to 12 (continuous trace). This peak of highly buoyant material (~18% sucrose) corresponds to a peak of specific [³H]ryanodine binding (closed circles). This radioactive peak disappeared in the presence of excess cold ryanodine (data not shown) and therefore our observations are consistent with the existence of specific [³H]ryanodine binding sites on the high density material recovered from the sucrose gradient as previously described (Lai et al. 1988a; Meissner et al. 1988; Picher et al. 1997). SDS-PAGE and Coomassie blue staining of the gradient fractions revealed the presence of high molecular weight protein bands of Mr > 450 000, which co-migrated with the peak of bound [³H]ryanodine and were absent from the fractions on each side of the peak. Western blot analysis revealed a faint band immunodetected by the RyR3 antibody, while greater immunoreactivity was consistently observed with the RyR1 antibody (Fig. 4, third lanes). A screening effect attributable to a high concentration of NaCl (1 M) in the purification and loading buffer might explain why the RyR3 isoform was not detected. Otherwise, under non-reducing conditions, it could correspond to a multimeric migration.

Membrane reconstitution

The functional properties of the purified RyR protein complexes were also tested upon reconstitution into planar lipid bilayers. Single channel activities were recorded in asymmetrical (50/250 mM) KCl buffer, with K⁺ as the current carrier. In Fig. 3B, the lower traces displayed a large
conducting (850 pS) channel, highly activated by 10 μM Ca\(^{2+}\) and 2 mM ATP, whose gating was poorly voltage-sensitive with \(P_o\) values oscillating between 0.78 and 0.92.

Figure 5A shows recordings obtained upon reconstitution of the purified channel complex protein at various voltages, in asymmetrical 50 mM trans/250 mM cis KCl buffer, in the presence of 10 μM free Ca\(^{2+}\), 2 mM ATP, and 20 mM K-HEPES (pH 7.4). The kinetics of the current fluctuations were relatively insensitive to voltage changes across the PLB, a feature typical of RyR Ca\(^{2+}\)-release channels.

![Western blot analysis of the diaphragm, tracheal and skeletal muscle SR membranes.](image)

**Table 1.** \(^{3}H\)-ryanodine binding properties of various canine striated muscles.

<table>
<thead>
<tr>
<th>Property</th>
<th>Cardiac (n = 7)</th>
<th>Diaphragm (n = 8)</th>
<th>Skeletal(^a) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_D), nM</td>
<td>2.5</td>
<td>6.3</td>
<td>10</td>
</tr>
<tr>
<td>(B_{max}), pmol/mg protein</td>
<td>2</td>
<td>1.1</td>
<td>8</td>
</tr>
<tr>
<td>IC(_{50}), nM ryanodine</td>
<td>5.2</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>EC(_{50}), μM (Ca sensitivity)</td>
<td>2</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>

Note: The experiments were performed and data analyzed as described previously (Picher et al. 1997).

**Fig. 2.** Western blot analysis of the diaphragm, tracheal and skeletal muscle SR membranes. Microsomal fractions isolated from canine diaphragm (Dia), tracheal smooth muscles (Tr), and rabbit white skeletal (SK) muscles (20 μg protein/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunostaining was performed with either anti-RyR3 (left) or anti-RyR1 (right) polyclonal antibodies (see Materials and methods). In both striated muscles, thin reactive bands were consistently detected with the anti-RyR3, and relatively large immunoreactive bands with the anti-RyR1 antibody. Arrowheads represent the positions of a >450 kDa protein band (RyR) recognized by the specific antibodies in the diaphragm and skeletal muscle SR membranes. The tracheal microsomal fraction (middle) as well as the molecular weight standard lane (STD; far right) are shown as negative controls.

**Fig. 3.** Purification and reconstitution of the diaphragm ryanodine receptors. (A) Protein (UV absorption in arbitrary units) and RyR-sedimentation (cpm/μL) profiles following centrifugation of solubilized diaphragm SR membranes on a 5–25% sucrose gradient. The closed circles represent the total \(^{3}H\)ryanodine bound in each fraction with a specific peak in fraction 9 to 11, which co-localized with a small bump of high buoyancy proteins detected by the UV cell. This peak was abolished in the presence of excess cold ryanodine. (B) Upon reconstitution of the purified RyRs into planar lipid bilayers, single channel activities were recorded at various potentials, in asymmetrical 50/250 mM KCl, plus 10 μM free Ca\(^{2+}\), 2 mM ATP and 20 mM K-HEPES (pH 7.4). The kinetics of the current fluctuations were relatively insensitive to voltage changes across the PLB, a feature typical of RyR Ca\(^{2+}\)-release channels.

2 mM ATP, as quantified in Fig. 5C. Such behaviors have already been reported for the native Ca\(^{2+}\)-release channels from fast twitch (Lai et al. 1988; Meissner et al. 1989) and cardiac (Lai et al. 1988b) muscle, as well as for the purified and reconstituted RyR1 isoforms (Picher et al. 1997).

**Fig. 5A**

**Fig. 5B**

**Fig. 5C**

**Typical conducting properties**

We compared the conductance values of the various isoforms within the same species, despite some difficulty in defining the exact molecular identity of the reconstituted

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canine SR release channels (as in the case of multiple channel recordings) regardless of the tissue or SR preparation used. Data collected in our laboratory over a long period of time allowed us to compile the conducting properties of the three RyR-isoforms for three widely-used charge carriers, namely, Ca\(^{2+}\), K\(^{+}\), and Cs\(^{+}\). Table 2 summarizes the mean conductance values of the three RyR-isoforms derived from either canine diaphragm (RyR1 and RyR3) or cardiac SR (RyR2). Ca\(^{2+}\) as well as the Cs\(^{+}\) conductance of the RyR1 and RyR3 isoforms, which were differentiated based on their gating in 10 \(\mu\)M free Ca\(^{2+}\), were only slightly different (115 vs. 112 pS and 370 vs. 330 pS, respectively). However, these values were consistently higher than those calculated for their cardiac RyR2 counterpart, even when K\(^{+}\) was used as charge carrier through the corresponding purified and reconstituted canine isozymes. These results demonstrate how difficult it is to discriminate between the two SR RyR isoforms found in the diaphragm by measuring only their unitary conductances.

**Discussion**

Our study demonstrates that it is possible, using functional reconstitution experiments, to study the single channel properties of the native and purified type-3 Ca\(^{2+}\)-release channel (RyR3) isoform derived from the SR membrane of a mammalian diaphragm, despite its low occurrence. The biophysical as well as the biochemical measurements attest that this type of intracellular Ca\(^{2+}\) channel displays intrinsic features that are quite different from the well-characterized RyR1 present in all skeletal muscles (Lai et al. 1988a; Picher et al. 1997; Takeshima et al. 1989; Wagenknecht et al. 1997). For the first time, the binding properties of different SR membrane preparations, as well as the unitary
are much more sensitive to Ca\(^{2+}\) removal than the typical al. 1988). The contractile properties of the diaphragm fibers of the diaphragm SR and the functional necessity for great Ca\(^{2+}\) distance of extracellular Ca\(^{2+}\) for optimum ECC coupling in diaphragm and cardiac muscles was documented many years ago (Viires et al. 1988). Thus the data obtained here at the membrane and molecular levels correlate well with the observations made at the tissue level. It also advocates the need for more detailed basic electrophysiological studies.

**Table 2. Mean unitary conductances of the ryanodine receptor isoforms from canine diaphragm and cardiac muscles under various ionic conditions.**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>RyR1</th>
<th>RyR2</th>
<th>RyR3</th>
</tr>
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<tbody>
<tr>
<td>Native Ca(^{2+})</td>
<td>115 ± 11 (17)</td>
<td>80 ± 7 (12)</td>
<td>112 ± 6 (5)</td>
</tr>
<tr>
<td>Native Cs(^{+})</td>
<td>370 ± 23 (9)</td>
<td>311 ± 10 (10)</td>
<td>330 ± 17 (6)</td>
</tr>
<tr>
<td>Purified and reconstituted K(^{+})</td>
<td>740 ± 30 (7)</td>
<td>500 ± 21 (5)</td>
<td>800 ± 25 (4)</td>
</tr>
</tbody>
</table>

Note: Native or purified ryanodine receptors were prepared from the SR of canine diaphragm (RyR1 and RyR3) or cardiac (RyR2) muscles, respectively. Conditions were as follows: Native Ca\(^{2+}\), asymmetrical Ca\(^{2+}\) buffer 50 mM trans/0.01 mM cis; Native Cs\(^{+}\), symmetrical Cs\(^{+}\) buffer 250/250 mM trans/cis; Purified and reconstituted K\(^{+}\); symmetrical K\(^{+}\) buffer 200/200 mM trans/cis. n are the values in parentheses.

*Mean unitary conductance values in picoSiemens.

**Molecular electrophysiology**

The membrane reconstitution technique allowed us to study the differential behavior of the two SR Ca\(^{2+}\)-release channels from canine diaphragm. Picher et al. (1997) have previously described the basic biophysical and biochemical properties of the RyR1 isoform in this preparation, but only three studies have dealt with the functional characteristics and role of the RyR3 isoform (Chen et al. 1997), and only two dealt with the diaphragm (Sonleitner et al. 1998; Murayama et al. 1999). Most of our knowledge derives from immunological and molecular biology approaches (Bertocchini et al. 1997; Tarroni et al. 1997). Thus, there was a specific requirement to study the electrophysiological properties of this infrequently observed channel, seen in less than one tenth of successful single or multiple channel recordings.

Apart from its immunological reactivity (Figs. 2 and 4) with two specific independent antibodies raised against peptides derived from the initially cloned or purified RyR3, this channel receptor could also be differentiated from the typical RyR1 and RyR2 (Picher et al. 1997; Rousseau and Meissner 1989; Tripathy and Meissner 1996) according to its Ca\(^{2+}\)-dependent activation. This activations was shifted towards higher [Ca\(^{2+}\)] with no sign of Ca\(^{2+}\) inactivation under high Ca\(^{2+}\) and voltage-clamp conditions (Fig. 1). This Ca\(^{2+}\)-sensitivity appears to be characteristic of the RyR3 derived from the canine diaphragm SR, and mimics the Ca\(^{2+}\)-dependent behaviors described for the cloned, expressed, and reconstituted human RyR3 (Chen et al. 1997), as well as the behavior ascribed to the native bovine RyR3 isoform (Sonleitner et al. 1998). Such behavior disappears in RyR3 knockout mice (Sonleitner et al. 1998). In our hands, the native RyR3-channels do not display any Ca\(^{2+}\)-dependent inactivation at high free [Ca\(^{2+}\)] (＞pCa = 3), which is different from the result reported previously for recombinant and expressed RyR3 (Chen et al. 1997). However, the behavior of the less abundant SR channel was somewhat similar to the behavior reported for the cardiac channel (Meissner et al. 1988; Rousseau and Meissner 1989). These results suggest that RyR3 may function as a high-threshold Ca\(^{2+}\)-induced Ca\(^{2+}\)-release channel. Moreover, its Ca\(^{2+}\)-dependent behavior was very different from that described for the typical RyR1 channel isoforms, which fully activate at 10–30 μM and inactivate at higher [Ca\(^{2+}\)] as reported by various groups, including this...
laboratory (Meissner et al. 1989; Picher et al. 1997; Sonnleitner et al. 1998). Hence, it is unlikely that the population of RyR3-release channel recorded and reported herein could correspond to a sub-population of damaged RyR1 because limited proteolysis fully activates the RyR1 isoform at 2.5 μM free [Ca\(^{2+}\)] (Meissner et al. 1989).

In addition, the unitary conductance values measured for RyR3, using three cations (Ca\(^{2+}\), Cs\(^{+}\), K\(^{+}\)) as charge carriers, were different from those of the so-called skeletal (RyR1) and cardiac (RyR2) SR Ca\(^{2+}\)-release channels from the same species (summarized in Table 2). The mean Ca\(^{2+}\) conductance (112 pS) calculated for the canine RyR3 isoform is large when compared with the typical conductance of sarcolemmal or T-tubule L-type Ca\(^{2+}\) channels (16–20 pS), but is in good agreement with the recently calculated value (105 pS) for the bovine RyR3 isoform (Sonnleitner et al. 1998). Moreover, a 777 pS K\(^{+}\)current was reported for the cloned and reconstituted RyR3 isoform (Chen et al. 1997), which compares well with the 800 pS reported herein (Table 2, third row). Notably, the RyR3 unitary conductances were not significantly different from those determined for the RyR1 isoform, but were consistently higher than the RyR2 conductances (Table 2, second row) when measured under identical in vitro conditions, including planar lipid bilayers of similar composition. Another consistent observation made in our study was the occurrence of long-lasting subconducting states (Fig. 1A). This behavior was not observed in the other studies (Chen et al. 1997; Sonnleitner et al. 1998) and might be related to putative changes in the association of the RyR3 homotetramers with regulatory proteins, such as FKBP-12, triadin, and/or junctin (Mackrill 1999).

Although difficult to quantify in detail due to the infrequent occurrence of single channel traces and the short duration of data recordings, the kinetics of the reconstituted native, as well as purified, RyR3 channels appear poorly voltage-dependent (Fig. 3B and 5C), a hallmark of Ca\(^{2+}\)-release channels (Picher et al. 1997; Tripathy and Meissner 1996). RyR3 gating was slightly different from the gating of the typical RyR1 and RyR2 isoforms under identical experimental conditions. These observations confirm the analysis performed by Chen et al. (1997) on cloned, expressed, and reconstituted RyR3 protein complexes. This group also reported the ATP and caffeine activation, as well as the Mg\(^{2+}\) and ruthenium red inhibition, of the RyR3 channels. Their results correlate well with the previous observations made by Murayama and Ogawa using \(^{[H]}\)ryanodine binding and selective immuno precipitation of the RyR3 (Murayama and Ogawa 1999).

Perspectives

Several features beyond the scope of the present work remain to be ascertained for RyR3. For instance, the direct effects of ryanodine on diaphragm RyR3 have not been illustrated; it has recently been shown that 10 μM ryanodine largely modified the conducting gating, and pharmacological behavior of cloned, expressed, and reconstituted human uterine RyR3 (Chen et al. 1997), exactly as described earlier for the two other isoforms (Picher et al. 1997; Rousseau et al. 1987). The putative effect of phosphorylation (Stern et al. 1997) or oxidation (Zhang et al. 1999) under steady state conditions should also be verified. Still, the most interesting avenues involve assessing the co-expression and functional interactions of RyR3 with accessory proteins (Mackrill 1999) such as FKBP-12, triadin, junctin, or calsequestrin, all of which are efficient modulators of the rapid Ca\(^{2+}\) release processes via skeletal RyR1 or cardiac RyR2 isoforms (Mackrill 1999; Wagenknecht et al. 1997; Zhang 1999). The precise in situ location of the RyR3 in either longitudinal or junctional SR remains to be determined. Moreover, it was not possible to delineate a specific or differential distribution of this specific RyR3 isoform in the diaphragm SR fractions (Murayama and Ogawa 1997; Pichet et al. 1997). This limitation might be related to the larger number of type-1 RyR that was consistently observed in the membrane fractions studied (Figs. 2 and 4). All these aspects will have to be addressed to envision the putative role of RyR3 in the generation of calcium sparks from the diaphragm SR (Schneider 1994; Stern et al. 1997).

Physiological considerations

What of complementary or respective roles for the two isoforms? Several reasons or determinants might justify the functional co-expression of two ryanodine receptors, namely RyR1 and RyR3, in the mammalian diaphragm. Dual expression of these two isoforms could be required for optimal excitation-contraction coupling (ECC) as discussed above (Murayama and Ogawa 1997), while a sequential and/or differential expression could be involved in the constant regeneration or the ontogeny of this unique respiratory muscle. The latter possibility has been contemplated by various authors (Bertocchini et al. 1997; Sutko and Airey 1996). One group was able to document the differential expression of the RyR isoforms during the development of skeletal muscles, including the diaphragm, by probing total RNA (Bertocchini et al. 1997; Tarroni et al. 1997), while another established a comparison with the sequential expressions of a number of contractile and Ca\(^{2+}\)-handling proteins during development (Sutko and Airey 1996). From a functional point of view, RyR1 likely represents the most specialized and evolved Ca\(^{2+}\)-release channel and thus is the prime candidate to support the rapid Ca\(^{2+}\)-release from the junctional SR upon voltage-dependent activation of the alpha-1 subunit of the DHP-receptors (Schneider 1994; Stern et al. 1997) during tidal breathing. The RyR3 isoform could participate in the intrinsic adaptive processes that occur in diaphragm muscle cells during neurologically driven respiratory cycles (Coirault et al. 1994; Herve et al. 1988).

The lower occurrence of the RyR3 isoform in the diaphragm and several other tissues, such as brain and cerebellum, coupled to its atypical Ca\(^{2+}\)-dependent behavior as well as its different time-related expression, could be related to its putative role in cellular growth or plasticity during ontogenesis (Tarroni et al. 1997). Consequently, RyR3 could be involved in the early events that facilitate SR development in maturing myotubes, which would be compatible with its early expression in embryonic tissues (Tarroni et al. 1997). In other tissues expressing RyR3, such as uterine and vascular smooth muscles, this isoform could be sensitive to up and
down regulations as a function of the prevailing physiological or pathological states (Chen et al. 1997).

In summary

The results of the present work provide further insight into the single channel properties of the mammalian RyR3. The canine, rabbit (Murayama and Ogawa 1997), and bovine (Sonnelitter et al. 1998) diaphragm muscles express two types of ryanodine receptor Ca"^2+ release channels, RyR1 and RyR3, whose binding, conducting, and gating behaviors reveal subtle as well as major differences, mainly in their Ca"^2+-dependent activation and inactivation, as reported elsewhere (Picher et al. 1997; Sonnelitter et al. 1998). We suggest that due to its basic functional properties, the less abundant, large conducting RyR3 isoform may contribute to either the adaptive ECC (Herve et al. 1988; Stern et al. 1997; Sutko and Airey 1996) or to the development (Takeshima et al. 1989) of the vital skeletal muscle, in a different though complementary fashion than the highly specialized RyR1 isoform. Further functional and biochemical studies are warranted to elucidate the putative intrinsic interactions with accessory proteins and exact physiological role of this mixed population of RyRs in mammalian diaphragm (and other tissues) under various pathological conditions.

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References


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