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Characterization of the Ryanodine Receptor and SERCA in Fetal, Neonatal, and Adult Rat Hearts

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The mammalian heart is known to undergo significant mechanical changes during fetal and neonatal development. The objective of this study was to define the ontogeny of the ryanodine receptor/Ca^{2+} release channel and SERCA that play the major roles in excitation-contraction coupling. Whole ventricular homogenates of fetal (F) (19 and 22 days in gestation), postnatal (N) (1 and 7 days postnatal), and adult (A) (5 weeks postnatal) Sprague-Dawley rat hearts were used to study [^{3}H]ryanodine binding and oxalate-supported $^{45}Ca^{2+}$ uptake. For the ryanodine receptor, the major findings were: (1) The ryanodine receptor density, as determined by maximal [^{3}H]ryanodine binding (B_{max}), increased 3 fold between the F22 and A periods (0.26 ± 0.1 vs. 0.73 ± 0.07 pmoles/mg protein, $p<0.01$), whereas there was no significant change during the F22 and N1 development phases (0.26 ± 0.1 vs. 0.34 ± 0.01). (2) Affinity of the ryanodine receptor to ryanodine did not significantly change, as suggested by the lack of change in the K_d during the development and maturation. For SERCA, changes started early with an increased rate of Ca^{2+} uptake in the fetal periods (F19: 8.1 ± 1.1 vs. F22: 19.3 ± 2.2 nmoles/g protein/min; $p<0.05$) and peaked by 7 days (N7) of the postnatal age (34.9 ± 2.1). Thus, we conclude that the quantitative changes occur in the ryanodine receptor during myocardial development. Also, the maturation of the Ca^{2+} uptake appears to start earlier than that of the Ca^{2+} release.

Keywords: Excitation-contraction coupling; Ontogeny, Ca^{2+} release channel; Ca^{2+}-ATPase

Introduction

In the adult mammalian heart, the Ca^{2+} release from sarcoplasmic reticulum (SR) through the ryanodine receptor/Ca^{2+} release channel and Ca^{2+} uptake into SR by SERCA (sarco(endo)plasmic reticulum Ca^{2+}-ATPase) are the two major mechanisms that control cytoplasmic Ca^{2+} concentrations, and thereby cardiac contraction and relaxation (Feher and Fabiato, 1990; Coronado *et al.*, 1994; Meissner, 1994; Zucchi and Ronca-Testoni, 1997). However, much less is known of the functional and biochemical roles of the two SR proteins during fetal and neonatal heart development.

The development of the mammalian heart is accompanied by mechanical changes, such as increased contractility and speed of relaxation, as well as the appearance of ryanodine-sensitive phasic contractions (Nakanishi *et al.*, 1988; Klitzner and Friedman, 1989; Kaufman *et al.*, 1990; Ostadalova *et al.*, 1993). These mechanical changes can be attributed, at least in part, to the increased amounts of SR (Page *et al.*, 1974; Wibo *et al.*, 1991) and expression levels of SR proteins (Anger *et al.*, 1994; Brillantes *et al.*, 1994; Fitzgerald *et al.*, 1994; Ramesh *et al.*, 1995). The results have shown less negative inotrophic effects by ryanodine in fetal and neonatal hearts than in adults. This suggests that the myocardial contraction in the early stage of development is largely dependent on the trans-sarcolemmal Ca^{2+} influx rather than the Ca^{2+} release from the SR (Klitzner, 1991; Tanaka and Shigenobu, 1989). The trans-sarcolemmal Ca^{2+} fluxes in the immature hearts could be controlled by the Na^+-Ca^{2+} exchanger and dihydropyridine receptor (DHPR) (Mahony, 1996). Until now, there have been no systematic studies on the activities of the ryanodine receptor and SERCA during fetal and neonatal cardiac development.

This study examined detailed fetal and neonatal development, focusing on the functional alterations of the ryanodine receptor and SERCA in a rat's heart. From this study, we conclude that a quantitative up-regulation occurs in both the ryanodine receptor and SERCA during fetal and

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neonatal development, but the maturation of SERCA appears to start earlier than that of the ryanodine receptor.

Materials and Methods

Animal procedure Sprague-Dawley rats (Charles River), weighing 120-140 g (5 weeks old), served as the young adult group (A). From rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.), hearts were removed and placed in ice cold 0.9% NaCl. After atrial tissues, visible fat and connective tissues were trimmed off, ventricular tissues were used for the preparation of whole homogenate and determination of the wet and dry weights (Ramesh *et al.*, 1995). Timed pregnant Sprague-Dawley rats (12 weeks old) at 19 and 22 days of gestation (term) (F19 and F22) were killed by CO_2 inhalation. The fetuses were removed by hysterectomy and weighed to confirm gestational age. Neonatal rats at the ages of 1 and 7 days (N1 and N7) were euthanized by CO_2 inhalation, and the hearts were removed and weighed. The removed hearts were immersed in an ice-cold 0.9% NaCl solution. On the basis of the previous results that showed no biochemical differences (Ramesh et al, 1995), we did not distinguish the left and right ventricles.

Whole homogenate preparation The rat heart ventricular tissues that were isolated from the five different age groups were homogenized at 4°C in a ratio of 20-50 mg of tissue per ml of homogenization solution containing 20 mM MOPS (pH 7.4), 1 M KCl, 1 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 0.1 mM PMSF, and 10 μg/ml trypsin inhibitor, using a Polytron PT10 probe (Brinkmann) at the speed setting of 5 for 20 s (Venkat *et al.*, 1995). Aliquots of the homogenates were frozen in liquid nitrogen and kept at −80°C until use.

Ryanodine binding Equilibrium ryanodine binding to whole homogenate was measured by incubation in 250 μl reaction mixtures that contained 2 mg whole homogenate, 1 M KCl, 20 mM MOPS (pH 7.4), 2.5-20 nM [^{3}H]ryanodine (54.7 Ci/mmol, New England Nuclear Co.), 1 mM EGTA, and 0.98 mM $CaCl_2$ (3 μM free Ca^{2+}) for 2 h at 37°C (Fabiato and Fabiato, 1979; Kim *et al.*, 1994, Ramesh *et al.*, 1995; Lee *et al.*, 1991). The binding parameters were calculated by iterative computer fitting using an equation of $Y = B_{max} \times X/(K_d+X)$, where Y is the ryanodine bound to whole homogenate (pmol/mg protein), B_{max} is the maximal ryanodine binding (pmol/mg protein), X is the ryanodine concentration (nM), and K_d is the dissociation constant of ryanodine (nM).

The reaction mixtures were incubated for 2 h at 37°C. Our data on the time course of the ryanodine binding showed that the steady state of ryanodine binding was reached within 1 h of incubation, and was stable for at least 2 more hours under the experimental conditions described previously. At the end of incubation, 100 μl of the polyethylene glycol (PEG) solution (30% PEG, 1 mM EDTA and 50 mM Tris, pH 7.4) was added to each vial that held 250 μl of the reaction mixture and incubated for 5 min at room temperature (Campbell *et al.*, 1987). The vials were centrifuged for 5 mins at 14,000 rpm in an Eppendorf microcentrifuge. The pellets were washed twice with 0.4 ml of the binding buffer. One hundred ml of Soluene 350 (Packard Co.) was added to each vial, incubated at 70°C for 30 min, transferred to scintillation vials, and the radioactivity counted in 4 ml Picofluor (Packard Co.).

Oxalate-supported Ca^{2+} uptake Whole homogenates of the rat hearts were preincubated for 4 min at 37°C in a bath that contained (final concentrations) 100 mM KCl, 20 mM MOPS (pH 6.8), 10 mM NaN_3, and 500 μM ryanodine (Feher *et al.*, 1989). The uptake reaction was begun by the rapid sequential addition of 5 mM MgATP, 10 mM potassium oxalate, and 0.2 mM $^{45}CaCl_2$. Gelman prefilter and Millipore filter (0.45 μm) were used together to facilitate filtration. The rate of Ca^{2+} uptake was calculated from the linear regression of Ca^{2+} uptake that was determined at 1, 2, 4, 6, and 10 min.

Miscellaneous Protein concentrations of the whole homogenates were determined by the method of Lowry (Lowry *et al.*, 1951). All of the data were presented as means ± SE. The statistical significance was evaluated with the Student's unpaired t-test and one-way ANOVA. P values less than 0.05 were considered significant.

Results

Equilibrium ryanodine binding to whole homogenates of fetal, neonatal, and adult rat hearts Equilibrium [^{3}H]ryanodine binding has been widely used to characterize properties of the ryanodine receptor/Ca^{2+} release channel in muscle tissues (Campbell *et al.*, 1987; Lee *et al.*, 1991; Kim *et al.*, 1994; Ramesh *et al.*, 1995; Park *et al.*, 1998). In order to examine changes that might have occurred in the biochemical properties of the ryanodine receptor during fetal and neonatal heart development, the [^{3}H]ryanodine binding to whole homogenates of the fetal, neonatal, and adult rat heart was

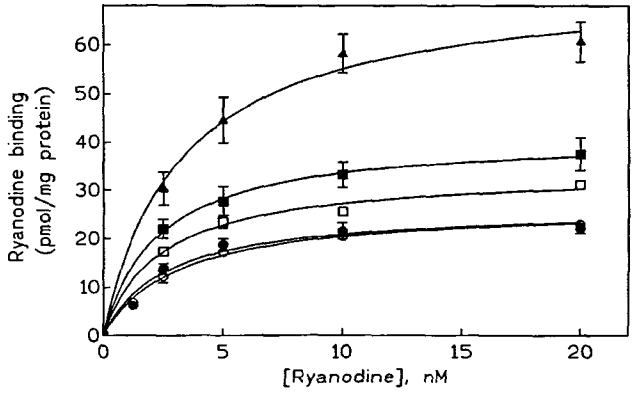

Fig. 1. Equilibrium ryanodine binding to the whole homogenate of fetal (F19, ○; F22, ●), neonatal (N1, □; N7, ■), and adult (▲) rat hearts. Ryanodine binding to whole homogenate was measured by incubation in a 250 μl reaction mixture that contained 2 mg whole homogenate, 1 M KCl, 20 mM MOPS (pH 7.4), 2.5-20 nM [^{3}H]ryanodine (54.7 Ci/mmol), 1 mM EGTA, and 0.98 mM $CaCl_2$ ($[Ca^{2+}]_{free}$ = 3 μM) for 2 h at 37°C, as described in "Materials and Methods". Each symbol represents mean ± SE from 3 sets of animals.

Table 1. Characteristics of ryanodine binding and Ca^{2+} uptake of whole ventricular homogenates from fetal (F19 & F22), neonatal (N1 & N7), and adult (A) rat hearts.

	F19	F22	N1	N7	A
B_{max} (pmol/mg protein)	0.26 ± 0.07*	0.26 ± 0.10*	0.34 ± 0.01	0.41 ± 0.03	0.73 ± 0.07
Kd (nM)	3.0 ± 0.3	2.6 ± 0.3	2.6 ± 0.4	2.5 ± 0.1	3.9 ± 1.0
Ca^{2+} uptake rate (nmole/g protein/min)	8.1 ± 1.1*	19.3 ± 2.2*	25.0 ± 1.8	34.9 ± 2.1	27.6 ± 1.1

*Denotes a statistically significant difference ($p<0.05$) between the fetal and neonatal or adult groups (One way ANOVA). Each values represent means ± SE from 3 sets of animals.

conducted at various ryanodine concentrations (Fig. 1). The [^{3}H]ryanodine binding per mg protein of the whole homogenate increased markedly by increased ryanodine concentrations, and reached a steady state at 10-20 mM [^{3}H]ryanodine (Fig. 1). The binding parameters were calculated by an iterative computer fitting using rectangular hyperbola curves. The density of the ryanodine receptor in rat heart tissues, calculated as the maximal [^{3}H]ryanodine binding (B_{max}) per mg protein, increased 3 fold between the fetal (F22, ●) and adult (A, ▲) periods (0.26 ± 0.10 vs. 0.73 ± 0.07 pmoles/mg protein, $p<0.01$), whereas there was no significant change during the fetal (F22, ●) and early postnatal (N1, □) development phases (0.26 ± 0.10 vs. 0.34 ± 0.01) (Fig. 1 and Table 1). On the other hand, the affinity of the ryanodine binding (K_d) did not change significantly during the developments (Fig. 1 and Table 1).

Oxalate-supported Ca^{2+} uptake in the presence of 500 μM ryanodine In the presence of oxalate, Ca^{2+} forms a Ca^{2+}-oxalate precipitate in the SR lumen, and thereby the Ca^{2+} uptake capacity is considerably increased by lowering the free Ca^{2+} concentration inside the SR lumen (Feher *et al.*, 1989; Ramesh *et al.*, 1995). Since this amplification process is a unique property of SR, the oxalate supported Ca^{2+} uptake experiments have been useful to determine the activity of SERCA in whole homogenates of muscle tissues. Ryanodine activates the ryanodine receptor at submacromolar concentrations, whereas it inhibits the receptor/channel at higher concentrations (Coronado *et al.*, 1994; Meissner 1994; Zucchi and Ronca-Testoni, 1997). As shown by Feher *et al.* (1989), the total Ca^{2+} uptake capacity in SR can be measured in the presence of 500 mM ryanodine, since the ryanodine-sensitive Ca^{2+} efflux can be completely blocked in the presence of 500 μM ryanodine.

In order to evaluate the capacity of the Ca^{2+} uptake that is mediated by SERCA during fetal and postnatal development, the oxalate-supported Ca^{2+} uptake (Feher *et al.*, 1989) was carried out in the presence of 500 μM ryanodine using whole homogenates (Fig. 2 and Table 1). The rate of Ca^{2+} uptake during the initial 10 min reaction time, a measure of the relative activity of SERCA, were calculated by linear regression. The rate of Ca^{2+} uptake in the presence of 500 μM ryanodine was increased more than two times during the period between F19 (○) and F22 (●), and peaked by 7 days (N7, ■) of postnatal age (34.9 ± 2.1 nmol Ca^{2+}/g protein/min). However, the rate of Ca^{2+} uptake decreased 17% in the adult heart (27.6 ± 1.1).

Comparison of the functional expressions of the ryanodine receptor and SERCA during myocardial development In order to compare the functional expressions of the ryanodine receptor and SERCA during the myocardial development in rats, B_{max} of the ryanodine binding and the Ca^{2+} uptake rate of SERCA (Table 1) were plotted as % of adult value vs. post conceptional age (Fig. 3). The graphs generally showed that the functional expression of SERCA occurred more rapidly than that of the ryanodine receptor (Fig. 3). At the 29th post conceptional day (7 days postnatal), SERCA showed the peak activity value (117% of the adult value), whereas ryanodine receptor showed only 50% of the adult value. This result is similar to the previous Western blot data of the rat heart primary culture that showed the faster maturation rate of SERCA than the ryanodine receptor (Park *et al.*, 1998).

Discussion

Mammalian hearts undergo significant physiological alterations during fetal and neonatal development (Nakanishi *et al.*, 1988; Klitzner and Friedman, 1989; Kaufman *et al.*, 1990; Klitzner, 1991; Ostadalova *et al.*, 1993). The alterations include an increased amount of cytosolic Ca^{2+} transients, increased tension development, and speed of relaxation (Klitzner and Friedman, 1989; Kaufman *et al.*, 1990; Ostadalova *et al.*, 1993). The goal of this study was a systematic characterization of the two essential excitation-contraction coupling proteins (ryanodine receptor and SERCA) in fetal (F19 and F22), neonatal (N1 and N7), and adult (A) rat heart tissues. The ability of using rat cardiac whole homogenates to assess functions of the ryanodine receptor and SERCA (Ramesh *et al.*, 1995) at different developmental stages was useful, since isolation of the SR vesicles from fetal and neonatal rat hearts was extremely difficult, due to their small sample sizes. Whole homogenates were directly used for this study to assess the functional capacities of the ryanodine receptor and SERCA (Ramesh *et al.*, 1995).

Ryanodine is a plant alkaloid and has dual functions on the ryanodine receptor. Because of its high affinity to the

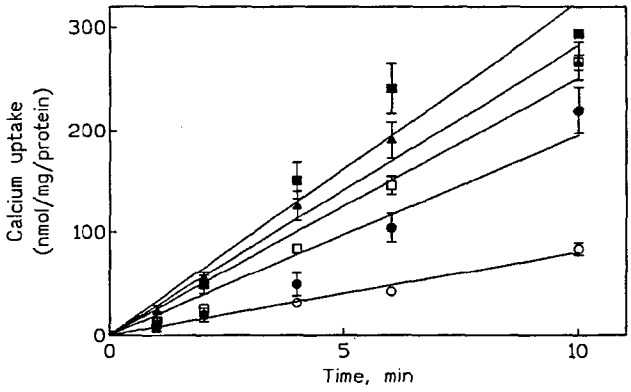

Fig. 2. Oxalate-supported Ca^{2+} uptake in fetal (F19, ○; F22, ●), neonatal (N1, □ ; N7, ■), and adult (▲) rat hearts. Oxalate-supported Ca^{2+} uptakes into SR in whole homogenates of fetal, neonatal, and adult rat hearts were measured in a reaction mixture that contained 5 mg tissue/ml, 100 mM KCl, 20 mM MOPS (pH 6.8), 5 mM NaN_3, with 500 μM ryanodine, 5 mM $MgCl_2$, 5 mM ATP, 10 mM potassium oxalate, and 200 uM $^{45}CaCl_2$ at 37°C, as described in "Materials and Methods". The rate of Ca^{2+} uptake was calculated from the linear regression of the Ca^{2+} uptake that was determined at 1, 2, 4, 6, and 10 min. Each symbol represents mean ± SE from 3 sets of animals.

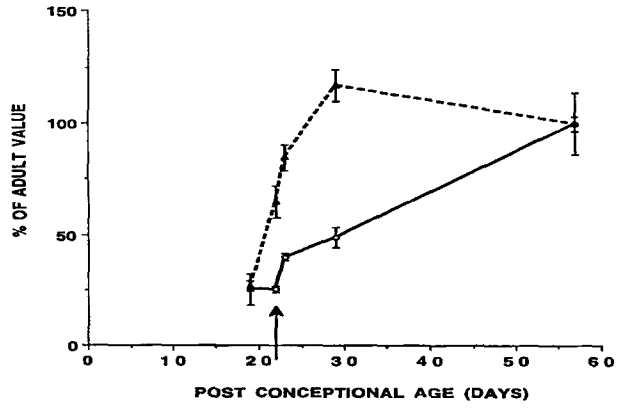

Fig. 3. Comparison of functional capacities of the ryanodine receptor (○) and SERCA (▲) at different stages of myocardial development. Ryanodine binding and oxalate-supported Ca^{2+} uptake were carried out, as described in "Materials and Methods". The B_{max} of ryanodine binding (Table 1) and the rate of Ca^{2+} uptake (Table 1) at different post conceptional ages (days) were expressed as % of adult values. The arrow indicates the birthday. Each symbol represents mean ± SE from 3 sets of animals.

ryanodine receptor, equilibrium ryanodine binding studies have been used to evaluate the density and functional states of the ryanodine receptor (Lee *et al.*, 1991; Kim *et al.*, 1994; Ramesh *et al.*, 1995). The results of the equilibrium ryanodine binding studies showed that the affinity of ryanodine binding (K_d) was similar among the tested groups, whereas the maximal number of ryanodine binding (B_{max}) increased during the neonatal stage (Table 1). This suggests that only quantitative changes could occur during the heart development. The results of the low B_{max} (25% of the adult value) during the fetal stages, and the gradual increment of the values after birth, could explain the physiological data that showed the excitation-contraction coupling in the fetal heart was largely dependent on the trans-sarcolemmal Ca^{2+} influx, rather than the Ca^{2+} efflux from the SR through the ryanodine receptor (Fabiato and Fabiato, 1978; Ostadalova *et al.*, 1993; Su and Chang, 1993; Mahony, 1996).

The time courses of the oxalate-supported Ca^{2+} uptake are generally linear where the total Ca^{2+} in the reaction mixture is not rate limiting (Feher *et al.*, 1989; Ramesh *et al.*, 1995). Therefore, the initial rates of Ca^{2+} uptake were determined to deduce the SERCA activities (Feher *et al.*, 1989; Ramesh *et al.*, 1995). Table 1 and Fig.2 show that the rates of Ca^{2+} uptake continuously increased during the fetal and neonatal stages (F19-N7). On the other hand, the ryanodine receptor matured slowly and continuously increased until the adult stage. This result is consistent with the previous Western blot data of the rat heart primary culture that showed the faster maturation rate of SERCA than the ryanodine receptor (Park *et al.*, 1998). The relatively slower production of the ryanodine receptor compared with SERCA during heart development could be related with the delayed onset of biogenesis of the junctional SR, where the ryanodine receptor is primarily located (Page *et al.*, 1974).

Previously, Fabiato and Fabiato (1978), who observed calcium-induced cyclic contraction in newborn heart cells, but not in those of the near-term fetus, attributed the difference to the lack of a transverse tubule-junctional SR system in the fetal heart (Page *et al.*, 1974). Wibo *et al.* (1991) found about a 2-fold increase in the density of the ryanodine receptor between 1 and 30 days of postnatal growth, which paralleled the maturation of the junctional SR and transverse-tubules in the rat ventricle. Our results (Figs. 1-3 and Table 1), combined with the report of Wibo *et al.* (1991), suggest that the density of the ryanodine receptor is closely related with the maturation of junctional SR, where the ryanodine receptor is located. The earlier onset of the expression of SERCA than that of the ryanodine receptor during myocardial development (Fig. 3) could be attributed to the faster onset of biogenesis of longitudinal SR (where SERCA is primarily located) than that of junctional SR (Crowe and Bastin, 1977). The lower density of the ryanodine receptor (25% of the adult value) could limit the functional role of SERCA during the fetal stage.

These findings suggest that the increased cytosolic [Ca^{2+}] transient, tension development, and contractility during fetal and neonatal development are due, at least in part, to the progressive development of the ryanodine receptor and SERCA in SR. However, the maturation of the SR Ca^{2+} uptake appears to start earlier than that of the SR Ca^{2+} release.

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