Rapid Calcium Release from Cardiac Sarcoplasmic Reticulum Vesicles Is Dependent on Ca$^{2+}$ and Is Modulated by Mg$^{2+}$, Adenine Nucleotide, and Calmodulin

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A subpopulation of canine cardiac sarcoplasmic reticulum vesicles has been found to contain a "Ca$^{2+}$ release channel" which mediates the release of intravesicular Ca$^{2+}$ stores with rates sufficiently rapid to contribute to excitation-contraction coupling in cardiac muscle. Ca$^{2+}$ release behavior of passively and actively loaded vesicles was determined by Millipore filtration and with the use of a rapid quench apparatus using the two Ca$^{2+}$ channel inhibitors, Mg$^{2+}$ and ruthenium red. At pH 7.0 and 5-20 mM external Ca$^{2+}$, cardiac vesicles released half of their 45Ca$^{2+}$ stores within 20 ms. Ca$^{2+}$-induced Ca$^{2+}$ release was inhibited by raising and lowering external Ca$^{2+}$ concentration, by the addition of Mg$^{2+}$, and by decreasing the pH. Calmodulin reduced the Ca$^{2+}$-induced Ca$^{2+}$ release rate 3–6-fold in a reaction that did not appear to involve a calmodulin-dependent protein kinase. Under various experimental conditions, ATP, and the nonhydrolyzable ATP analog, adenosine 5′-(β,γ-methylene)triphosphate (AMP-PCP), and caffeine stimulated 45Ca$^{2+}$ release 2–500-fold. Maximal release rates (t$\_50$ = 10 ms) were observed in media containing 10 mM Ca$^{2+}$ and 5 mM AMP-PCP or 10 mM caffeine. An increased external Ca$^{2+}$ concentration (≥1 mM) was required to optimize the 45Ca$^{2+}$ efflux rate in the presence of 8 mM Mg$^{2+}$ and 5 mM Ca$^{2+}$. These results suggest that cardiac sarcoplasmic reticulum contains a ligand-gated Ca$^{2+}$ channel that is activated by Ca$^{2+}$, adenine nucleotide, and caffeine and inhibited by Mg$^{2+}$, H$^+$, and calmodulin.

EXPERIMENTAL PROCEDURES

Materials—Troponin inhibitor protein (Tnl) was the generous gift of Dr. James Potter (University of Miami Medical School, FL). [3H]Ryoadinone was kindly provided by Dr. John Setkog (University of Texas Health Sciences Center at Dallas, TX). Ca$^{2+}$ was purchased from ICG Pharmaceuticals, Irvine, CA; ruthenium red from Fluka, Hauppauge, NY; and ATP, the ATP analog, AMP-PCP, and calmodulin from Sigma. All other materials were of reagent grade.

Preparation of Membranes—Cardiac muscle SR vesicles were isolated from the ventricular tissue of dogs. Animals were anesthetized with a sublethal dose of thuyamyl (30 mg/kg) followed by removal of the heart via median sternotomy. Excised hearts were immediately placed in ice-cold 0.3 M sucrose solution. Muscle was minced and placed in ice-cold 0.3 M sucrose, 1 mM diisopropyl fluoride, 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM EGTA, 5 mM ATP, 20 mM K/Hepes, pH 7.4, at 4 °C for 60 s in a Waring blender. The homogenate was centrifuged for 20 min at 7,500 rpm (9,200 × g) in a GSA rotor in a Sorvall RC-2 centrifuge. A crude microsomal fraction was obtained from the supernatant by centrifugation for 60 min at 33,000 rpm (90,000 × g) in Beckman Type 50 or TI-45 rotors. The pellets were resuspended in approximately 15 ml of 0.3 M sucrose containing 0.4 M KCl, 0.1 mM MgCl$_2$, 100 μM CaCl$_2$, 100 μM EGTA, and 5 mM K/Hepes, pH 6.8, quickly frozen, and stored at -135 °C.

The crude microsomal fraction was further subfractionated on a sucrose gradient as follows. The membranes derived from three dog hearts were rapidly thawed and placed in a Beckman Ti-14 zonal
**Ca**<sup>2+</sup> Release by Cardiac Sarcomplasmic Reticulum

rotor on top of a linear 20-45% (w/w) sucrose gradient containing 0.4 M KCl, 0.1 mM MgCl<sub>2</sub>, 100 mM EGTA, 100 μM CaCl<sub>2</sub> and 5 mM K/PIPES, pH 6.8. After centrifugation for 24 min at 40,000 rpm, membranes sedimenting between 19-25% (Fraction I), 25-28% (Fraction II), 28-31% (Fraction III), 31-34% (Fraction IV), and 34-40% (Fraction V) were collected, diluted to 1.5 volumes of 0.4 M KCl, and sedimented by centrifugation for 60 min at 30,000 rpm in Beckman Type 35 or Ti-45 rotors. The pellets were resuspended at a protein concentration of 5-10 mg/ml in 0.5 M sucrose, 5 mM K/PIPES, pH 6.8, quickly frozen, and stored at -15°C before use.

Heavy rabbit skeletal muscle SR Ca<sup>2+</sup> release vesicle fractions were recovered from the 30-45% region of a sucrose gradient that contained membranes sedimenting at 2,600-35,000 x g (13).

**[Ca]<sup>2+</sup> Flux Measurements with Passively Loaded Vesicles—**Ca<sup>2+</sup> efflux from vesicles passively loaded with Ca<sup>2+</sup> was determined with the use of an Update System 1000 Chemical Quench apparatus (Madison, WI) and by Millipore filtration (13, 15). Unless otherwise indicated, vesicles (2-10 mg of protein/ml) were passively loaded for 60 min at 23°C in a medium containing 1.1 mM CaCl<sub>2</sub>, 100 μM EGTA, 100 mM KCl, 1 mM diisopropyl fluorophosphate, and 20 mM K/PIPES, pH 7.0. 45Ca<sup>2+</sup> efflux behavior of the vesicles was measured by diluting vesicles 5-30-fold into iso-osmotic unlabeled release media containing varying concentrations of free Ca<sup>2+</sup>, adenosine nucleotide, and Mg<sup>2+</sup>. In the rapid quench experiments, 45Ca<sup>2+</sup> efflux was inhibited at time intervals ranging from 25 to 1600 sec by the addition of 10 mM Mg<sup>2+</sup>, 5 mM EGTA, and 10 μM ruthenium red (final concentrations). Extravesicular 45Ca<sup>2+</sup> was separated by placing the vesicles on 0.45-μm Millipore filters followed by rapid rinsing with 100 mM KCl, 10 mM Mg<sup>2+</sup>, 0.1 mM EGTA, 100 μM ruthenium red, 20 mM K/PIPES, pH 7.0. Radioactivity retained by the filters on the filters was determined by liquid scintillation counting.

45Ca<sup>2+</sup> flux measurements were carried out at least in duplicate with two or more time points. For a given preparation the standard errors were ±15% or less.

**Incubation of Passively Loaded Vesicles with Calmodulin—**Vesicles (1 mg of protein/ml) were initially incubated for 10 min at 34°C in a medium containing 20 mM K/PIPES, pH 7.0, 0.1 M KCl, 100 μM Ca<sup>2+</sup>, 100 μM EGTA, 1 mM Mg<sup>2+</sup>, 10 mM KCl, and 0.1 M EGTA. Vesicles were diluted 100-fold into a medium containing 10 mM Mg<sup>2+</sup> and 10 μM ruthenium red (Fig. 1A). This allows one to determine the amounts of 45Ca<sup>2+</sup> (22 nmol/mg protein) trapped by the vesicles in the incubation medium. When vesicles were diluted into a medium which contained 10 μM free Ca<sup>2+</sup>, about half of this amount was released in less than 30 s. Most of the 45Ca<sup>2+</sup> remaining with the vesicles in the 10 μM Ca<sup>2+</sup> release medium was present in a releasable form, since less than 1 nmol of 45Ca<sup>2+</sup>/mg protein was retained when the Ca<sup>2+</sup>-ionophore A23187 (2 μg/ml) was present. Consequently, Fraction IV appeared to be made up of two subpopulations of vesicles, only one of which was capable of rapidly releasing its 45Ca<sup>2+</sup> stores in the 10 μM Ca<sup>2+</sup> medium. Among the five fractions, the 31-40% sucrose gradient fractions (Fractions IV and V) contained the highest percentage of vesicles displaying Ca<sup>2+</sup> release activity (Table I). In comparison, the activity of the Ca<sup>2+</sup>-ATPase, measured in the

### RESULTS

**Isolation of Cardiac Ca**<sup>2+</sup> Release Vesicles—A crude microsomal fraction of canine cardiac muscle was separated into five fractions on a sucrose gradient (Table I). The Ca<sup>2+</sup> release activities of the five vesicle fractions were determined as outlined for Fraction IV in Fig. 1. Vesicles were passively loaded with 1 mM Ca<sup>2+</sup> by incubation for 60 min at 23°C and then either diluted into a medium which inhibited or activated the Ca<sup>2+</sup> release channel of cardiac SR, 45Ca<sup>2+</sup> efflux was slow when the vesicles were placed into a medium containing 10 mM Mg<sup>2+</sup> and 10 μM ruthenium red (Fig. 1A); This allows one to determine the amounts of 45Ca<sup>2+</sup> (22 nmol/mg protein) trapped by the vesicles in the incubation medium. When vesicles were diluted into a medium which contained 10 μM free Ca<sup>2+</sup>, about half of this amount was released in less than 30 s. Most of the 45Ca<sup>2+</sup> remaining with the vesicles in the 10 μM Ca<sup>2+</sup> release medium was present in a releasable form, since less than 1 nmol of 45Ca<sup>2+</sup>/mg protein was retained when the Ca<sup>2+</sup>-ionophore A23187 (2 μg/ml) was present. Consequently, Fraction IV appeared to be made up of two subpopulations of vesicles, only one of which was capable of rapidly releasing its 45Ca<sup>2+</sup> stores in the 10 μM Ca<sup>2+</sup> medium. Among the five fractions, the 31-40% sucrose gradient fractions (Fractions IV and V) contained the highest percentage of vesicles displaying Ca<sup>2+</sup> release activity (Table I). In comparison, the activity of the Ca<sup>2+</sup>-ATPase, measured in the

### Table I

| Density (%) sucrose | I | II | III | IV | V |
|---------------------|---|----|-----|----|---|
| 19-25               | 10 ± 2.5 | 14 ± 4 | 21 ± 4 | 17 ± 8 | 28 ± 12 |
| 25-28               | 20 ± 7   | 24 ± 3 | 31 ± 15| 34 ± 10|
| 28-31               | 25 ± 10  | 30 ± 8 | 36 ± 12| 50 ± 15|
| 31-34               | 27 ± 12  | 30 ± 10| 31 ± 13| 40 ± 15|
| 34-40               | 28 ± 15  | 30 ± 12| 32 ± 14| 41 ± 15|

### Enzymatic and Ca<sup>2+</sup> release properties of sucrose gradient membrane fractions of canine cardiac muscle

Canine cardiac membranes were subsectioned into five membranous fractions on a sucrose gradient (cf. "Experimental Procedures"). Ca<sup>2+</sup> release properties of vesicles passively loaded with 1 mM Ca<sup>2+</sup> were determined as described in the legend to Fig. 1. The total amount of 45Ca<sup>2+</sup> trapped by the vesicles was determined by diluting vesicles 100-fold into a release medium containing 10 mM Mg<sup>2+</sup> plus 10 μM ruthenium red. The percentage of 45Ca<sup>2+</sup> release indicates the portion of trapped 45Ca<sup>2+</sup> that was rapidly released in the 10 μM free Ca<sup>2+</sup> medium. Ca<sup>2+</sup>-ATPase activity was determined as described under "Experimental Procedures." The data are the average of five preparations ± S.E.

| Density (%) sucrose | I | II | III | IV | V |
|---------------------|---|----|-----|----|---|
| 19-25               | 29 ± 6  | 30 ± 5 | 25 ± 4 | 22 ± 4 | 17 ± 4 |
| 25-28               | 26 ± 6  | 30 ± 5 | 25 ± 4 | 22 ± 4 | 17 ± 4 |
| 28-31               | 27 ± 6  | 30 ± 5 | 25 ± 4 | 22 ± 4 | 17 ± 4 |
| 31-34               | 28 ± 6  | 30 ± 5 | 25 ± 4 | 22 ± 4 | 17 ± 4 |
| 34-40               | 29 ± 6  | 30 ± 5 | 25 ± 4 | 22 ± 4 | 17 ± 4 |

**[H]ryanodine Binding—**Cardiac vesicles (0.5 mg of protein in 0.25 ml) were incubated at 37°C in a medium containing 20 mM K/PIPES, pH 7.0, 0.1 M KCl, 1 mM diisopropyl fluorophosphate, 0.1 mM EGTA, 0.1 mM Ca<sup>2+</sup>, and [H]ryanodine (specific activity, 60.8 Ci/mmol). After 2 and 4 h, an aliquot of the vesicles (0.2 mg of protein) was sedimented by centrifugation for 30 min at 90,000 × g in a Beckman Airfuge. Pellets were briefly washed at 4°C and radioactivity associated with the vesicles and radioactivity remaining in the supernant were determined by liquid scintillation counting to obtain bound and free [H]ryanodine ([H]ryanodine was added to a concentration of 5 nM, greater concentrations were prepared as admixtures of labeled and unlabeled ryanodine.

**Biochemical Assays—**Protein was determined by the Lowry method using bovine serum albumin as a standard. "Basic" and Mg<sup>2+</sup>-dependent, Ca<sup>2+</sup>-stimulated ATPase activities were determined in the presence of the ionophore A23187 (2 μg/ml) at 32°C as previously described (13). Free Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations were calculated according to a computer program using binding constants published by Fabiato (19); free divalent cation concentrations in the presence of AMP-PCP (10) were estimated using the ATP binding constants published by Fabiato.
Effective inhibition of ⁴⁶Ca²⁺ release from cardiac vesicles required the presence of both Mg²⁺ and ruthenium red in the quench solution. Omission of either 10 μM ruthenium red or 10 μM Mg²⁺ from the channel inhibiting medium resulted in incomplete inhibition of rapid ⁴⁶Ca²⁺ efflux. Release of more than one-half of the ⁴⁶Ca²⁺ stores in the Ca²⁺-permeable vesicle population occurred within 30 s, i.e. when the first time point was taken using the Millipore filtration technique. In the presence of adenine nucleotide, an additional requirement was that the free Ca²⁺ concentration was decreased to below 10⁻⁷ M Ca²⁺ during the quench step. This was achieved by including in the quench solution the Ca²⁺ chelating agent EGTA.

Fig. 1A shows that an appreciable fraction of the rapidly releasable ⁴⁶Ca²⁺ remained with the vesicles when ⁴⁶Ca²⁺ efflux was inhibited at 50 and 140 ms by the addition of the two channel inhibitors, Mg²⁺ and ruthenium red. In Fig. 1B, rapid ⁴⁶Ca²⁺ efflux was stopped at time intervals ranging from 25 to 500 ms. In the 10 μM Ca²⁺ medium, the Ca²⁺ releasing vesicle released its ⁴⁶Ca²⁺ stores with a half-time of about 25 ms. ⁴⁶Ca²⁺ efflux was slowed about 5-fold by the addition of 0.7 mM Mg²⁺ to the 10 μM Ca²⁺ medium. In the presence of 10 μM Ca²⁺ and 5 mM Mg²⁺-AMP-PCP (0.7 mM free Mg²⁺), ⁴⁶Ca²⁺ was released with a rate comparable to that in the Mg²⁺- and nucleotide-free release medium. Taken together, the data of Fig. 1 suggest that cardiac sarcoplasmic reticulum vesicles contain a Ca²⁺ release channel, which in the presence of micromolar external Ca²⁺, mediates the release of Ca²⁺ with rates sufficiently rapid to contribute to excitation-contraction coupling. This channel appears to be inhibited by Mg²⁺ and activated by adenine nucleotides.

In Fig. 2, vesicles were passively loaded for varying times with 1 mM ⁴⁶Ca²⁺. After incubation of 1 min to 4 h at 23 °C, vesicles were diluted into Ca²⁺ release blocking medium to estimate the amounts of ⁴⁶Ca²⁺ that were accumulated by all vesicles. Dilution into the Ca²⁺ release promoting medium was used to determine the fraction of vesicles that lacked the Ca²⁺ release channel. Fig. 2 shows that ⁴⁶Ca²⁺ influx was a rapid process with a half-time of about 2 min. The difference presence of the ionophore A23187, was highest in the 19–28% sucrose gradient fractions (Fractions I and II).

The time course of the rapid release phase in Fig. 1A was determined by inhibiting ⁴⁶Ca²⁺ efflux at short time intervals by the addition of Mg²⁺ and ruthenium red using a rapid quench apparatus (15). In preliminary experiments it was found that the cardiac channel was less sensitive than the skeletal channel to inhibition by Mg²⁺ and ruthenium red.

![Fig. 1. Measurement of ⁴⁶Ca²⁺ efflux. A, cardiac SR vesicles (5 mg of protein/ml; Fraction IV in Table I) were passively loaded with 1 mM ⁴⁶Ca²⁺ as described under "Experimental Procedures." ⁴⁶Ca²⁺ efflux was initiated by diluting vesicles 100-fold into release media containing either 10 mM Mg²⁺, 1 mM EGTA plus 10 μM ruthenium red (RR) (C) or 1 mM EGTA plus 0.95 mM Ca²⁺ (10 μM free Ca²⁺ after the addition of the vesicles) (D). ⁴⁶Ca²⁺ efflux was terminated by placing vesicles on 0.45-μ Millipore filters and rinsed with a free Ca²⁺ medium as well as amounts not readily released in the 10 μM Ca²⁺ release medium (10 nmol/mg of protein) were obtained by back extrapolation to the time of vesicle dilution. B, rapid initial ⁴⁶Ca²⁺ efflux rates were determined with the use of an Update System 1000 Chemical Quench apparatus (15). Vesicles (2 mg of protein/ml) passively loaded with 1 mM ⁴⁶Ca²⁺ were diluted with 4 volumes of release medium containing 0.5 mM EGTA, 0.24 mM Ca²⁺ and 0 (O) or 0.875 mM Mg²⁺ (△). ⁴⁶Ca²⁺ release in the presence of 5 mM Mg²⁺ and 5 mM nucleotide was measured by adding 4 volumes of 10.4 mM Mg²⁺ plus 11.25 mM AMP-PCP in a second mixing step at 72 ms to 10 μM Ca²⁺, 0.7 mM Mg²⁺ release medium (D). Rapid ⁴⁶Ca²⁺ efflux was inhibited at the indicated times (50 and 140 ms in A; 25–500 ms in B) by the addition of 4 volumes of a quench solution containing Mg²⁺, EGTA, and ruthenium red (final concentrations 10 mM, 5 mM and 10 μM, respectively). Vesicles were subsequently placed on 0.45-μ Millipore filters and rinsed with a medium containing 10 mM Mg²⁺, 0.1 mM EGTA, and 10 μM ruthenium red. The amount of ⁴⁶Ca²⁺ remaining in the vesicles was determined by back extrapolation to the time of addition of the quench solution.

FIG. 2. Dependence of ⁴⁶Ca²⁺ retention by the vesicles on incubation time. Cardiac SR vesicles (Fraction IV in Table I) were incubated for the indicated times with 1 mM ⁴⁶Ca²⁺. Vesicles were then diluted 200-fold into media containing 10 mM Mg²⁺, 1 mM EGTA, and 10 μM ruthenium red (RR) (O) or 1 mM EGTA and 0.97 mM Ca²⁺ (10 μM free Ca²⁺ after the addition of the vesicles) (C). Amounts of ⁴⁶Ca²⁺ retained by the vesicles were obtained by back extrapolation to the time of vesicle dilution (cf. Fig. 1A). The lower curve indicates the amount of ⁴⁶Ca²⁺ rapidly released in the 10 μM free Ca²⁺ medium ( }).
in the two uptake curves, i.e. the amount of \(^{45}\text{Ca}^2+\) that was released in the 10 \(\mu\text{M}\ \text{Ca}^2+\) medium, remained fairly constant with time, suggesting that a similar number of vesicles containing viable \(\text{Ca}^2+\) release channels were present during the 4-h incubation period. Comparison of the release activity of passively and actively loaded vesicles (see below) also suggested that the \(\text{Ca}^2+\) permeability of the vesicles remained largely unaltered during incubation for 1–2 h at 23 °C. In other experiments it was found that the \(\text{Ca}^2+\) pump mediated \(\text{Ca}^2+\) loading rate of the vesicles (0.5 \(\mu\text{mol}/\text{mg of protein}/\text{min}\) was independent of the time of vesicle incubation (not shown).

\[^3\text{H}\]Ryanodine binding and \(^{45}\text{Ca}^2+\) flux measurements have indicated that ryanodine specifically binds to the skeletal and cardiac SR Ca\(\text{^2+}\) release channels (20–22). Cardiac preparations have been reported by Pessah et al. (20) to bind 0.5 pmol of ryanodine/mg of protein with a \(K_D\) of 36 nM and 1.7 pmol/mg of protein with a \(K_D\) of 340 nM. The Ca\(\text{^2+}\) releasing membrane fractions obtained in this study (Fractions IV and V of Table I) contained an appreciably higher concentration of specific high-affinity ryanodine binding sites (9 ± 3 pmol/mg of protein). Scatchard plot analysis indicated the presence of a single high-affinity receptor site with a \(K_D\) of about 7 nM (Fig. 3). Specific high-affinity \[^3\text{H}\]ryanodine binding to membranes displaying a low Ca\(\text{^2+}\) release activity (Fraction I of Table I) amounted to 2–3 pmol/mg of protein (not shown).

**Inhibition of \(^{45}\text{Ca}^2+\) Release—Ca\(\text{^2+}\)-induced \(^{45}\text{Ca}^2+\) release is partially inhibited by 1 \(\mu\text{M}\) ruthenium red, 1 \(\mu\text{M}\) neomycin, and 50 \(\mu\text{M}\) tetracaine (Fig. 4). Preincubation of the vesicles with the inhibitors was required to observe the extent of inhibition seen in Fig. 4. Without prior exposure, a large fraction of the intravesicular \(^{45}\text{Ca}^2+\) was rapidly released before the three compounds were capable of exerting their inhibitory effects.

The effect of varying external Mg\(\text{^2+}\) concentration on \(^{45}\text{Ca}^2+\) release is shown in Fig. 5. Ca\(\text{^2+}\)-induced \(^{45}\text{Ca}^2+\) efflux was half-maximally inhibited at \(3 \times 10^{-3} \text{M} \text{Mg}^2+\). In the presence of 10 \(\mu\text{M}\ \text{Mg}^2+\) the \(t_s\) of \(^{45}\text{Ca}^2+\) release increased to about 1 s. Analysis of the efflux data in the form of a Hill plot yielded an apparent \(n\) value of 1.5 for \(\text{Mg}^2+\) inhibition of \(^{45}\text{Ca}^2+\) release (not shown). A Hill coefficient of 1.5 is in accord with the existence of multiple interacting Mg\(\text{^2+}\) binding sites. Due to the complexity of the system, other explanations cannot, however, be ruled out at present.

**Dependence of the \(^{45}\text{Ca}^2+\) efflux rate on Mg\(\text{^2+}\) concentration.** Cardiac SR vesicles (2 mg of protein/ml) passively loaded with 1 mM \(^{45}\text{Ca}^2+\) were diluted into media containing 10 \(\mu\text{M}\) free \(\text{Ca}^2+\) and the indicated concentrations of Mg\(\text{^2+}\). \(^{45}\text{Ca}^2+\) efflux rates from the Ca\(\text{^2+}\)-permeable vesicle population were determined as indicated in the legend to Fig. 1.
were treated with D-glucose and hexokinase and passively loaded with 1 mM $^{45}$Ca²⁺ in the absence (O) or presence of 6 μM calmodulin (C) or 1.2 μM calmodulin and 5 μM free Ca²⁺ (E, F) or 10 mM Mg²⁺, 1 mM EGTA plus 10 μM ruthenium red (RR). (*), $^{45}$Ca²⁺ release rates from the Ca²⁺-permeable vesicle fraction in the 5 μM Ca²⁺ release medium were determined as indicated in the legend to Fig. 1.

**TABLE II**

**Effects of troponin inhibitor protein and calmodulin on the Ca²⁺ release behavior of cardiac vesicles**

Cardiac SR vesicles (2 mg/ml; Fraction V in Table I) were treated with D-glucose and hexokinase and passively loaded with 1 mM $^{45}$Ca²⁺ in the presence of the indicated concentrations of TnI and calmodulin as described under "Experimental Procedures." After the addition of vesicles, release media contained 0 (O, •) or 1.2 μM (E, F) calmodulin and 5 μM free Ca²⁺ (E, F) or 10 mM Mg²⁺, 1 mM EGTA plus 10 μM ruthenium red (RR) (•). $^{45}$Ca²⁺ release rates from the Ca²⁺-permeable vesicle fraction in the 5 μM Ca²⁺ release medium were determined as indicated in the legend to Fig. 1.

| Additions to vesicle medium | $^{45}$Ca²⁺ efflux |
|----------------------------|-------------------|
| None                       | 105               |
| 1 μM calmodulin            | 24                |
| 5 μM TnI, 1 μM calmodulin  | 21                |

and absence of calmodulin suggested that the effect of calmodulin was to slow down the release of $^{45}$Ca²⁺ from all Ca²⁺-release vesicles rather than to inhibit a selected fraction of the vesicles. Variation of the calmodulin concentration in vesicle and release media indicated that the Ca²⁺ release rate was half-maximally reduced at 0.1–0.2 μM calmodulin and maximally reduced at a calmodulin concentration of 1–3 μM (not shown). For comparison, the calmodulin concentration of heart has been estimated to be 2–3 μM (24).

Cardiac vesicles were incubated with the calmodulin-binding component of TnI²⁺ to assess possible inhibition of $^{45}$Ca²⁺ release by an impurity present in the commercial preparation of calmodulin or by endogenous calmodulin. Table II shows that TnI was effective in neutralizing the inhibitory effect of 1 μM calmodulin in that it maintained the release rate at a value seen in the absence of added calmodulin. Inhibition of the channel by another component present in the calmodulin preparation appears therefore to be unlikely. In the absence of added calmodulin, TnI increased the release rate of cardiac vesicles by about 15%. This result raises the possibility that isolated cardiac vesicles contain small amounts of calmodulin which inhibit the channel.

**pH Dependence of $^{45}$Ca²⁺ Release and Exchange**—The pH dependence of $^{45}$Ca²⁺ efflux from cardiac vesicles was measured in a medium containing 5 μM or 1 mM free Ca²⁺ (Fig. 7). In the presence of a maximally activating Ca²⁺ concentration of 5 μM (see below), vesicles released half their $^{45}$Ca²⁺ within 20 ms at pH 7.0. At pH 6.0, $^{45}$Ca²⁺ release was about 50-fold reduced, occurring with a half-time of 1 s. Cardiac vesicles were equilibrated with 1 mM $^{45}$Ca²⁺ by incubating for 1 h (Fig. 2). An increase in external Ca²⁺ concentration to 1 mM in the release medium therefore allowed measurement of the Ca²⁺ release behavior of the vesicles under $^{45}$Ca²⁺-$^{40}$Ca²⁺ exchange conditions. Calcium exchange proceeded with about a 5-fold lower rate than $^{45}$Ca²⁺ release at pH values below 7. The calcium exchange rate steadily increased from pH 6 to 8, without appearing to reach a maximal value at pH 8.

**Activation of $^{45}$Ca²⁺ Release by Ca²⁺, Nucleotides, and Caffeine**—The $^{45}$Ca²⁺ release behavior of canine cardiac vesicles has been studied in the presence of Ca²⁺, ATP (AMP-PCP), and caffeine, and compared with the release behavior of rabbit skeletal muscle SR Ca²⁺ release vesicles (Table III). At pH 7.0 in 10⁻⁶ M free Ca²⁺, $^{45}$Ca²⁺ release was slow, requiring a $t_{1/2}$ of 25 and 8 s for cardiac and skeletal vesicles, respectively. An increase in free Ca²⁺ to 2 μM and addition of the nonhydrolyzable ATP analog AMP-PCP increased the release rate by a factor of about 1000 in both preparations. In the presence of micromolar Ca²⁺ and millimolar nucleotide, nearly optimal release rates were observed since the cardiac and skeletal channels are essentially open all the time, as shown previously in single channel measurements (6, 17). Intermediate release rates were obtained when the two channels were activated at 10⁻⁶ M Ca²⁺ by either ATP or caffeine or by 10⁻⁴ M Ca²⁺ alone.

**Fig. 7. Dependence of the $^{45}$Ca²⁺ efflux rate on pH**. Cardiac SR vesicles (Fraction V in Table I) were passively loaded with 1 mM $^{45}$Ca²⁺ by incubating vesicles for 60 min at 23°C in a medium containing 0.1 M KCl and either 20 mM K/Pipes (pH 6.0–7.0) or 20 mM K/Hepes (pH 7.0–8.0). Vesicles were diluted into media containing 5 μM (O) or 1 mM free (O) Ca²⁺, after the addition of the vesicles. $^{45}$Ca²⁺ efflux rates from the Ca²⁺-permeable vesicle fraction were determined as described in the legend to Fig. 1.
Several important differences were noted to exist between cardiac and skeletal SR. At 10^{-5} M external Ca^{2+}, the t_{0.9} of 45Ca^{2+} release was 20 ms for cardiac vesicles, as compared to 600 ms for skeletal vesicles. Caffeine mimicked the effects of external Ca^{2+} in that it was more effective in stimulating 45Ca^{2+} release from cardiac than skeletal vesicles in the low Ca^{2+} medium. At micromolar Ca^{2+}, 10 mM caffeine, like 5 mM nucleotide, optimally stimulated 45Ca^{2+} efflux from cardiac vesicles, whereas in skeletal vesicles it was without a significant effect. In contrast, adenine nucleotides were more effective in stimulating 45Ca^{2+} release from skeletal vesicles than from cardiac SR. At 10^{-4} M external Ca^{2+}, the 45Ca^{2+} release was 20 ms for cardiac vesicles, as compared to 10^{-2} fold increase from skeletal vesicles. Combination of Mg^{2+} and ATP greatly reduced the 45Ca^{2+} efflux, whereas in skeletal vesicles it was without a significant effect. In contrast, adenine nucleotides were more effective in stimulating 45Ca^{2+} release from skeletal than cardiac vesicles. Addition of 5 mM ATP to the 10^{-3} M Ca^{2+} medium was without an appreciable effect on Ca^{2+} release from cardiac vesicles. Similarly, 5 mM inositol 1,4,5-triphosphate, another potential activator of the Ca^{2+} release channel (25-27), did not increase 45Ca^{2+} efflux when added to the 10^{-9} M Ca^{2+} release medium (not shown).

Ca^{2+} Dependence of Ca^{2+} Release—We have attempted to mimic the ionic conditions in relaxed and contracted muscle by determining the Ca^{2+} dependence of Ca^{2+} release from cardiac vesicles in the presence and absence of Mg^{2+} and the nonhydrolyzable ATP analog AMP-PCP. Canine myocardium contains about 5 mM ATP (28). The free Mg^{2+} concentration of cardiac muscle is not known but has been estimated to range from about 0.2 to 4 mM in skeletal muscle (29, 30). In the absence of Mg^{2+} and nucleotide, a maximal release rate was measured in media containing about 5-20 μM free Ca^{2+} (Fig. 8). 45Ca^{2+} release was slow at nanomolar Ca^{2+} and was severalfold reduced at millimolar concentrations of Ca^{2+}. Effects of external Ca^{2+} on 45Ca^{2+} efflux in Fig. 8 can be most easily explained by assuming that the channel possesses activating and inhibitory Ca^{2+} binding sites. Adenine nucleotide modified Ca^{2+}-induced Ca^{2+} release from cardiac SR in two ways. One effect of AMP-PCP was to increase the maximally observable Ca^{2+} release rate. In Fig. 8, the t_{0.9} of 45Ca^{2+} release was decreased from about 25 to 15 ms by 5 mM AMP-PCP. A second effect of AMP-PCP was to shift the Ca^{2+} activating curve to the left by lowering the half-maximally activating Ca^{2+} concentration from about 2 × 10^{-6} M to 8 × 10^{-7} M. Variation of AMP-PCP concentration at 3 × 10^{-7} M Ca^{2+} indicated that a high concentration of nucleotide (5-10 mM range) was required to optimally stimulate 45Ca^{2+} release. At 3 × 10^{-7} M Ca^{2+}, the 45Ca^{2+} efflux rate was half-maximally accelerated by 1-2 mM AMP-PCP (not shown).

Mg^{2+} affected the channel in an opposite manner. Ca^{2+}-
vesicles were rinsed 10 mM during rinsing with the 10 mM 45Ca2+ was released from vesicles that were actively lowered from about 1 to 0.1 μM, because the cardiac Ca2+ stores from passively loaded vesicles. At least two lines of evidence suggest that the channel is localized in sarcoplasmic reticulum as opposed to another cellular structure. First, passively loaded "release" vesicles from cardiac membranes demonstrated 45Ca2+ release behavior similar to vesicles actively loaded via the SR Ca2+ pump. Second, Ca2+ release from cardiac vesicles qualitatively resembled Ca2+ release from heavy skeletal muscle SR vesicles. Both channels were activated by external Ca2+ and adenine nucleotide and inhibited by Mg2+ and calmodulin. Another argument strongly favoring localization of the Ca2+ channel in the SR membrane is that Ca2+ release from skeletal and cardiac SR vesicle fractions used in this study was similarly affected by the plant alkaloid ryanodine (22).

The specificity of 45Ca2+ uptake and release was assessed by actively loading vesicles in the presence of 0.1 μM NaCl and by preincubation with 1 μM ryanodine. In the presence of 0.1 mM NaCl, 45Ca2+ uptake by sarcosomal vesicles is minimal due to the immediate release of any accumulated Ca2+ through the Na+/Ca2+ exchange system (32). In the experiments of Fig. 9, substitution of 0.1 mM KCl by 0.1 mM NaCl did not significantly reduce the amount of 45Ca2+ retained by vesicles loaded in the presence of 0.3 μM free Ca2+ and rinsed with the channel inhibiting medium, suggesting that 45Ca2+ uptake by contaminating sarcosomal vesicles was negligible. Low concentrations of ryanodine "open" the cardiac Ca2+ release channel and thereby render passively loaded vesicles permeable to Ca2+ in Ca2+ release channel activating and inhibiting media (22). In actively loaded vesicles, preincubation with 1 μM ryanodine for 5 min at 37 °C reduced 1.5-2-fold the amount of 45Ca2+ that was retained by vesicles loaded at 0.3 μM free Ca2+ and rinsed with the channel inhibiting medium. Only a small fraction of the sequestered 45Ca2+ was released when ryanodine-treated vesicles were rinsed with channel activating medium, suggesting that ryanodine rendered Ca2+ release channel containing vesicles permeable to 45Ca2+. Possible Ca2+ uptake by contaminating mitochondria was blocked by actively loading vesicles in the presence of 5 mM NaN3, an inhibitor of the mitochondrial electron transport chain, and carbamyl cyanide N-chlorophenylhydrazine, a potent inhibitor of oxidative phosphorylation.

Data obtained with actively and passively loaded vesicles are in good general agreement. At free Ca2+ concentrations of 1 μM and more, Ca2+ release vesicles are incapable of sequestering significant amounts of 45Ca2+, because the cardiac Ca2+ release channel is sufficiently activated to cause the rapid release of the transported 45Ca2+. On the other hand, because of channel inactivation, the Ca2+ transport rate exceeds the release rate at submicromolar Ca2+ concentrations in the presence of Mg2+ and nucleotide, with the result that significant amounts of the transported 45Ca2+ are retained by Ca2+ release vesicles.

**DISCUSSION**

Results presented here show that a subpopulation of canine cardiac SR vesicles contains a ligand-gated Ca2+ release channel that mediates the rapid release of intravesicular Ca2+ stores from passively loaded vesicles. At least two lines of evidence suggest that the channel is localized in sarcoplasmic reticulum as opposed to another cellular structure. First, passively loaded "release" vesicles from cardiac membranes demonstrated 45Ca2+ release behavior similar to vesicles actively loaded via the SR Ca2+ pump. Second, Ca2+ release from cardiac vesicles qualitatively resembled Ca2+ release from heavy skeletal muscle SR vesicles. Both channels were activated by external Ca2+ and adenine nucleotide and inhibited by Mg2+ and calmodulin. Another argument strongly favoring localization of the Ca2+ channel in the SR membrane is that Ca2+ release from skeletal and cardiac SR vesicle fractions used in this study was similarly affected by the plant alkaloid ryanodine (22).

Ca2+-induced calcium release from passively and actively loaded cardiac vesicles has been previously observed. 45Ca2+ efflux was stimulated at micromolar Ca2+ and was sensitive to inhibition by ruthenium red (33-35). However, the release rates reported were several orders of magnitude slower than those observed in the present study. In a preliminary report, Ca2+ release peaked at 0.2 and 1 μM external Ca2+, suggesting the possible involvement of two components in the regulation of the cardiac Ca2+ release channel (36). The presence of a specific Ca2+ permeability mechanism in a subpopulation of cardiac SR vesicles has also been suggested in studies with the plant alkaloid ryanodine (20-22, 35, 37-40). This compound profoundly alters the Ca2+ uptake and release properties of isolated cardiac and skeletal SR vesicle fractions by specifically binding to the Ca2+ release channels.

Cardiac SR Ca2+ release vesicle fractions used in this study were prepared according to a protocol similar to the one used for the isolation of heavy SR junctional derived Ca2+ release vesicles from rabbit skeletal muscle (13). Canine ventricular tissue was homogenized in buffered 0.3 M sucrose solution and a Ca2+ release vesicle fraction was isolated in the presence of the protease inhibitor diisopropyl fluorophosphate by differential and sucrose gradient centrifugation. Between 40 and 65% of the vesicular Ca2+ could be rapidly released from the 30-40% sucrose gradient fractions, as compared to 70-90% from heavy skeletal muscle SR vesicles. [H]Ryanodine binding measurements indicated a similar number of specific high-affinity binding sites (7-14 pmol/mg protein) as reported for skeletal SR junctional derived vesicles (4-20 pmol/mg protein; Refs. 20 and 21). In contrast, Pessah et al. (20) obtained only 0.5 pmol of high-affinity binding sites/mg of protein using a purified cardiac SR preparation.

Ca2+ release behavior of skeletal muscle SR vesicles has been extensively studied using rapid flow and rapid quench techniques (11, 12, 14-16), while comparable detailed studies with cardiac SR vesicle fractions have not been previously reported to our knowledge. The nonlinear 45Ca2+ efflux curves on the semilog plots of this study indicate that Ca2+ release from cardiac vesicles is more difficult to evaluate by kinetic analysis than that from skeletal Ca2+ release vesicle fractions. The latter display a higher Ca2+ release activity and more importantly, appear to be made up of a fairly homogenous population of vesicles (41), with the result that 45Ca2+ efflux
can be reasonably well fitted by a single exponential function and thus approximated by first-order kinetics. Data of the present study nevertheless show that, in a qualitative manner, the cardiac and skeletal channels are similarly affected by Ca\(^{2+}\), Mg\(^{2+}\), H\(^+\), adenine nucleotide, and calmodulin. In both systems, regulation of the channel can be summarized by the equation shown below.

\[
\text{Ca}^{2+} + \text{ATP} \xrightarrow{\text{Mg}^{2+}, \text{H}^+} \text{open} \\
\text{Mg-ATP, calmodulin}
\]

The cardiac and skeletal channels are opened for intermittent times in the presence of micromolar calcium and millimolar adenine nucleotide. The presence of both ligands is required to keep the two channels open essentially all the time (6, 17). The equation also depicts Mg\(^{2+}\) and H\(^+\) as inhibitors of the channel.

In muscle, regulation of the channel is more complex than indicated by the above equation in that channel behavior is modulated by Mg-ATP and calmodulin (enclosed in box). Most of the ATP in muscle is present in the form of a Mg-ATP complex which modulates the Ca\(^{2+}\) dependence of 45Ca\(^{2+}\) release by skeletal (15) and cardiac (Fig. 8) SR vesicles. Furthermore, the rate of Ca\(^{2+}\) release from the Ca\(^{2+}\)-permeable subpopulation of skeletal (16) and cardiac (Fig. 6) SR vesicles is decreased by a factor of 2–6 by exogenously added calmodulin. Inhibition occurred in the absence of added ATP and therefore does not appear to involve a calmodulin-dependent phosphorylation reaction. Instead experiments with rabbit skeletal muscle SR vesicles have suggested that calmodulin slows down 45Ca\(^{2+}\) release by directly interacting in a rapid, reversible, Ca\(^{2+}\)-dependent manner with the channel (16). The physiological function of calmodulin in excitation-contraction is difficult to define at present. One proposal is that calmodulin, by partially inhibiting release of Ca\(^{2+}\) from SR, compensates for possible increases in the resting Ca\(^{2+}\) level during increased muscle activity.

Several important differences exist in the extent to which the cardiac and skeletal channels are activated by Ca\(^{2+}\) and adenine nucleotide and inhibited by Mg\(^{2+}\). Micromolar concentrations of Ca\(^{2+}\) stimulate cardiac 45Ca\(^{2+}\) release in the absence of Mg\(^{2+}\) and nucleotide to as much as 50% of the optimal rate, whereas only 1–3% of the optimal rate could be achieved in skeletal vesicles in the absence of nucleotide and Mg\(^{2+}\) (15). A similar discrepancy in the rates of 45Ca\(^{2+}\) release was observed for vesicles diluted into low Ca\(^{2+}\) media containing 10 mM caffeine (Table III). In contrast, adenine nucleotides were more effective in stimulating 45Ca\(^{2+}\) release from skeletal than from cardiac vesicles. This difference in effectiveness was particularly apparent at low Ca\(^{2+}\) concentrations. At 10–8 M Ca\(^{2+}\), ATP was minimally effective in activating the cardiac channel. By comparison, in skeletal muscle, 5 mM ATP was effective in stimulating 45Ca\(^{2+}\) release at 10–3 M Ca\(^{2+}\) to as much as 15% of the optimal rate (Table III). Another significant difference is that 45Ca\(^{2+}\) efflux from cardiac vesicles is less sensitive to Mg\(^{2+}\) inhibition than that from skeletal vesicles. Thus, in the presence of nucleotide, it was possible to observe at high concentrations of inhibitory Mg\(^{2+}\) (3 mM), vesicular 45Ca\(^{2+}\) release rates which would appear to be significant to excitation-contraction coupling (Fig. 8). In order to observe similar rapid release rates from skeletal vesicles, it was necessary to decrease the free Mg\(^{2+}\) concentration in the presence of nucleotide to 5 × 10–4 M or lower (15).

A Ca\(^{2+}\) concentration of 1 mM and higher was required to optimally activate the cardiac Ca\(^{2+}\) release channel in the presence of adenine nucleotide and Mg\(^{2+}\) (Fig. 8). This Ca\(^{2+}\) concentration is 2–3 orders of magnitude higher than the free Ca\(^{2+}\) concentration measured in contracting muscle (42), thus raising the question of whether cardiac SR in muscle is capable of rapid Ca\(^{2+}\)-induced Ca\(^{2+}\) release in the presence of physiological concentrations of nucleotide and Mg\(^{2+}\). It can be argued, however, that Ca\(^{2+}\) concentrations in excess of measured values may exist in the junctional area during surface membrane Ca\(^{2+}\) channel activation, considering that extracellular Ca\(^{2+}\) is 1–2 mM. In this regard it is of interest that recent sucrose gradient centrifugation studies with vesicle fractions have suggested co-migration of the dihydropyridine labeled cardiac surface membrane channel and the SR junctional feet (43). This would suggest, that as in skeletal muscle (3, 21, 35, 41), surface membrane and SR Ca\(^{2+}\) channels are in close proximity.

It has been proposed that physiological release of Ca\(^{2+}\) from sarcoplasmic reticulum is induced by a second messenger such as Ca\(^{2+}\) (3, 5) or inositol 1,4,5-triphosphate (25–27), "depolarization" of the SR membrane (3), changes in membrane surface charge (44, 45), or a pH gradient (46). Among these various putative effectors we have found Ca\(^{2+}\) to cause the most dramatic changes in the Ca\(^{2+}\) release behavior of cardiac SR vesicle fractions. In our bilayer (17) and vesicle flux experiments, we have been unable to demonstrate activation of the cardiac SR Ca\(^{2+}\) channel by inositol 1,4,5-triphosphate. Also, the cardiac channel displays only a weak voltage dependence in planar lipid bilayers (17). Furthermore, changes in membrane surface charge, brought about by the addition to nanomolar Ca\(^{2+}\) release medium of 20 μM tetracyclenohrom- or tetracylenohlorinum (45), did not stimulate Ca\(^{2+}\) release by more than a factor of five. A similar result was seen upon the creation of a pH gradient, brought about by diluting vesicles from pH 6.5 to 7.5 and from pH 7.5 to 6.5. On the other hand, a raise of external Ca\(^{2+}\) from 10–9 to 10–5 M Ca\(^{2+}\) increased the rate of 45Ca\(^{2+}\) release from cardiac SR vesicles by a factor of 1000 (Table III, Fig. 8).

The cardiac and skeletal Ca\(^{2+}\) channels display a similar dependence on Ca\(^{2+}\), peaking at micromolar external free Ca\(^{2+}\) in the absence of Mg\(^{2+}\) and adenine nucleotide. Addition of adenine nucleotide and Mg\(^{2+}\) shifts the Ca\(^{2+}\) concentration required for maximal Ca\(^{2+}\) release from about 2–20 μM Ca\(^{2+}\) to about 100–1000 μM (Fig. 8, Ref. 15). Also, in the skeletal system, the Hill plot n value of Ca\(^{2+}\) activation increases from 1 to 2 in the presence of adenine nucleotide and Mg\(^{2+}\). A consequence of this increase in cooperativity is that the skeletal channel is rendered sensitive to external Ca\(^{2+}\) in a quite narrow concentration range of about 10–9 to 10–4 M (15). Similarly, 45Ca\(^{2+}\) release from cardiac vesicles could be nearly fully inhibited or activated in the presence of Mg\(^{2+}\) and adenine nucleotide by varying the external Ca\(^{2+}\) concentration from about 10–6 to 10–1 M (Fig. 8). One important conclusion that can be drawn from these data is that under ionic conditions resembling those in muscle, i.e. in the presence of Mg\(^{2+}\) and nucleotide, the cardiac channel, like the skeletal channel, displays a strict dependence on Ca\(^{2+}\). In comparison, ATP and Mg\(^{2+}\) may play a more limited role in regulating Ca\(^{2+}\) release; our data suggest that ATP and Mg\(^{2+}\) act as allosteric effectors of the channel by modulating the channel's sensitivity to external Ca\(^{2+}\). This interpretation is supported by the fact that, in contrast to Ca\(^{2+}\), the concentrations of Mg\(^{2+}\) and nucleotide are thought to remain fairly constant during all phases of muscle activity.

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