The Role of Dicarboxylic Anion Transport in the Slower Ca^{2+} Uptake in Fetal Cardiac Sarcoplasmic Reticulum

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ABSTRACT. Sarcoplasmic reticulum- (SR-)mediated Ca^{2+} transport is slower in the fetal heart compared with the adult. Virtually all previous studies of cardiac SR Ca^{2+} transport were performed in the presence of oxalate, a dicarboxylic anion that is cotransported with Ca^{2+} in skeletal muscle SR. If anion transport is developmentally regulated in cardiac SR, this could explain, in part, the previously reported results. The purposes of this study were to establish the presence of an SR dicarboxylic anion transport process in the rabbit heart and to determine if the perinatal changes in SR Ca^{2+} transport occur in a dicarboxylic anion-dependent and/or independent manner. In isolated fetal and adult rabbit cardiac SR membranes, we measured Ca^{2+} ATPase rates and $^{45}Ca^{2+}$ uptake in the presence of the dicarboxylic anions maleate and succinate compared with the zwitterionic buffer PIPES, to which cardiac SR is essentially impermeable. We also measured ^{14}C-succinate uptake by fetal and adult SR membranes. Anion-independent Ca^{2+} ATPase activity and net $^{45}Ca^{2+}$ uptake were significantly lower in the fetal SR membranes than in the adult. Maleate and succinate increased the Ca^{2+} ATPase rates in the fetal and adult SR, but the effect was significantly greater in the adult. Maleate and succinate stimulated earlier attainment of maximal net Ca^{2+} uptake in the fetal and adult SR, suggesting that these dicarboxylic anions stimulated the rate of Ca^{2+} accumulation. Maleate and succinate significantly increased the maximal net Ca^{2+} uptake in the adult SR, but not in the fetus. The percentage of stimulation of Ca^{2+} uptake by maleate and succinate was similar in the fetal and adult SR. Dicarboxylic anion transport, as estimated by ^{14}C-succinate uptake, was significantly lower in the fetus. The previously reported slower Ca^{2+} uptake rate in the fetus is related to dicarboxylic anion-dependent as well as independent mechanisms. The results provide firm support for the presence of a cardiac SR dicarboxylic anion transport process in rabbits that is developmentally regulated. These results also support the previously reported developmental regulation of the SR Ca^{2+} pump. (*Pediatr Res* 32: 664–668, 1992)

Abbreviations

SR, sarcoplasmic reticulum
Ca^{2+}, calcium
PIPES, 1,4-piperazine ethanesulfonic acid

The maximum rate of relaxation is slower in the developing mammalian heart than in adults of the same species (1). In the adult heart, relaxation results primarily from an ATP-dependent Ca^{2+} sequestration by the SR Ca^{2+} pump, which moves Ca^{2+} from the cytosol into the SR lumen (2–4). The slower relaxation in the fetus has been associated with slower SR Ca^{2+} transport (5–8), which has been related, at least partly, to less expression of SR Ca^{2+} pumps (5, 8, 9).

ATP-dependent Ca^{2+} uptake in isolated cardiac SR membranes is responsive to several factors, including the density of Ca^{2+} pumps and other SR proteins such as phospholamban, and the sensitivity of the Ca^{2+} pump to Ca^{2+}, ATP, pH, and temperature. In skeletal muscle SR membranes, Ca^{2+} uptake also is sensitive to the nature of the anion that is present in the uptake reaction (10–13). Dicarboxylic anions, such as maleate, succinate, and oxalate, are cotransported with Ca^{2+} across skeletal muscle SR membranes (11, 12), where they augment SR Ca^{2+} transport by chelating or precipitating intravesicular Ca^{2+}, maintain a low, free intravesicular Ca^{2+} concentration, and thereby prevent an inhibition of Ca^{2+} ATPase activity (14). The presence of dicarboxylic anion transport also has been established in canine cardiac SR, although the data are limited (15–17).

It has been noted that the stoichiometry of oxalate-supported Ca^{2+} transport/Ca^{2+} ATPase activity is lower in fetal than in adult ovine cardiac SR membranes (5). Virtually all of the measurements that have demonstrated slower Ca^{2+} uptake rates in fetal SR were performed in the presence of oxalate, a dicarboxylic anion (5–8). We hypothesized that cardiac SR dicarboxylic anion transport may be developmentally regulated, which would result in a slower Ca^{2+} uptake rate when the reactions are performed in the presence of dicarboxylic anions. In this report, we present evidence for developmental regulation of dicarboxylic anion transport in rabbit cardiac SR membranes. These data also demonstrate that the previously reported slower Ca^{2+} transport in the fetus (5–8) may result in part from lower dicarboxylic anion transport, as well as from a lower anion-independent Ca^{2+} transport.

MATERIALS AND METHODS

All of the protocols used in this study were performed according to the guidelines in the *NIH Guide for The Care and Use of Laboratory Animals*, with the highest standards of humane care. The protocols were approved by the Committee on Animal Experimentation at Baylor College of Medicine.

Isolation and characterization of SR membranes. Cardiac SR vesicles were isolated at the same time from New Zealand White rabbit fetuses at 29 d of gestation (term = 31 d) and their mothers by a previously described modification of the method of Harigaya and Schwartz (8, 18). Briefly, the mothers were anesthetized with ketamine, xylazine, and acepromazine. Fifteen to 20 min later, the mothers underwent cervical subluxation. The fetal and ma-

ternal hearts were removed rapidly, rinsed free of blood, and trimmed. The left ventricular free wall and septum were minced and homogenized in 10 volumes of 10 mM $NaHCO_3$ and 10 mM Tris maleate (pH 6.8, 4°C, for 15 s three times at 5 800 rpm with a PT3000 Brinkmann Polytron, Brinkmann Instruments Co., Westbury, NY). The homogenate was centrifuged for 5 min at 650 × g and for 15 min at 4 000 × g. The supernatant was centrifuged for 20 min at 8 000 × g, and the resulting supernatant was centrifuged at 40 000 × g for 30 min. The pellet was resuspended in 0.8 mL/g wet heart weight of 600 mM KCl and 10 mM Tris maleate (pH 6.8, 4°C) with a Teflon-glass homogenizer and centrifuged at 40 000 × g for 30 min. The final pellet was resuspended in 20 mM Tris maleate (pH 6.8, 4°C). Protein concentrations were determined by the method of Lowry (19) with BSA as a standard. The SR membranes were frozen rapidly and stored at −70°C. Previous experiments have determined that there was no independent effect of the anesthetic or freezing, and that there were no differences in SR membranes isolated from hearts from nonpregnant adult rabbits and the pregnant mothers (8).

The yield of SR membranes was calculated by dividing the mg of SR membranes that were isolated by the wet weight of left ventricular free wall and septum used for the isolation. The fold enrichment of SR membranes that was accomplished by the isolation procedure was determined by dividing the oxalate-supported $^{45}Ca^{2+}$ uptake rate (see below) in the isolated SR membranes by the rate in fresh tissue homogenates (20). We estimated the amount of contaminating sarcolemma in SR membranes by calculating the recovery of total ouabain-sensitive Na^+,K^+ ATPase activity (21) in SR membranes as a percentage of the total activity in the homogenate protein. All reported Na^+,K^+ ATPase activities represent the highest values obtained after preincubation of protein with a range of saponin concentrations (22). We estimated the amount of contaminating mitochondria in SR membranes by calculating the recovery of the total azide-sensitive Mg^{2+} ATPase (see below) in SR membranes as a percentage of the total activity in the homogenate protein (23). All assays of SR function reported in this study were performed in 10 mM sodium azide to inhibit the contaminating mitochondrial activity (23).

$^{45}Ca^{2+}$ and ^{14}C-succinate uptake. Oxalate-supported $^{45}Ca^{2+}$ uptake was determined in 40 mM of PIPES (pH 6.8, 30°C), 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium azide, 40 μM $^{45}CaCl_2$ (Ca^{2+}_{free} = 16 μM; sp act = 8 000–10 000 cpm/nmol, New England Nuclear, Boston, MA), 5 mM sodium oxalate, 2 mM P-enolpyruvate, 8.75 U of pyruvate kinase, 1 mM Na_2ATP, and 20 μg of SR protein/mL.

To check for an anion-dependent effect on Ca^{2+} uptake, we compared the effects of the dicarboxylic anions maleate and succinate (11, 12) with the zwitterionic buffer PIPES, to which cardiac SR is essentially impermeable (17). These $^{45}Ca^{2+}$ uptake reactions, in the absence of oxalate, were performed in 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium azide, 20 μM $^{45}CaCl_2$ (Ca^{2+}_{free} = 18 μM), 1 mM Na_2ATP, 50 μg/mL of protein, and 40 mM of PIPES, maleate, or succinate (pH 6.8, 30°C). ^{14}C-succinate uptake was measured in the same conditions except for the substitutions of ^{14}C-succinate (sp act, 800–1 000 cpm/nmol; ICN Pharmaceuticals, Covina, CA) and unlabeled Ca^{2+} for unlabeled succinate and $^{45}CaCl_2$, respectively.

All reactions were performed above a Ca^{2+}_{free} concentration of 10 μM, which we have previously determined as the threshold for maximal activation of the rabbit SR Ca^{2+} pump (8). Ca^{2+}_{free} concentrations were calculated with a computer program (24). The uptake reactions were performed with a vacuum filtration technique (0.45 μm HAWP #025 00 filters, Millipore Corp., Bedford, MA). Timed aliquots were rapidly filtered before and after initiation of a reaction by the addition of 1 mM Na_2ATP, and the filters were immediately washed with 5 mL of an iced solution of 100 mM KCl and the appropriate anion (40-mM Pipes, maleate or succinate, or 5 mM sodium oxalate, pH 6.8).

The filters were dried, and then counted in a liquid scintillation counter. The specific radioactivity for all of the assays was calculated by subtracting the nonspecific binding to filters and protein (i.e. no ATP present), from the total radioactivity after the reaction was initiated. Oxalate-dependent Ca^{2+} uptake rates were calculated from the time-points when uptake was linear. The correlation coefficients were >0.99. We were unable to manually measure an adequate number of time-points to calculate rates of $^{45}Ca^{2+}$ uptake in the presence of maleate, succinate, and Pipes, and the ^{14}C-succinate uptake rate. For these assays, we estimated the uptake "rates" by statistically comparing net Ca^{2+} uptake at t = 1 min, and by visually comparing the apparent rates during the earliest time-points as the uptake reactions attained their maxima.

Ca^{2+} ATPase activity. Ca^{2+}-dependent ATPase rates were measured at 30°C with a linked enzyme system of pyruvate kinase-lactate dehydrogenase by continuously monitoring NADH oxidation at 340 nm (25). The reaction was performed in 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium azide, 20 μM $^{45}CaCl_2$ (Ca^{2+}_{free} = 18 μM), 2 mM P-enolpyruvate, 400 μM NADH, 8.75 units of pyruvate kinase, 12.75 units of lactate dehydrogenase, 1 mM Na_2ATP, 20 μg of SR protein/mL, and 40 mM of PIPES, maleate, or succinate (pH 6.8). The reactions were performed in the presence and absence of 2 mM EGTA, and the difference was calculated as the Ca^{2+} ATPase activity. The ATP-dependent activity measured in EGTA is reported as the Mg^{2+} ATPase activity.

PAGE. We used Coomassie brilliant blue staining of SDS-PAGE slab gels to compare the SR protein profile in the different isolations, and to determine whether our SR membrane isolations were qualitatively similar to previously published developmental data (5, 6). Aliquots of SR were solubilized in 1% SDS, 10 mM DTT, 12.5% glycerol, 0.001% bromphenol blue (tracking dye) in 31 mM Tris-HCl (pH 8.8) at 80°C for 5 min. Electrophoresis was performed at room temperature in 5–15% gradient gels (26).

Data analysis. All experiments were repeated with at least five to eight different preparations of SR membranes. Data are presented as mean ± SEM or as representative individual experiments when appropriate. When the analyses compared two data groups, we used t tests to examine for significant differences (p < 0.05). When the analyses compared more than two groups, we used analysis of variance. When the computed F ratio of an analysis of variance exceeded the critical F ratio (at p = 0.05), the Student-Newman-Keuls test was used to identify the group(s) that were significantly different.

RESULTS

SR Ca^{2+} pump activity in isolated rabbit cardiac SR membranes. The Ca^{2+} ATPase and oxalate-dependent Ca^{2+} uptake rates were significantly lower in fetal than in adult SR membranes (Table 1). This was evident even though the preparative techniques used for membrane isolation had been optimized for these activities (8). The stoichiometry of the rates of oxalate-dependent $^{45}Ca^{2+}$ uptake to Ca^{2+} ATPase (in the presence of the Ca^{2+} ionophore A23187) was significantly lower in the fetus (Table 1). These data are in agreement with previous reports of developmental trends in sheep and rabbits (5–8).

SR anion cotransport during cardiac development. To identify an anion-dependent developmental effect on SR, we compared the effects of the dicarboxylic anions maleate and succinate with the nontransportable zwitterionic PIPES. Compared with 40 mM of PIPES, 40 mM of maleate or succinate significantly increased the Ca^{2+} ATPase rate in fetal and adult SR membranes (Fig. 1). The percentage of increase in the Ca^{2+} ATPase rate with maleate or succinate was significantly greater in the adult SR (Fig. 1). The stimulation of Ca^{2+} ATPase activity required 20 mM maleate or succinate in both age groups and was saturated at 40 mM (data not shown).

Table 1. *Data from fetal and maternal cardiac SR membranes**

	Fetal	Adult
ATPase rate		
Mg^{2+}-dependent	104 ± 10†	196 ± 20
Ca^{2+}-dependent	159 ± 16†	294 ± 31
Ca^{2+}-dependent 2 μM A23187	343 ± 33†	604 ± 73
% increase with A23187	117 ± 13	106 ± 14
$^{45}Ca^{2+}$ uptake rate	51 ± 8†	276 ± 26
Uptake:ATPase	0.15 ± 0.02†	0.46 ± 0.05

* Values are means ± SEM ($n = 7$). ATPase and $^{45}Ca^{2+}$ uptake rates (nmol/mg/min) were measured as described in Materials and Methods, in 40 mM of PIPES (pH 6.8). $^{45}Ca^{2+}$ uptake was determined in the presence of 5 mM of oxalate. The uptake to ATPase ratio was calculated with the Ca^{2+} ATPase rate in the presence of 2 μM A23187. The Ca^{2+} ionophore A23187 had no effect on the Mg^{2+} ATPase rate.
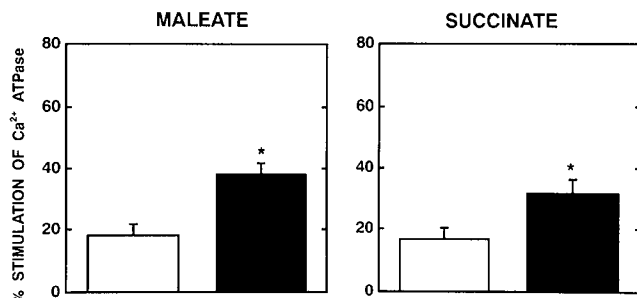
† Significantly different from adults at $p < 0.01$.

Fig. 1. The effect of dicarboxylic anions on Ca^{2+} ATPase activity. Ca^{2+}-dependent ATPase rates were measured in fetal (*open bars*) and maternal (*black bars*) cardiac SR as described in Materials and Methods. The fetal and maternal control Ca^{2+}-dependent ATPase rates in 40 mM of PIPES were 159 ± 16 and 294 ± 31 nmol/mg/min, respectively (Table 1). Substitution of 40 mM maleate or succinate significantly increased the Ca^{2+}-dependent ATPase activity in both age groups at $p < 0.01$. *, % increase is significantly greater in the adults compared with the fetuses at $p < 0.01$.
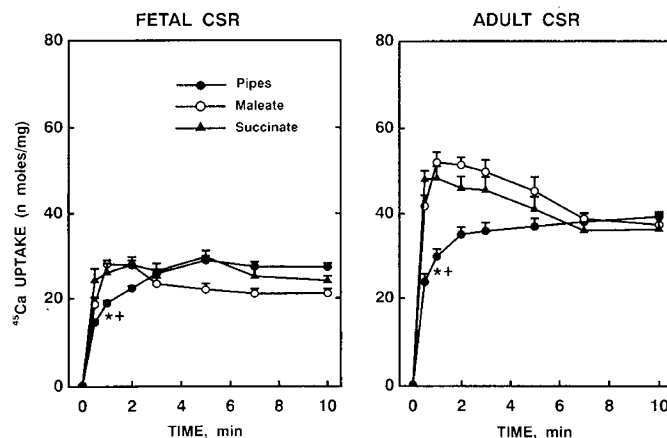

Fig. 2. The effect of dicarboxylic anions on oxalate-independent $^{45}Ca^{2+}$ uptake activity. ATP-dependent Ca^{2+} uptake in the absence of oxalate was measured as described in Materials and Methods. *CSR*, cardiac sarcoplasmic reticulum. + and * indicate that maleate and succinate significantly ($p < 0.01$) increased Ca^{2+} uptake at t = 1 min, compared with Pipes.

Maximal dicarboxylic anion-independent Ca^{2+} uptake was lower in the fetal SR than in the adult (Fig. 2, $p < 0.01$). Forty mM of maleate or succinate significantly increased the maximal Ca^{2+} uptake in the adult SR (Fig. 2, $p < 0.01$), whereas there was no significant effect on maximal uptake in fetal SR. However, maleate and succinate stimulated the earlier attainment of the maximal Ca^{2+} uptake in fetal as well as adult SR (Fig. 2), which

suggests that the rate of net Ca^{2+} uptake was stimulated by maleate and succinate in both the fetal and adult SR membranes. The apparent rate of dicarboxylic anion-stimulated $^{45}Ca^{2+}$ uptake in this early portion of the time course appears to be lower in the fetus (Fig. 2). One min after the reaction was initiated with ATP, the percentage of stimulation of Ca^{2+} uptake by maleate and succinate was similar in the fetal and adult SR membranes.

^{14}C-succinate uptake was significantly lower in fetal than in adult SR membranes (Fig. 3, $p < 0.01$ at t = 1 min). This was evident throughout the time course for ^{14}C-succinate uptake. The apparent rate of ^{14}C-succinate uptake in this early portion of the time-course appears to be lower in the fetus (Fig. 3).

Relative purity of SR membrane preparations. Gradient SDS-PAGE demonstrated several differences in the protein profiles of the fetal and adult rabbit SR membranes (Fig. 4). The abundance of SR Ca^{2+} pumps appears to be lower in the fetal SR membranes. Proteins of approximately 450 kD, 50–55 kD, and 25–30 kD also appeared to be of lower abundance in the fetal SR membranes, whereas a 55- to 60-kD protein appeared to be present at greater abundance (Fig. 4). These results are in general agreement with previously published data from fetal and adult sheep (5, 6).

The SR protein yield was significantly lower in the fetal SR (Table 2). There was no significant difference in fold enrichment by the isolation procedure, or in the percentage of contamination with sarcolemma or mitochondria (Table 2). The contamination of the SR membranes with sarcolemma and mitochondria was estimated at <1% and <3%, respectively, of the cardiac homogenates (Table 2). All reactions were performed in 10 mM sodium azide, thereby avoiding any possible influence of this residual activity on our results (23).

In agreement with previously published data (7), we found that adult rabbit cardiac SR membranes contain a higher proportion of Mg^{2+}-dependent ATPase activity (Table 1) than typically is found in adult dog or sheep cardiac SR membranes (5, 27). However, the Mg^{2+} ATPase rates were not sensitive to the presence of dicarboxylic anions in the fetal or adult rabbit cardiac SR membranes (data not shown). Similar results have been noted in chicken and rabbit skeletal muscle membrane populations wherein the Mg^{2+}-dependent ATPase activity has been localized to a t-tubular protein (28, 29).
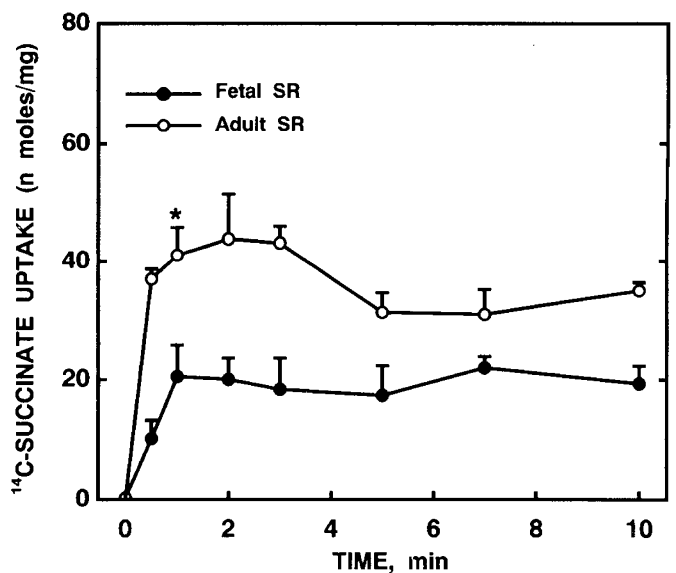

Fig. 3. Developmental differences in uptake of ^{14}C-succinate. ^{14}C-succinate uptake was measured under the same conditions as in Figure 3 (see Materials and Methods), except that ^{14}C-succinate and nonradioactive $CaCl_2$ were substituted. *, significantly ($p < 0.01$) lower uptake in the fetuses compared with the adults at t = 1 min.
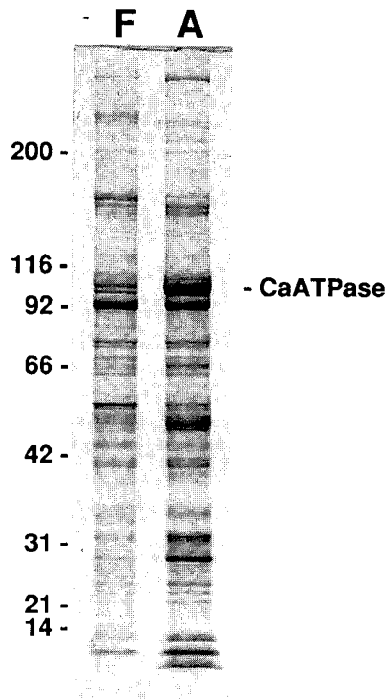
Fig. 4. PAGE of fetal and adult SR. SDS-PAGE of 25 μg of SR was performed as described in Materials and Methods on a 5–15% gradient slab gel. Molecular weight markers (in kD) are located to the right of the gel lanes, and the location of the SR Ca^{2+} pump is noted by CaATPase to the left of the gel lanes. F and A are fetal and adult SR, respectively.

Table 2. Relative purity of SR membrane preparations*

	Fetus	Adult
Yield (mg/g)	0.21 ± 0.03†	0.38 ± 0.05
Fold enrichment	18 ± 2	20 ± 3
% Contamination of SR membranes with		
Sarcolemma (Na⁺,K⁺ ATPase)	0.7 ± 0.1	0.9 ± 0.1
Mitochondria (Azide-sensitive Mg²⁺ ATPase)	2.9 ± 0.5	2.6 ± 0.3

* Values represent means ± SEM ($n = 5$–6). The variables were measured as in Materials and Methods. The percentages of recovery of sarcolemma and mitochondria are calculations based on the measurements of ouabain-inhibitable Na⁺,K⁺ ATPase, and azide-sensitive Mg²⁺ ATPase (see Materials and Methods).

† Significantly different from adults at $p < 0.02$.

DISCUSSION

SR-mediated anion transport has been demonstrated previously in skeletal muscle by a variety of techniques. These include the identification of anion channel activity by incorporation of SR protein into planar lipid bilayers (30–32), the demonstration of phosphate transport in cholate-solubilized SR Ca^{2+} pump that was reconstituted into proteoliposomes (33), and the recent demonstration of differential sensitivity of SR Ca^{2+} and oxalate transport to FITC (13). Using FITC to modify the skeletal SR Ca^{2+} pump under a variety of conditions, the data led us to suggest that skeletal muscle SR-mediated dicarboxylic anion transport activity is mediated by the Ca^{2+} pump protein, in a domain that is distinct from the domains that are responsible for ATP-dependent Ca^{2+} transport (13).

Before the present study, there was limited direct information available to support the concept of dicarboxylic anion transport in cardiac SR membranes (15–17). The present demonstration of dicarboxylic anion-stimulated increases in Ca^{2+} ATPase activity and $^{45}Ca^{2+}$ uptake and the demonstration of ^{14}C-succinate uptake clearly establish the presence of a dicarboxylic anion transport mechanism in rabbit cardiac SR. Furthermore, it is

apparent that the cardiac anion transport mechanism has properties similar to those of the skeletal muscle SR dicarboxylic anion cotransporter wherein anion and Ca^{2+} transport are invariably linked (10–13). However, we have not yet determined whether dicarboxylic anions are cotransported with Ca^{2+} in rabbit cardiac SR (i.e. the relationship between Ca^{2+} and anion flux). From the available data, we are unable to localize this cardiac anion transport mechanism to a specific SR protein like the SR Ca^{2+} pump.

Our data indicate that SR-dependent dicarboxylic anion transport, anion enhancement of the apparent rate of net Ca^{2+} transport, the stimulation of the Ca^{2+} ATPase rate by dicarboxylic anions, and anion-independent Ca^{2+} transport all are lower in the developing heart. It is apparent that the previously reported slower SR Ca^{2+} uptake rate in the fetus (5–8) can be attributed to lower anion transport as well as to slower anion-independent Ca^{2+} transport (Figs. 2 and 3). The slower anion-independent Ca^{2+} transport is consistent with our recently published demonstration of lower expression of SR Ca^{2+} pumps (8), as well as with available morphometric data (34). It is evident that there is developmental regulation of both the SR dicarboxylic anion transport mechanism as well as the Ca^{2+} pump in the rabbit heart (8). Although both processes develop roughly in parallel, the mechanism(s) responsible for this developmental regulation remain unknown, and it is not known if they are regulated independently or by a single process.

Our results indicate that the slower cardiac relaxation in the developing heart is temporally related to at least two developmentally regulated SR-mediated activities, Ca^{2+} and anion transport. Other unknown factors also may be involved. Although it is possible that developmental regulation of the SR Ca^{2+} pump and/or of anion transport activity may form an important part of the subcellular basis of the perinatal change in mammalian cardiac relaxation, these data do not allow us to identify a cause-and-effect relationship or to specifically exclude additional mechanisms. The recent demonstration of increased Na⁺-Ca²⁺ exchanger activity and immunoreactive protein content in the fetal rabbit heart compared with the adult (35) raises the possibility that the slower cardiac relaxation in the fetus may relate to a dependency on alternate mechanisms of Ca^{2+} egress from the cytosol (2).

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