Identification of the Ca^{2+} -release activity and ryanodine receptor in sarcoplasmic-reticulum membranes during cardiac myogenesis

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Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release activities were identified in sarcoplasmic-reticulum (SR) vesicles isolated from adult- and fetal-sheep hearts. Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release appear to proceed via the same channels, since both phenomena are similarly inhibited by Ruthenium Red. Ca^{2+} release from fetal SR vesicles is inhibited by higher concentrations of Ruthenium Red than is that from adult membranes. Both fetal and adult SR vesicles bind ryanodine. Fetal SR shows higher ryanodine-binding capacity than adult SR vesicles. Scatchard analysis of ryanodine binding revealed only one high-affinity binding site (K_d 6.7 nM) in fetal SR vesicles compared with two distinct binding sites (K_d 6.6 and 81.5 nM) in the adult SR vesicles. SR vesicles isolated from fetal and adult hearts were separated on discontinuous sucrose gradients into light (free) and heavy (junctional) SR vesicles. Heavy SR vesicles isolated from adult hearts exhibited most of the Ca^{2+} release activities. In contrast, Ca^{2+} -induced Ca^{2+} release, pH-induced Ca^{2+} release and ryanodine receptors were detected in both light and heavy fetal SR. These results suggest that fetal SR may not be morphologically and functionally as well differentiated as that of adult cardiac muscle and that it may contain a greater number of Ca^{2+} -release channels than that present in adult SR membranes.

INTRODUCTION

In the adult sheep heart the sarcoplasmic reticulum (SR) constitutes a distinct intracellular membrane compartment that regulates the contraction–relaxation cycle of muscle [1–3]. The SR consists of two morphologically well defined structures: longitudinal (free) SR and terminal cisternae (junctional) SR [4]. Release of Ca^{2+} from the SR membrane system plays an important role in triggering muscle contraction, whereas relaxation occurs when the Ca^{2+} is re-accumulated within the lumen of the SR. Ca^{2+} uptake is mediated by a membrane-bound Ca^{2+} -transporting ATPase protein [1,3,5]. By comparison with the process of Ca^{2+} uptake, the mechanism of Ca^{2+} release from SR is less well understood [2,6].

The physiological role of SR in the developing heart is not well established. It has been suggested that changes in the regulation of the intracellular Ca^{2+} concentration may contribute to the decreased mechanical properties of the fetal heart [7–9]. Recently we have studied the cardiac SR membrane during myogenesis [10] and showed that Ca^{2+} -uptake and Ca^{2+} -ATPase activities are low in fetal SR compared with the adult controls, although the apparent affinities for Ca^{2+} are similar [10]. These results correlate well with the relatively low amounts of 110 kDa Ca^{2+} -ATPase protein band observed in the fetal SR vesicles [10]. Mahoney & Jones [11] have also published similar observations. The observed developmental changes in the Ca^{2+} -transport properties of cardiac SR membrane may also be related to age-dependent changes in the properties of the SR Ca^{2+} -release channel. The maturation of the Ca^{2+} -release system during the differentiation of the cardiac SR has not been studied.

Ryanodine (a neutral alkaloid isolated from the higher plant *Ryania speciosa*) has been used to modulate Ca^{2+} transport across SR of skeletal and cardiac muscle. This drug influences efflux of Ca^{2+} from SR vesicles of skeletal muscle [12–16] and stimulates significantly Ca^{2+} uptake by junctional SR vesicles of skeletal and cardiac origin [14,17]. Furthermore, high concentrations of ryanodine block Ca^{2+} -induced Ca^{2+} release [15,18], and hence this drug is a powerful biochemical tool to probe the Ca^{2+} -release mechanism. Recent studies using radiolabelled ryanodine have shown that the drug binds to SR isolated from skeletal [19,20] and cardiac muscle [20]. It has been suggested that ryanodine interacts with the Ca^{2+} -release channel of the SR [15,19–20]. A ryanodine receptor has recently been isolated from the skeletal-muscle junctional SR vesicles [22,23].

Here we demonstrate that a Ca^{2+} -release channel is present at early stages of cardiac SR development in the sheep. In isolated SR vesicles, Ca^{2+} release was stimulated by both micromolar Ca^{2+} concentrations and by increasing the pH of the external medium. A specific ryanodine receptor was identified in the adult and fetal SR vesicles. The number of ryanodine-binding sites in the fetal SR was shown to be greater than that present in the adult membranes.

EXPERIMENTAL PROCEDURES

Materials

Phenylmethanesulphonyl fluoride, A23187 and Pipes were obtained from Sigma. $^{45}\text{CaCl}_2$ and $[^3\text{H}]$ ryanodine were from New England Nuclear. Ruthenium Red was from Fluka A.G., Buchs, Switzerland. Hepes was

obtained from Boehringer-Mannheim. Ryanodine was generously provided by Dr. K. P. Campbell (University of Iowa, Iowa City, IA, U.S.A.). Gel-electrophoresis reagents and molecular-mass markers were purchased from Bio-Rad.

Preparation of SR membranes

SR membranes were isolated from 100–105- and 140–145-day-old fetal hearts (full term is approx. 146 days) and from adult sheep hearts by using the procedure of Chamberlain *et al.* [24]. SR vesicles were suspended in 290 mM-sucrose/100 mM-KCl/10 mM-imidazole, pH 6.7, and stored frozen in liquid N₂. In some experiments SR vesicles isolated from adult and 140–145-day-old fetal hearts were further separated by a sucrose-density-gradient centrifugation using an SW27 rotor at 100 000 *g* for 2 h at 4°C. The discontinuous sucrose gradient consisted of 25, 28, 36 and 45% (w/v) sucrose in 400 mM-KCl/0.1 mM-EGTA/0.1 mM-CaCl₂/10 mM-Pipes, pH 7.0. Membrane vesicles were collected from sucrose interfaces, diluted 1 in 5 and pelleted at 120 000 *g* for 60 min. These SR membrane subfractions were suspended in 290 mM-sucrose/100 mM-KCl/10 mM-imidazole, pH 6.7, and stored frozen in liquid N₂. Light and heavy SR vesicles were collected at the 25/28%- and 28/36%-sucrose interfaces respectively.

Ca²⁺-release measurements

⁴⁵Ca²⁺ release rates from vesicles passively loaded with ⁴⁵Ca²⁺ were determined by Millipore filtration. Vesicles at the protein concentration of 5 mg/ml were incubated

for 2 h at room temperature in a loading buffer [20 mM-Pipes (pH 6.8)/100 mM-KCl/5 mM-⁴⁵Ca²⁺]. When the effects of ryanodine on Ca²⁺ efflux were studied, ryanodine was added (final concn. 500 μM) after the initial 2 h of Ca²⁺ loading, followed by incubation for an additional 1 h at room temperature. ⁴⁵Ca²⁺ release (efflux) from vesicles was assessed by diluting the vesicles 20-fold into appropriate media. For pH-induced Ca²⁺ release the dilution medium contained 20 mM-Hepes, pH 8.0, 1 mM-EGTA and 100 mM-KCl, whereas for Ca²⁺-induced Ca²⁺ release the dilution medium contained 20 mM-Pipes, pH 6.8, 0.3 mM-EGTA, 0.25 mM-Ca²⁺ and 100 mM-KCl; the free Ca²⁺ concentration was 5 μM. After Millipore filtration, the radioactivity retained by the vesicles was determined by liquid-scintillation counting. Data for three different SR preparations are presented.

[³H]Ryanodine binding to SR vesicles

SR vesicles (2 mg/ml) isolated at different stages of heart development were incubated with different concentrations of [³H]ryanodine (sp. radioactivity 55.0 Ci/mmol) in a solution containing 10 mM-Pipes, pH 7.4, 500 mM-NaCl and 50 μM-CaCl₂ for 2 h at 37 °C. The unbound ryanodine was separated from the protein-bound drug by Millipore filtration, followed by three 5 ml washes with an ice-cold solution containing 10 mM-Pipes, pH 7.1, 200 mM-NaCl and 50 μM-CaCl₂. Radioactivity retained on the filters was determined by liquid-scintillation counting. Non-specific binding was determined in the presence of 10 μM-unlabelled ryanodine and did not exceed 10–15% of the total binding.

Fig. 1. Ca²⁺-induced Ca²⁺ release (a) and pH-induced Ca²⁺ release (b) from SR vesicles isolated from adult sheep hearts

Ca²⁺-induced Ca²⁺ release and pH-induced Ca²⁺ release from SR vesicles were measured as described in the Experimental section. ○, Vesicles diluted into a medium containing 1 mM-EGTA, pH 6.8; ◇, vesicles diluted into a medium containing 5 μM free Ca²⁺ (a) or 1 mM-EGTA, pH 8.0 (b); ●, same as ◇, but in the presence of 1 μM-Ruthenium Red; ▲, same as ○, but in the presence of 5 μM-ionophore A23187. Amounts of ⁴⁵Ca²⁺ retained by the vesicles were obtained by back-extrapolation of the time of vesicles dilution.

Fig. 2. Ca^{2+} -induced Ca^{2+} release (a) and pH-induced Ca^{2+} release (b) from SR vesicles isolated from 140-145-day-old sheep hearts

○, Vesicles diluted into a medium containing 1 mM-EGTA, pH 6.8; ◇, vesicles diluted into a medium containing 5 μ M free Ca^{2+} (a) or 1 mM-EGTA, pH 8.0 (b); ◆, ●, vesicles diluted into a medium containing 1 μ M (◆)-Ruthenium Red or 50 μ M (●)-Ruthenium Red; ▲, same as ○, but in the presence of 5 μ M-ionophore A23187.

Fig. 3. Ca^{2+} -induced Ca^{2+} release (a) and pH-induced Ca^{2+} release (b) from SR vesicles isolated from 100-105-day-old fetal-sheep hearts

○, Vesicles diluted into a medium containing 1 mM-EGTA, pH 6.8; ◇, vesicles diluted into a medium containing 5 μ M free Ca^{2+} (a) or 1 mM-EGTA, pH 8.0 (b); ◆, ●, vesicles diluted into a medium containing 1 μ M (◆)-or 50 μ M (●)-Ruthenium Red; ▲ same as ○, but in the presence of 5 μ M-A23187.

Miscellaneous procedures

Protein concentrations were determined by the method of Lowry *et al.* [25], with bovine serum albumin as a standard. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [26]. Free Ca^{2+} concentrations were determined by using a Ca^{2+} /EGTA buffer, using the EGTA- Ca^{2+} association constant reported by Fabiato & Fabiato [27]. The calculation of free Ca^{2+} was accomplished by the use of a computer program which corrected for ionic strength, pH and the Mg^{2+} , EGTA, K^{+} and ATP concentrations.

RESULTS

Measurements of $^{45}Ca^{2+}$ release from passively loaded adult and fetal SR vesicles

Figs. 1, 2 and 3 show that SR vesicles isolated from adult, 140-145-day-old and 100-105-day-old fetal hearts contain a Ca^{2+} -release activity. SR vesicles isolated from adult hearts released most of the $^{45}Ca^{2+}$ within the first few seconds when diluted into Ca^{2+} -release medium containing 5 μ M-free Ca^{2+} (Fig. 1a). Ca^{2+} release was much more rapid after diluting the Ca^{2+} -loaded vesicles into pH 8.0 medium (Fig. 1b). In SR vesicles isolated from adult hearts, both Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release were inhibited by 1 μ M-Ruthenium Red. SR vesicles isolated from fetal hearts also exhibited Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release (Figs. 2 and 3). However, Ca^{2+} release in fetal vesicles was not blocked in the presence of 1 μ M-Ruthenium Red, but was blocked in the presence of 50 μ M-Ruthenium Red (Figs. 2 and 3).

Subfractionation of adult and fetal SR vesicles

SR membranes exhibit morphological and functional subspecialization [4,28-30]. The SR membrane preparations used in our studies contain a mixture of both free (light) and junctional (heavy) vesicles. We have separated adult and fetal SR vesicles on a discontinuous sucrose

gradient and collected light SR vesicles (banding at the 25/28%-sucrose interface) and heavy SR vesicles (banding at the 28/36%-sucrose interface) in order to localize the Ca^{2+} -release activity in the vesicle subpopulations. SR subfractions were analysed for their protein composition, Ca^{2+} -release activity and ryanodine binding.

In all SR subfractions isolated from adult and fetal hearts a major protein band could be identified as a 110 kDa Ca^{2+} -ATPase (Fig. 4). As determined by densitometric scanning of Coomassie Blue-stained SDS/polyacrylamide gels, the content of the 110 kDa band was approx. 35-40% of the cardiac SR protein from adult hearts and approx. 25-30% of the protein in total

Fig. 4. Gel-electrophoretic pattern of sucrose-gradient-SR subfractions isolated from adult (a) and 140-145-day-old fetal-sheep heart (b)

SDS/10% (w/v)-polyacrylamide-gel electrophoresis was performed as described by Laemmli [26]. L and H are light and heavy SR vesicles collected from the 25/28%- and 28/36%-sucrose interfaces respectively.

Fig. 5. Ca^{2+} -induced Ca^{2+} release (a,c) and pH-induced Ca^{2+} release (b,d) from adult SR vesicles fractionated on sucrose density gradients

L (a,b), light SR vesicles (25/28 % sucrose interface); H (c,d), heavy SR vesicles (28/36 %-sucrose interface). $\circ$, Vesicles diluted into a medium containing 1 mM-EGTA, pH 6.8; $\diamond$, vesicles diluted into a medium containing 5 μM free Ca^{2+} (a,c) or 1 mM-EGTA, pH 8.0 (b,d); $\bullet$, $\blacklozenge$, vesicles diluted into a medium containing 1 μM -Ruthenium Red ($\bullet$) or 500 μM -ryanodine ($\blacklozenge$); $\blacktriangle$, same as $\circ$, but in the presence of 5 μM -ionophore A23187.

Fig. 6. Ca^{2+} -induced Ca^{2+} release (a,c) and pH-induced Ca^{2+} release (b,d) from SR vesicles isolated from 140–145-day-old fetal-sheep hearts and further fractionated on a sucrose density gradient

L (a,b), light SR vesicles (25/28 %-sucrose interface); H (c,d), heavy SR vesicles (28/36 %-sucrose interface). $\circ$, Vesicles diluted into a medium containing 1 mM-EGTA, pH 6.8; $\diamond$, vesicles diluted into a medium containing 5 μM free Ca^{2+} (a,c) or 1 mM-EGTA, pH 8.0 (b,d); $\bullet$, $\blacklozenge$, vesicles diluted into a medium containing 50 μM -Ruthenium Red ($\bullet$) or 500 μM -ryanodine ($\blacklozenge$); $\blacktriangle$, same as $\circ$, but in the presence of 5 μM -ionophore A23187.

SR vesicles, in agreement with our previous observations [10].

Cardiac calsequestrin (CS), a 55 kDa Ca^{2+} -binding protein was identified in the light and heavy SR vesicles, by both its interaction with a specific anti-CS serum and its characteristic blue staining with Stains-All (results not shown). CS was enriched in heavy SR compared with light vesicles isolated from adult hearts: 5–6 and 9–10 % of the membrane proteins in the light (L) and heavy (H) SR fractions respectively (Fig. 4a). The amount of CS was similar in the heavy and light SR vesicles isolated from fetal hearts (Fig. 4b).

Ca^{2+} -induced Ca^{2+} release was localized almost exclusively to heavy SR vesicles isolated from adult hearts (Fig. 5). Light vesicles showed very little Ca^{2+} -induced Ca^{2+} release (Fig. 5). pH-induced Ca^{2+} release was also observed mostly in heavy SR vesicles isolated from adult hearts. In contrast, in SR vesicles isolated from fetal hearts, Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release were found in both the light and heavy fractions (Fig. 6). Both Ca^{2+} -induced Ca^{2+} release and pH-induced Ca^{2+} release were inhibited in the presence of Ruthenium Red or ryanodine.

Ryanodine binding to fetal and adult SR vesicles

Table 1 shows the binding of [^3H]ryanodine to crude, light and heavy SR vesicles isolated from adult and fetal hearts. SR vesicles isolated from fetal hearts bound a greater amount of ryanodine than did vesicles isolated from adult hearts (Table 1 and Fig. 7). This binding was fully inhibited in the presence of 100 μM -Ruthenium Red (results not shown) indicating, in agreement with previous observations [20,21], that ryanodine and Ruthenium Red bind to the same, or closely related, receptor sites. Heavy SR vesicles isolated from adult hearts bound more ryanodine than did light SR vesicles (Table 1). In contrast, light and heavy SR vesicles isolated from fetal hearts showed no significant difference in the amount of specific binding of ryanodine (Table 1).

Scatchard-plot analysis of ryanodine binding revealed that ryanodine binding to fetal SR vesicles was to a single class of high-affinity receptors (K_d 6.7 nM) (Fig. 7b). Adult SR vesicles, however, yielded non-linear Scatchard plots compatible with the presence of two receptor sites having greatly different affinities for the ligand (K_d 6.6 and 81.5 nM) (Fig. 7a).

Table 1. Specified binding of [³H]ryanodine to cardiac SR vesicles

SR vesicles before (crude SR) and after (light and heavy SR) sucrose-gradient separation were incubated with [³H]-ryanodine as described in the Experimental section. All binding assays were performed under equilibrium conditions with 20 nM-[³H]ryanodine. Non-specific binding (about 10 % of the total binding) in the presence of 10 μ M-ryanodine was subtracted. The values in parentheses denote the number of determinations. Significance: statistically different from light SR vesicles (**P* < 0.005); unpaired Student's *t* test analysis of variance followed by an adjusted *t* test.

Fraction	Age ...	Specific binding (pmol/mg of protein) (mean $\pm$ S.D.)	
		Adult	140–145-Day-old
Crude membranes		0.80 $\pm$ 0.10 (3)	2.20 $\pm$ 0.20 (3)
Light SR vesicles (25/28 %-sucrose interface)		0.31 $\pm$ 0.12 (3)	1.80 $\pm$ 0.25 (3)
Heavy SR vesicles (28/36 %-sucrose interface)		0.75 $\pm$ 0.15 (3)*	1.70 $\pm$ 0.25 (3)

DISCUSSION

In the present study we have identified Ca²⁺-induced Ca²⁺ release and pH-induced Ca²⁺-release activities in SR vesicles isolated from adult and fetal sheep hearts. Ca²⁺-induced Ca²⁺ release and pH-induced Ca²⁺ release appear to proceed via the same pathway, since both phenomena are characterized by similar inhibition by Ruthenium Red.

It is difficult, as yet, to speculate on the physiological role of the pH-induced Ca²⁺ release observed in cardiac SR vesicles. The physiological significance of the pH regulation of Ca²⁺ release from SR would depend on the actual change in cytoplasmic pH that occurs during a muscle contraction–relaxation cycle. Such a change was suggested previously by Shoshan *et al.* [31] for skeletal-muscle SR membrane. The significance of pH-induced Ca²⁺ release from cardiac SR membrane awaits further studies, although it provides a powerful tool for the identification and characterization of the SR Ca²⁺-release channel.

Ca²⁺ release in fetal SR vesicles is inhibited by higher concentrations of Ruthenium Red than is that in adult membranes. This suggests that the fetal SR Ca²⁺-release channel exhibits a different affinity for Ruthenium Red. We therefore studied the binding of [³H]ryanodine to both fetal and adult SR vesicles. Ryanodine is a potent inhibitor of Ca²⁺ release from cardiac and skeletal-muscle SR [15,18]. Ruthenium Red competes with ryanodine for the same, or closely related, binding site(s) on the SR membrane [15,17,19,20,32]. It has been speculated that ryanodine and Ruthenium Red may bind to a receptor which may constitute part of the SR Ca²⁺ channel or even represent the channel itself [15,19,23].

Both fetal and adult SR vesicles bind ryanodine. Fetal SR shows higher binding when compared with adult vesicles, indicating that SR vesicles isolated from fetal hearts are a richer source of ryanodine receptor. It is possible that the ryanodine binding part of the Ca²⁺ channel develops earlier than, or independently of, the functional Ca²⁺-release channel. This may be analogous to the development of the fast Na²⁺ channel. Renaud *et al.* [33] reported that, early in development, the fast Na⁺ channel exists mostly in a non-functional form, which is nevertheless able to bind tetrodotoxin, a Na⁺-channel-specific toxin. During the course of development

Fig. 7. Specific binding of [³H]ryanodine to adult and fetal SR vesicles

Saturation isotherms and Scatchard plots are shown. [³H]Ryanodine binding was carried out as described in the Experimental section. Non-specific binding to adult and fetal SR vesicles varied between 10 and 15 % of the total binding. For Scatchard insets, *B*_{max}, *B*₁, *B*₂ (maximum binding capacity) and bound ligand are given in units of pmol/mg of protein and *K*_d, *K*₁, *K*₂ (dissociation constant) are given in nM. Values are averages for three experiments with three different SR preparations, the S.E.M. being about 8 %.

the fast Na⁺ channel undergoes a transformation into a physiologically responsive, functional, form.

Scatchard analysis of ryanodine binding to SR revealed that only one high-affinity binding site is present in fetal SR vesicles, compared with two distinct binding sites present in the adult SR vesicles. Similar non-linear Scatchard plots were also observed previously for rabbit cardiac SR vesicles [20], but not for rabbit skeletal-muscle SR vesicles [20,21]. Fetal cardiac SR vesicles (the present study) and skeletal-muscle SR vesicles [20,21] yield similar linear Scatchard plots. These results suggest that the ryanodine receptor in fetal cardiac SR may be structurally similar to the skeletal-muscle receptor. Although this conclusion is speculative, it was previously reported that the skeletal and cardiac muscle forms of the SR Ca²⁺-transporting ATPase may be expressed at the same time during the course of muscle development [34].

It is well known that the SR exhibits morphological [4] and functional [30] subspecialization and that membrane vesicle preparations contain a mixture of both free (light) and junctional (heavy) vesicles [28]. In the present study we have further separated SR vesicles isolated from fetal and adult hearts on discontinuous sucrose gradients in order to separate light (free) and heavy (junctional) SR vesicles. In agreement with previous observations for adult skeletal- and cardiac-muscle SR vesicles [18,35], the Ca²⁺-release activities reside mostly in the heavy (junctional) SR from adult heart. This fraction was identified as heavy SR by its relatively high concentration of CS and ryanodine receptors, both known to be reliable markers for heavy SR vesicles isolated from skeletal muscle SR [15,23,29]. Sucrose-density-gradient centrifugation of fetal SR vesicles did not produce a clear structural or functional separation of free and junctional SR. CS appeared to be present in both light and heavy SR fraction in relatively similar amounts. There was no enrichment of ryanodine-binding sites in the heavy SR vesicles isolated from fetal hearts. Ca²⁺-induced Ca²⁺ release and pH-induced Ca²⁺ release were detected in both light and heavy fetal SR. These results suggest that fetal SR may not be as functionally and morphologically differentiated as the SR of adult cardiac muscle.

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