Characterization of Junctional and Longitudinal Sarcoplasmic Reticulum from Heart Muscle*

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Longitudinal tubules and junctional sarcoplasmic reticulum (SR) were prepared from heart muscle microsomes by Ca++-phosphate loading followed by sucrose density gradient centrifugation. The longitudinal SR had a high Ca++ loading rate (0.93 ± 0.08 μmol·mg⁻¹·min⁻¹) which was unchanged by addition of ruthenium red. Junctional SR had a low Ca++ loading rate (0.16 ± 0.02 μmol·mg⁻¹·min⁻¹) which was enhanced about 5-fold by ruthenium red. Junctional SR had feet structures observed by electron microscopy and a high molecular weight protein with M₉ of 340,000, whereas longitudinal SR was essentially devoid of both. Thus, these subfractions have similar characteristics to longitudinal and junctional terminal cisternae of SR from fast twitch skeletal muscle. Ryanodine binding was localized to junctional cardiac SR as determined by [³H]ryanodine binding. Scatchard analysis of the binding data showed two types of binding (high affinity, Kₐ~ 7.9 nm; low affinity, Kₐ~ 1 μM), contrasting with skeletal muscle SR, where only one site with Kₐ~50 nM was observed. The ruthenium red enhancement of Ca++ loading rate in junctional cardiac SR was blocked by pretreatment with low concentrations of ryanodine as reported for junctional terminal cisternae of skeletal muscle SR. The Ca++ loading rate of junctional cardiac SR was enhanced by preincubation with high concentrations of ryanodine. The apparent inhibition constant (Kᵢ~ 7 nm) and stimulation constant (Kₛ~ 1.1 μM) for ryanodine on junctional SR corresponded to the Kₐ for high affinity binding (Kₐ~ 7.9 nm) and low affinity binding (Kₐ~ 1.1 μM), respectively. These results suggest that high affinity ryanodine binding is a specific ligand for the Ca++ release channels of the junctional cardiac SR. The characteristics of the Ca++ release channels of junctional cardiac SR appear to be similar to that of skeletal muscle SR, but the Ca++ release channels of cardiac SR are more sensitive to ryanodine.

Muscle contraction and relaxation are regulated by the myoplasmic free calcium ion concentration which in turn is regulated by membranes. In order to define the machinery involved in skeletal muscle contraction and relaxation, our laboratory has concentrated on isolating and characterizing specific types of muscle membranes. This approach resulted in the isolation of plasmalemma, triads (the junctional association of transverse tubule and sarcoplasmic reticulum), and the longitudinal tubules and terminal cisternae, the two main types of sarcoplasmic reticulum (SR) (1). The longitudinal tubules consist essentially of the Ca++ pump membrane which is capable of energized Ca++ uptake enabling muscle to relax. The terminal cisternae consist of two types of membranes, the Ca++ pump membrane (~80% of the membrane) and the junctional face membrane with well defined structures which, in situ, are junctionally associated with the transverse tubule to form the triad junction. The depolarization of transverse tubule leads to the release of Ca++ from the terminal cisternae. Recently, we found that ryanodine acts pharmacologically as a specific ligand for the Ca++ release channels which are localized to the junctional terminal cisternae (2). This led to the isolation of the ryanodine receptor and the finding that it is identical to the feet structures (3).

In heart muscle, some of the Ca++ which induces muscle contraction must first enter the cell via the slow inward Ca++ current. The elevation in Ca++ concentration then triggers Ca++ release from the SR. This type of release is referred to as "Ca++-induced Ca++ release" (4, 5). A key question to be resolved is whether the basic machinery for Ca++ release in heart is similar to that in skeletal muscle. In order to characterize the molecular machinery for Ca++ release in SR in heart and to compare it with that in skeletal muscle, it is necessary to isolate this apparatus. Previously, we have isolated highly purified and stable SR from heart (6). This study is concerned with the subfractionation and characterization of junctional terminal cisternae and longitudinal tubules from canine heart.

MATERIALS AND METHODS

Ryanodine was obtained from Penick Corp. (Lindhurst, NJ) and [³H]ryanodine (70 Ci/mmol) was prepared and purified as described previously (2). Ruthenium red was obtained from Sigma and used without purification, but solutions were corrected for stated purity. Molecular weight standards for SDS-PAGE were from Bio-Rad. All other chemicals were reagent grade or the best available.

Preparation of Subfractions of Cardiac SR—All operations were carried out in the cold except where otherwise noted. Cardiac microsomes were isolated from dog heart ventricles by the procedure of Chamberlin et al. (6). Briefly, sliced ventricles (30–32 g of portion) were homogenized in 5 volumes (v/w) of 0.29 M sucrose, 0.5 mM dithiothreitol, 3 mM NaN₃, 10 mM imidazole·HCl, pH 6.9, in a 250-ml flask using a VirTis "45" homogenizer at 21,500 rpm for 15 s

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‡ Supported by an Investigatorship of the American Heart Association, Tennessee Affiliate.

1 The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethylenedinitroxyethylene}tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RR, ruthenium red; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
followed by 35 s at 35,800 rpm. The homogenates were centrifuged 15 min at 5,000 rpm (3,800 × gmax) in a Beckman JA-14 rotor. The supernatants were filtered through four layers of cheesecloth, adjusted to 140 ml with fresh homogenization solution, and centrifuged 15 min at 13,600 rpm (27,000 × gmax) in a JA-14 rotor. After filtration through cheesecloth, the supernatants from the second centrifugation were resuspended in 0.3 M KCl, 10 mM MgCl2, 2 mM EGTA, 10 mM EGTA, 25 mM potassium phosphate, pH 7.4, and incubated at 37 °C for 10 min. The suspension was then immediately centrifuged for 30 min at 46,000 rpm (218,000 × gmax) in a Beckman 70 Ti rotor. The pellets from 25 ml of the suspension (50 mg of protein) were resuspended in buffer A (150 mM KCl, 5 mM ATP, 5 mM MgCl2, 2 mM CaCl2, 2 mM EGTA, 50 mM potassium phosphate, pH 7.4) and layered on a discontinuous sucrose gradient consisting of 7.5 M sucrose, 0.65 M KCl, 0.5 mM dithiothreitol, 3 mM NaN3, 10 mM imidazole-HCl, pH 6.7 (about 22 ml/30 g portion), and 0.3 M sucrose, 0.3 M KCl, and 20 mM Hepes, pH 7.0, and stored frozen at −10844°C.

Electron Microscopy—Samples suspended and incubated at 4 °C for 1 h in 1 ml of 5 mM imidazole-HCl, pH 7.4, were resuspended at 30 min at 35,000 rpm (395,000 × gmax) in a Beckman TL 100.2 rotor using a TLA 100.2 rotor. The pellets were fixed at 4 °C in 1% OsO4, 0.2% gluteraldehyde, 8% sucrose, 100 mM cacodylate, pH 7.2–7.3. The samples were washed and additionally fixed with 1% OsO4 in 2.4 mM CaCl2, 60 mM NaCl, 100 mM Veronal acetate, pH 7.2, for 2 h at 4 °C and further processed as described previously (10, 11). Intact heart tissue was fixed and stained in situ as described previously (10).

RESULTS

Subfractions of Cardiac SR—Cardiac microsomes were subfractionated by active loading of the vesicles with Ca2+ in the presence of phosphate and ATP and subsequent separation by centrifugation on a sucrose step gradient. The pelleting in the bottom of the gradient through 1.6 M sucrose showed high Ca2+ loading rate which is not enhanced by addition of ruthenium red (7 μM) (longitudinal SR in Table I). Fractions 2 and 3 at the interfaces of 0.6–0.8 M and 0.8/1.1 M sucrose, respectively, had low Ca2+ loading rates and high ATPase activity (junctional SR in Table I).

Ca2+ Loading Assay—Ca2+ loading was measured with a Hewlett-Packard UV/visible spectrophotometer model 8450A or diode array spectrophotometer model 8451A by using antipyrylazo III as the metallochromic indicator and measuring the difference in absorbance between 710 and 790 nm at 37 °C. The Ca2+ loading assay medium in 1 ml contained 100 μM potassium phosphate buffer, pH 7.0, 1 mM MgCl2, 5 mM NaN3, 0.2 mM antipyrylazo III, and about 50 μg of protein. Na2ATP (1 mM) was then added, and the reaction was initiated by addition of 50 μM CaCl2. In some experiments, ruthenium red (7 μM) was added 70–80 s thereafter (2).

(→ RR/− RR) + RR− RR

+ RR− RR

π2 + RR− RR

Table I

<table>
<thead>
<tr>
<th>Fracrtion</th>
<th>Ca2+ loading rate</th>
<th>(→ RR/− RR) + RR− RR</th>
<th>Ratio</th>
<th>[3H]Ryanodine binding</th>
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<tr>
<td>Microsomes</td>
<td>0.16 ± 0.02 (4)</td>
<td>0.84 ± 0.16 (4)</td>
<td>4.75</td>
<td>0.08 ± 0.02 (3)</td>
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<tr>
<td>Longitudinal SR</td>
<td>0.93 ± 0.08 (3)</td>
<td>1.01 ± 0.05 (3)</td>
<td>1.09</td>
<td>0.12 ± 0.02 (4)</td>
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<tr>
<td>Junctional SR</td>
<td>0.65 ± 0.02 (3)</td>
<td>0.91 ± 0.06 (3)</td>
<td>1.44</td>
<td>0.73 ± 0.12 ± 0.06 (3)</td>
</tr>
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</table>

* + RR refers to 7 μM ruthenium red.

**Ryanodine binding was measured at 210 nm ryanodine in the presence of 1 mM KCl and 25 μM CaCl2. Total binding is given after subtracting radioactivity binding to the 0.25-μm Millipore filter (see Fig. 2).”

**Fractions 2 and 3 gave essentially the same results. The values from fraction 3 are presented.”

Characterization of Functional and Longitudinal Cardiac SR
longitudinal and junctional terminal cisternae of skeletal muscle SR prepared by gradient centrifugation without calcium loading (12) where the Ca\(^{2+}\) release channel was localized to the junctional terminal cisternae (2).

The subfractions of cardiac SR were examined by electron microscopy and SDS-PAGE. Fractions 2 and 3 consisted of vesicles containing some "feet" structures (Fig. 1, B and C). In situ, the terminal cisternae of SR are junctionally associated with transverse tubules by way of these structures (Fig. 1A). Fraction 5 consisted of vesicles devoid of feet structures (Fig. 1D). SDS-PAGE showed that a high molecular weight protein with \(M_r\) of 340,000, which has been shown to be a constituent of the feet structures in the junctional terminal cisternae of skeletal muscle SR (3), was enriched in fractions 2 and 3 but not in fraction 5 (Fig. 2). Several other proteins including Ca\(^{2+}\) binding protein (calsequestrin) were also enriched in fractions 2 and 3, which were not present in fraction 5 (arrowheads in Fig. 2). Based on these morphological, compositional, and functional observations, we designate fraction 2 and 3 as junctional terminal cisternae (junctional SR) and fraction 5 as longitudinal cisternae (longitudinal SR). Fraction 1 was partially enriched with sarcolemmal and/or transverse tubule vesicles, based on morphology by electron microscopy, Ca\(^{2+}\) loading rate, and (Na\(^{+},K^{+}\))-ATPase activity. Fraction 4 contained a mixture of junctional and longitudinal activity and some aggregation of vesicles.

Since Ca\(^{2+}\)-phosphate loading was employed to subfractionate cardiac SR, the calcium remaining in the vesicles was estimated using \(^{45}\)Ca during preparation (Table II). When compared with the Ca\(^{2+}\)-oxalate loading method by Jones and Cala (13), calcium remaining in the vesicles was significantly lower with the Ca\(^{2+}\)-phosphate loading procedure. The calcium within the vesicles could further be removed by incubation.

Thin section microscopy, using tannic acid enhancement, can be used to discern cardiac sarcoplasmic reticulum from plasmalemma in that the cytoplasmic face of sarcoplasmic reticulum displays surface material and thereby has an asymmetric membrane appearance (6).

2 The Ca\(^{2+}\)-phosphate loading rate of fraction 1 was 0.084 ± 0.012 \(\mu\)mol Ca\(^{2+}\)/mg·min (mean ± S.E., \(n = 3\)). The rate was not changed by addition of ruthenium red (7 \(\mu\)M). The Ca\(^{2+}\) loading rates of fractions 3 (junctional SR) and 5 (longitudinal SR) are shown in Table I.

Ouabain-sensitive (Na\(^{+},K^{+}\))-ATPase activity was 55.3 \(\mu\)mol/mg·h in fraction 1. The fraction is approximately 30% enriched with regard to cardiac plasmalemma using a specific activity of 183 \(\mu\)mol/mg·h (24). The activities in fractions 3 and 5 were 14.0 and 5.8 \(\mu\)mol/mg·h, indicating 7.7 and 3.2% contamination, respectively, by cardiac plasmalemma. The (Na\(^{+},K^{+}\))-ATPase activity was determined as described previously with alamethicin (6).
Characterization of Junctional and Longitudinal Cardiac SR

Subfractions of cardiac SR by the phosphate (P_i) loading method were prepared as described under "Materials and Methods" except 46CaCl_2 was used instead of CaCl_2. Subfractions by oxalate loading method were prepared according to the method of Jones and Cala (13) using 46CaCl_2. Specific activity of 46Ca was 1716 cpm/nmol. Radioactivity remaining in the vesicles was determined using 0.22-μm Millipore filters (type GSWP). The vesicles (50 μg of protein) were placed on a Millipore filter and washed three times with 3 ml of 5 mM EGTA, 0.3 M KCl, 0.3 M sucrose, and 20 mM Hepes, pH 7.0. 46Ca remaining on the filter was counted. Fraction 5 in the P_i loading method and fraction D in the oxalate loading method were selected as junctional SR. Fraction 5 in the P_i loading method and fraction E in the oxalate loading method were used as longitudinal SR. The same microsomes were used for both subfractionation. The results from a typical experiment are presented.

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>P_i method</th>
<th>Oxalate method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Junctional SR</td>
<td>0.142</td>
<td>0.064</td>
</tr>
<tr>
<td>Longitudinal SR</td>
<td>2.63</td>
<td>0.954</td>
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</table>

^b Removal of calcium from the vesicles was performed as described under "Materials and Methods." Ca^2+ loading rate and stimulation of Ca^2+ loading by ruthenium red were not changed before and after incubation.

The value reported by Jones and Cala (13) was 0.24 μmol of Ca^2+ mg of protein.

The value reported by Jones and Cala (13) was 8.18 μmol of Ca^2+ mg of protein.

Fig. 2. SDS-polyacrylamide gel electrophoresis of cardiac membrane fractions. Electrophoresis was carried out on a 5-15% polyacrylamide gradient gel in SDS and stained with Coomassie Blue. Lane 1, molecular weight standard, α2-macroglobulin (nonreduced) (340,000); lane 2, cardiac microsomes (18 μg); lane 3, junctional SR (fraction 3) (18 μg); lane 4, longitudinal SR (fraction 5) (18 μg); lane 5, molecular weight standards, myosin (200,000), β-galactosidase (116,600), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000) from the top (TOP) to the bottom (DF). a, high molecular weight protein; b, Ca^2+ pump protein; c, calcium binding protein (calsequestrin). Proteins specific to junctional SR are indicated by arrowheads in lane 3, DF, dye front.

Fig. 3. Scatchard analysis of ryanodine binding to junctional SR. [3H]Ryanodine binding to junctional SR (0.1 mg/ml) was measured in 1 M KCl, 25 mM CaCl_2, 10 mM Hepes, pH 7.4, as described under "Materials and Methods." Ryanodine concentrations were varied from 3 nM to 2.4 μM. Total binding to junctional SR, after subtracting the radioactivity binding to 0.22-μm Millipore filters (type GSWP), was plotted. The radioactivity bound to the Millipore filters was usually 4-10% of the total binding to junctional SR at 6-100 nM of ryanodine, and 30-35% at 0.1-2.4 μM of ryanodine. The values are the mean of duplicate assays in which the difference from the mean was less than 10%. The correlation coefficients of high and low affinity binding sites were -0.985 and -0.789, respectively.

Different affinity sites of ryanodine binding were observed in junctional SR vesicles (Fig. 3), the effects of high and low concentrations of ryanodine on the Ca^2+ loading rate were examined and compared with [3H]ryanodine binding data in order to determine the function of each type of ryanodine binding on Ca^2+ release channels. Ruthenium red enhances...
Characterization of Junctional and Longitudinal Cardiac SR

Table III

<table>
<thead>
<tr>
<th>Ryanoctine binding</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (pmol/mg)</th>
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<tr>
<td>High affinity site</td>
<td>7.92 ± 1.2 (3)</td>
<td>5.1 ± 0.6 (2)</td>
</tr>
<tr>
<td>Low affinity site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM KCl</td>
<td>1.087 ± 7.43 (3)</td>
<td>32.8 ± 4.5 (2)*</td>
</tr>
<tr>
<td>No KCl</td>
<td>10.214 ± 303 (2)</td>
<td>32.8 ± 16.0 (2)*</td>
</tr>
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</table>

* KCl was omitted from the standard binding assay medium.

This value includes nonspecific binding as defined in Fig. 4B.

Fig. 4. Characterization of [3H]ryanodine binding to junctional SR at low and high concentrations of ryanodine. The time course of ryanodine binding was determined at ryanodine concentrations of 10 nM (A) and 1.5 µM (B). Junctional SR (0.1 mg/ml) was incubated at 37 °C in the standard assay buffer (1 mM KCl, 25 mM CaCl$_2$, 10 mM Hepes, pH 7.4) (A), the KCl-omitted (C), the EGTA (2 mM)-containing (X), and the excess unlabeled ryanodine (1 µM in A and 1.5 mM in B)-containing buffers (non-specific binding) (B). [3H]Ryanodine binding was measured as described under "Materials and Methods." The ryanodine binding to junctional SR, after subtracting the radioactivity bound to the Millipore filters, was plotted. The values are the mean of duplicate assays in which the difference from the mean was less than 10%.

The Ca$^{2+}$ loading rate of junctional SR about 5-fold (Table I), apparently by closing the Ca$^{2+}$ release channels. When junctional SR was preincubated with low concentrations of ryanodine (1-10 nM) at 37 °C for 30 min, stimulation of Ca$^{2+}$ loading by ruthenium red was blocked in a dose-dependent fashion, whereas the Ca$^{2+}$ loading rate without ruthenium red was not affected by preincubation with ryanodine (Fig. 5A). A double-reciprocal plot of the ruthenium red-stimulated Ca$^{2+}$ loading rate versus ryanodine concentration gave a straight line from which an apparent inhibition constant ($K_i$) was obtained (Fig. 5B). $K_i$ values for ryanodine are summarized in Table IV. There is essentially no change in the amount of bound ryanodine under conditions of the Ca$^{2+}$ loading assay (see legend to Fig. 5). Since the same conditions were used for ryanodine binding assay and preincubation of Ca$^{2+}$ loading, it is possible to compare the dose dependency of ryanodine on its effect in Ca$^{2+}$ loading with the ryanodine binding to junctional SR. The $K_i$ value (7.1 nM) was essentially the same as the $K_d$ value for the high affinity binding site (7.9 nM) (Tables III and IV), indicating that the action of ryanodine in blocking ruthenium red stimulation of Ca$^{2+}$ loading is referable to ryanodine binding to the high affinity site.

It is known that very high concentrations (>200 µM) of ryanodine enhance the Ca$^{2+}$ loading in junctional SR (13). Fig. 6A confirms this effect of ryanodine for our purified junctional SR; the Ca$^{2+}$ loading rate was stimulated by preincubation of junctional SR with high concentrations of ryanodine (0.5-10 µM). The apparent inhibition constant ($K_i$) for ryanodine was obtained in the double-reciprocal plot from the intersection of the line with the x axis. A typical experiment is presented. The RR-stimulated rate in samples preincubated with ryanodine was subtracted from the maximal RR-stimulated rate obtained in the absence of ryanodine, to give (Δ velocity) (2). The apparent inhibition constant ($K_i$) for ryanodine was obtained in the double-reciprocal plot from the intersection of the line with the x axis. A typical experiment is presented.

5. S. McGrew and S. Fleischer, unpublished observations.
Modulation of the function of cardiac junctional SR by ryanodine

Values are the mean ± S.E. from the number of separate preparations indicated in parentheses.

<table>
<thead>
<tr>
<th>Ryanoline action</th>
<th>$K_i$</th>
<th>$K_m$</th>
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<tr>
<td>Inhibition of RR stimulation of Ca$^{2+}$ loading</td>
<td>7.1 ± 2.1 (3)</td>
<td>1.11 ± 0.63 (3)</td>
</tr>
<tr>
<td>Stimulation of Ca$^{2+}$ loading</td>
<td>10.22 (1)</td>
<td></td>
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</tbody>
</table>

* Effect of ryanodine preincubation on ruthenium red stimulation of Ca$^{2+}$ loading in junctional SR was examined by the type of experiment shown in Fig. 5A. $K_i$ for ryanodine was determined from the double-reciprocal plot of (A velocity) versus ryanodine concentration as shown in Fig. 5B.

* Effect of ryanodine on Ca$^{2+}$ loading was examined by the type of experiment shown in Fig. 6A. $K_m$ for ryanodine was determined from the double-reciprocal plot of velocity versus ryanodine concentration as shown in Fig. 6B.

* KCl was omitted from the preincubation medium (see legends to Figs. 5 and 6).

**Fig. 6. Effect of high concentration of ryanodine on Ca$^{2+}$ loading in junctional SR.** A, Ca$^{2+}$ loading assay. Junctional SR (3 mg/ml) were preincubated for 30 min at 37 °C with various concentrations (0.5-10 mM) of ryanodine under the same conditions as Fig. 5. A aliquot (16.7 μl) of the sample was then added to 1 ml of Ca$^{2+}$ loading assay medium. Ca$^{2+}$ loading was measured as described in Fig. 5 except that ruthenium red was omitted. In the control study, junction SR was incubated with [3H]ryanodine under the preincubation conditions for 30 min and then diluted into the Ca$^{2+}$ loading assay medium in the presence of 50 μM CaCl$_2$ and 1 mM ATP. The amount of ryanodine binding was determined 5 min after the dilution. There was no significant change in ryanodine binding (less than 10%) before and after the dilution. B, double-reciprocal plot. The Ca$^{2+}$ loading rate (velocity) was determined from A. The apparent stimulation constant ($K_m$) for ryanodine was obtained from the double-reciprocal plot. A typical experiment is presented.

**Fig. 7. Modulation of ryanodine action on the Ca$^{2+}$ release channels by salt concentration.** Junctional SR (3 mg/ml) were preincubated at 37 °C for 30 min with 10 mM ryanodine (A) and 10 or 100 μM ryanodine in 25 mM CaCl$_2$ and 10 mM Hepes, pH 7.4, in the presence and absence of 1 mM KCl. An aliquot (16.7 μl) was added to 1 ml of Ca$^{2+}$ loading assay medium. The assay conditions for Ca$^{2+}$ loading in A and B are the same as those of Figs. 5 and 6, respectively. In the control (–Ry), junctional SR were preincubated without ryanodine in 1 mM KCl, 25 mM CaCl$_2$, and 10 mM Hepes, pH 7.4.

**Table IV**

<table>
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<tr>
<th>Ryanoline action</th>
<th>$K_i$</th>
<th>$K_m$</th>
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<tr>
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<td>7.1 ± 2.1 (3)</td>
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<tr>
<td>Stimulation of Ca$^{2+}$ loading</td>
<td>10.22 (1)</td>
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**Table V**

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<th>Preincubation conditions</th>
<th>Ryanoline binding</th>
<th>Ca$^{2+}$ loading rate</th>
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<tr>
<td></td>
<td>pmol/mg protein</td>
<td>μmol/mg min</td>
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<tr>
<td>Control</td>
<td>0</td>
<td>0.154</td>
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<tr>
<td>10 nM ryanodine</td>
<td>5.40</td>
<td>0.190*</td>
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<tr>
<td>No KCl</td>
<td>0.23</td>
<td>0.617*</td>
</tr>
<tr>
<td>10 μM ryanodine</td>
<td>9.0</td>
<td>0.569</td>
</tr>
<tr>
<td>No KCl</td>
<td>4.1</td>
<td>0.310</td>
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</table>

* Ca$^{2+}$ loading rate after addition of 7 μM ruthenium red.
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can also be obtained with 100 \mu M ryanodine in the absence of KCl (Fig. 7B). Since the apparent \( K_a \) is 10 \mu M, 1 M salt decreased the \( K_a \) and \( K_d \) by a factor of about 10 (Tables III and V).

**DISCUSSION**

Subfractions of cardiac SR referable to junctional and longitudinal SR have been purified and the ryanodine binding localized to the junctional cardiac SR. The fractions are similar to those of skeletal muscle SR with regard to morphology, localization of ryanodine binding to junctional SR, and functional characteristics (2, 12). Two types of binding are discerned, high affinity (\( K_a \approx 8 \) nM) and low affinity binding (\( K_a \approx 1 \) \mu M). The high affinity ryanodine binding can be correlated with blocking the ruthenium red stimulation of Ca\(^{2+}\) loading (\( K_a \approx 7 \) nM), whereas low affinity binding stimulates Ca\(^{2+}\) loading (\( K_a \approx 1.1 \) \mu M). Since ruthenium red and ryanodine (up to 300 \mu M) have little effect on the Ca\(^{2+}\) pump protein [(Ca\(^{2+}\),Mg\(^{2+}\)]-ATPase] (Table I) (2, 13), the effect of ryanodine on Ca\(^{2+}\) loading is referable to the direct modulation of Ca\(^{2+}\) release channels. Therefore, our results indicate that high affinity ryanodine binding locks the Ca\(^{2+}\) release channels in the open state and that low affinity binding closes the Ca\(^{2+}\) release channels of junctional cardiac SR.

Subpopulations of cardiac SR were first reported by Jones and Cala (13). In their study, Ca\(^{2+}\)-oxalate preloading was used for separation. They showed that a high concentration of ryanodine (300 \mu M) stimulates Ca\(^{2+}\) uptake in the "ryanodine-sensitive" vesicles, but not in the "ryanodine-insensitive" vesicles, and later found that a high molecular weight protein (M<sub>r</sub> 290,000-350,000) (14) and the Ca\(^{2+}\) binding protein (calseenestrin) are present in the ryanodine-sensitive vesicles but not in the ryanodine-insensitive vesicles (13). In our study, the subfractions of cardiac SR were isolated by Ca\(^{2+}\)-phosphate loading followed by sucrose density gradient centrifugation. The characteristics observed from the Ca\(^{2+}\)-oxalate loading method are also observed in this study. When the initial Ca\(^{2+}\) loading rates of the subfractions of SR obtained by the two methods are compared, they are comparable or higher by the Ca\(^{2+}\)-phosphate loading method. Both junctional SR fractions have similar stimulation of Ca\(^{2+}\) loading rate by ruthenium red or high concentration of ryanodine. An advantage of the Ca\(^{2+}\)-phosphate method is that calcium remaining within the vesicles is lower than that in the vesicles prepared by the Ca\(^{2+}\)-oxalate loading method (Table II). Furthermore, we provide methodology to decrease the preloaded calcium within the vesicles (Table II). This removal is achieved without loss of activity. In our study, the subfractions of cardiac SR were further characterized with regard to morphology (Fig. 1) and the differential effect of high and low affinity ryanodine binding on permeability to Ca\(^{2+}\) and hence the Ca\(^{2+}\) release channels (Figs. 3-7) (15). The high affinity binding locks the channels in the open state, whereas the low affinity binding closes them. The ryanodine receptor was localized to the terminal cisternae fraction which contains the foot structures and the high molecular weight polypeptide (M<sub>r</sub> 340,000).

Pharmacological effects of ryanodine on cardiac microsomes (16-18), purified SR (19), and ryanodine-sensitive vesicles subfractionated by Ca\(^{2+}\) oxalate loading (13) were reported from a number of laboratories. Some investigators found that ryanodine at very high concentrations (300-400 \mu M) increased Ca\(^{2+}\) uptake with little effect on the Ca\(^{2+}\) pump (13, 16, 19). Others reported that 1 \mu M of ryanodine increased passive Ca\(^{2+}\) efflux from the vesicles and that 300-400 \mu M of ryanodine decreased passive Ca\(^{2+}\) efflux (17, 18). Ryanodine binding has also been examined in a crude fraction of cardiac microsomes by Pessah et al. (20), and binding was interpreted in terms of two different affinities for ryanodine (\( K_a \) of 36 and 339 nM). In the present study, we correlated the relationship between the ryanodine binding in cardiac junctional SR to its modulation of the Ca\(^{2+}\) release channels. The different pharmacological effect of ryanodine on cardiac SR reported previously may be explained in terms of the two types of ryanodine binding, and their different action on the Ca\(^{2+}\) release channels of junctional SR is a function of concentration. The effective ryanodine concentrations reported varied somewhat with the study, the differences may be due in part to the conditions used such as Ca\(^{2+}\) and KCl concentrations, which modulate the ryanodine binding to junctional SR (Fig. 4, Tables III and V). Of the two types of ryanodine binding, low affinity binding to junctional SR showed a higher percentage of nonspecific binding (Fig. 4). The ryanodine binding at low affinity sites which has a good correlation with the pharmacological effect (closing the Ca\(^{2+}\) release channels) was the total binding including the nonspecific binding (Tables III and IV), indicating that the weaker binding at the low affinity sites closes the channels. The relationship between the two types of binding and the nature of the modulation of the channel needs further definition. Our working hypothesis is that the same channel machinery is involved. This is supported by the observation that the two types of binding are observed in the purified ryanodine receptors from heart (21).

In skeletal muscle SR, we have reported that the ryanodine binding is localized to junctional terminal cisternae (2). Ryanodine at low concentrations locks the Ca\(^{2+}\) release channels in the open state (\( K_a \approx 50 \) nM) (2) or opens the channels (at \( \approx 10 \) nM) (17). High concentrations of ryanodine (>10 or 300 \mu M) closes the channels (17, 18). These pharmacological actions of ryanodine on junctional terminal cisternae of cardiac SR are comparable with that from skeletal muscle, suggesting that the basic machinery for Ca\(^{2+}\) release is similar in both types of muscle. In both skeletal muscle and heart, the ryanodine receptor has been purified and shown to be equivalent to the feet structures of the junctional face membrane of terminal cisternae (3, 21). The high molecular weight protein with M<sub>r</sub> of 360,000 is the diagnostic constituent. A similar protein with M<sub>r</sub> of 340,000 is also enriched in junctional cardiac SR (Fig. 2) (13). The amount of high molecular weight protein, the percentage of junctional face membrane, and the \( B_{max} \) for the high affinity ryanodine binding are lower in junctional SR from heart as compared with skeletal muscle. The \( B_{max} \) is 5.1 versus 20 pmol/mg for heart and skeletal muscle junctional SR, respectively (this paper and (3)). Two differences exist between cardiac and skeletal muscle SR. 1) Cardiac SR are more sensitive (~7-fold) to ryanodine than skeletal muscle SR, even though for SR from both sources the ryanodine binding (\( K_a \)) and the pharmacological effect of low concentrations of ryanodine (\( K_a \)) are comparable (this study and Ref. 2). 2) The low affinity ryanodine binding was not observed in skeletal muscle SR (2, 20).

The pharmacological action of ryanodine on whole muscle appears to differ in heart versus skeletal muscle. Vertebrate
skeletal muscle responds to ryanodine with a slow irreversible contracture, whereas cardiac muscle responds with a progressive decline in contractile force (22, 23). It has also been noted that cardiac muscle is more sensitive to ryanodine \((10^{-7} \text{ to } 10^{-8} \text{ M})\) than skeletal muscle \((10^{-6} \text{ to } 10^{-5} \text{ M})\) (22). Since the major site of ryanodine action in muscle cells is believed to be in SR (24), the latter phenomenon can be explained in part by the different affinity of ryanodine binding in cardiac SR \((K_d \sim 8 \text{ nM})\) versus skeletal muscle SR \((K_d \sim 50 \text{ nM})\) (3) which corresponds to the pharmacologically greater sensitivity of ryanodine by isolated junctional cardiac SR than that in skeletal muscle SR \((K_i \sim 7 \text{ nM} \text{ versus } K_i \sim 50 \text{ nM})\) (this study and Ref. 2). However, the different affinities do not explain the basis for the different pharmacological actions in cardiac and skeletal muscle. The explanation may be related to the basic difference in the pools of the calcium fluxes which regulate muscle contraction and relaxation. In skeletal muscle, the \(Ca^{2+}\) is cycled between SR and the myoplasm, whereas in heart \(Ca^{2+}\) is also cycled in and out of the cell. The toxic action of ryanodine causes the \(Ca^{2+}\) not to remain in the SR. Thus, in skeletal muscle, the \(Ca^{2+}\) remains in the myoplasm, inducing contractures, whereas in heart it is extruded out of the cell resulting in reduced contractile force.

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