Steady-State mRNA Levels of the Sarcolemmal Na⁺-Ca²⁺ Exchanger Peak Near Birth in Developing Rabbit and Rat Hearts

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Abstract To functionally compensate for an underdeveloped sarcolemmal reticulum in immature cardiomyocytes, it has been proposed that the sarcolemmal Na⁺-Ca²⁺ exchanger may assume a more predominant role for regulating cytosolic Ca²⁺. Previous studies using sarcolemma prepared from developing rabbit hearts demonstrated that Na⁺-dependent Ca²⁺ uptake and exchanger protein content were highest at birth and declined postnatally. To further investigate the significance of the Na⁺-Ca²⁺ exchanger during normal myocardial development, steady-state mRNA levels of the cardiac Na⁺-Ca²⁺ exchanger were quantitated by Northern blot and slot-blot analyses using poly(A⁺) RNA isolated from rabbit and rat ventricles at various fetal and postnatal ages. Northern analyses were performed with a 1.35-kb guinea pig cardiac Na⁺-Ca²⁺ exchanger cDNA probe. Exchanger mRNA levels were quantitated by densitometric scans of the slot blots, and results were normalized by reprobing the same blots with ³²P 5'‑end-labeled oligo(dT). In both species, exchanger mRNA levels peaked near birth and declined postnatally. Maximal levels were approximately sixfold greater in the late fetal rabbit (gestational day 29) and eightfold greater in the early newborn rat (postnatal day 1) compared with adults of the respective species. The parallel changes in exchanger mRNA and protein levels suggest that developmental regulation of cardiac Na⁺-Ca²⁺ exchanger expression involves pretranslational control mechanisms. These results support the concept that during normal cardiac development, Na⁺-Ca²⁺ exchanger expression is maximal near the time of birth and then declines postnatally as Ca²⁺ regulation by the sarcoplasmic reticulum reaches functional maturity. (Circ Res. 1994;74:354-359.)

Key Words • fetus • newborn • cardiac development • Na⁺-Ca²⁺ exchanger

Fetal and newborn hearts from a variety of species exhibit profound differences in contraction and relaxation processes compared with adults. Many developmental changes in cardiac contractile function have been attributed to alterations in Ca²⁺ regulation by the cardiomyocyte. Ultrastructural and biochemical analyses indicate that Ca²⁺ regulation by the sarcoplasmic reticulum (SR) of the immature heart is deficient compared with that of the adult heart. To compensate for an underdeveloped SR, immature cardiomyocytes appear to be relatively more dependent on transsarcolemmal Ca²⁺ fluxes for directly regulating Ca²⁺ concentrations at the contractile proteins during excitation-contraction coupling.

The major Ca²⁺-regulatory proteins of the sarcolemma (SL) include the voltage-sensitive Ca²⁺ channels and the Na⁺-Ca²⁺ exchanger. Several studies indicate that voltage-sensitive Ca²⁺ channels are functionally diminished in early development of the chicken and rabbit heart, despite a relatively greater dependence on transsarcolemmal Ca²⁺ influx for contraction. It has been hypothesized that the Na⁺-Ca²⁺ exchanger may provide an alternative mechanism for Ca²⁺ influx in the newborn rabbit heart. In contrast to studies of the developing chicken and rabbit, Cohen and Lederer demonstrated that voltage-sensitive Ca²⁺ channel current density was higher in neonatal rat cardiomyocytes than in adult myocytes. They proposed that a greater Ca²⁺ current was required as a consequence of the deficient SR in the neonatal rat heart cells (based on ultrastructural analysis and lack of response to ryanodine or caffeine). The potential role of SL Na⁺-Ca²⁺ exchange was not explored, but it seems logical to postulate that Ca²⁺ influx via the exchanger may be greater in neonatal rat cardiomyocytes because of the reduced SR Ca²⁺ uptake capacity. Thus, cardiac Na⁺-Ca²⁺ exchange activity may be high at birth in both rabbits and rats as a compensatory mechanism for SR immaturity, even though Ca²⁺ channel ontogeny may differ in these two species.

Several indirect lines of evidence support the hypothesis that Na⁺-Ca²⁺ exchanger activity will be greatest at birth. Using perfused rabbit hearts, Nakanishi and Jarmakani demonstrated that the negative inotropic effect of high extracellular Na⁺ concentration was greater in newborns compared with adults. Although other factors could contribute to these findings, the results are at least consistent with a relatively greater functional response of Na⁺-Ca²⁺ exchange in the newborn. Vetter and colleagues have shown that Na⁺-dependent Ca²⁺ uptake was greater in crude membrane preparations from chicken and rat hearts during late fetal and early newborn periods than in adult preparations. More recently, we found that Na⁺-Ca²⁺ exchange activity and exchanger protein content in SL vesicles prepared from rabbit hearts were greatest during late fetal and early newborn periods of development. The correlation between exchanger activity and protein levels suggests that the increase in exchanger activity

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during the perinatal period is due to increased exchanger expression rather than direct or indirect functional alterations of the protein. However, comparison of results from experiments using SL vesicles prepared from different age groups may be affected if the enrichment of SL membrane subfractions changes with age.

To further evaluate our hypothesis that expression of the cardiac SL Na\(^{+}\)-Ca\(^{2+}\) exchanger is maximal near the time of birth and declines postnatally, we measured steady-state mRNA levels in rabbit and rat hearts. The purposes of the present study were not only to characterize normal developmental expression of the exchanger but also to provide initial insight into the contribution of translational and transcriptional control mechanisms to the developmental regulation of cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger expression.

**Materials and Methods**

**Animals**

Hearts were rapidly excised from pentobarbital-anesthetized (=50 mg/kg) rabbits and rats of several age groups. New Zealand White rabbits of either sex included 25-day and 29-day fetuses (term, 31 days) and 1-, 7-, and 14-day-old immature animals. Adults were sexually mature males (4 to 6 months old). Sprague-Dawley rats of either sex included 19-day fetuses (term, 21 days), 1-, 7-, 14-, and 21-day-old immature animals, and 3- to 4-month-old male adults. For each preparation, the ventricles were isolated, rinsed with ice-cold phosphate-buffered saline, rapidly frozen, and stored at \(-70^\circ\)C until used for further RNA isolation. Hearts from each age group were pooled to provide a sufficient amount of tissue for each time point. Experimental protocols for use of these animals were approved by the University of South Alabama Institutional Animal Care and Use Committee.

**RNA Isolation**

Total RNA was isolated from the ventricles using RNA STAT-60 (Tel-Test “B” Inc, Friendswood, Tex) based on the single-step method developed by Chomczynski and Sacchi.\(^{18}\) Samples were stored in 70% ethanol and 0.3 mol/L sodium acetate (pH 5.2) at \(-70^\circ\)C until further processing.

**Poly(A\(^+\)) Selection**

Poly(A\(^+\)) RNA was selected by affinity chromatography using oligo(dT)-cellulose based on the method of Aviv and Leder\(^{19}\) and modified for batch processing using Spin-X columns (Costar Corp, Cambridge, Mass). Briefly, total RNA (2 to 5 mg) was denatured at 70°C and loaded onto 50 to 100 mg oligo(dT)-cellulose (type 3, Becton Dickinson, Bedford, Mass) under high-salt conditions in buffer containing 10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L EDTA, and 0.1% sodium dodecyl sulfate (SDS) with 500 mmol/L NaCl. The cellulose was loaded onto the spin columns and washed by centrifugation (2000g) at room temperature with the same buffer except containing 150 mmol/L NaCl. Poly(A\(^+\)) RNA was eluted using buffer containing 10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L EDTA, and 0.05% SDS. Two sequences of purification were used. The final eluted poly(A\(^+\)) RNA was quantitated by UV absorbance at 260 nm, precipitated at \(-20^\circ\)C with 70% ethanol and 0.3 mol/L sodium acetate (pH 5.2) using 5 μg/mL glycogen carrier (Boehringer Mannheim, Indianapolis, Ind), and stored at \(-70^\circ\)C until further analysis.

**Northern Analysis**

Sample RNA was denatured with glyoxal, size-fractioned in a 1% agarose gel with 10 mmol/L sodium phosphate (pH 7.0) running buffer at 4°C, and transferred onto nylon membrane by capillary action with 10× standard saline citrate (SSC) (1× SSC contains 150 mmol/L NaCl and 15 mmol/L sodium citrate) according to the method of Thomas.\(^{20}\) RNA was then UV-cross-linked to nylon, and the glyoxylated was reversed with 20 mmol/L Tris base at 95°C. Membranes were prehybridized at 42°C for 4 to 6 hours in the presence of 50% formamide, 5× Denhardt’s solution (1× Denhardt’s solution contains 0.02% FicolI, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 6× SSPE buffer (1× SSPE buffer contains 150 mmol/L NaCl, 10 mmol/L sodium phosphate, and 1 mmol/L EDTA), 10% dextran sulfate, 1% SDS, and 100 μg/mL denatured sheared salmon-sperm DNA. Sequential hybridizations were performed under the same conditions for 12 to 18 hours with the addition of 50 μg/mL denatured sheared salmon-sperm DNA and excess (5 ng/mL) denatured probe. The probe was a 1.35-kb guinea pig cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger cDNA (generously provided by Dr K.D. Philipson, University of California, Los Angeles) that contained 1 kb of the 3′ coding region. The cDNA probe was random-prime-labeled with biotin-14-dCTP using the Random Prime Images labeling kit (US Biochemical Corp, Cleveland, Ohio) on the basis of methodology developed by Feinberg and Vogelstein.\(^{21,22}\) After hybridization, membranes were washed at a final stringency of 0.5× SSC at 65°C. The hybridized probe was detected by chemiluminescence using a Gene Images nonisotopic detection kit (US Biochemical) with XRP film (Eastman Kodak, Rochester, NY).

**Northern Slot-Blot Analysis**

Sample RNA was denatured with glyoxal, serially diluted with ice-cold 10× SSC, and applied to nylon using a slot filtration manifold (model PR648, Hoefer Scientific Instruments, San Francisco, Calif) under vacuum (10 mm Hg) with 10× SSC washes. After UV cross-linking, the glyoxylated was reversed, and the blots were prehybridized, hybridized, and washed under the same conditions as described above, except the exchanger probe was random-prime-labeled with \(^{32}\)P-dCTP and was added in excess (1×10\(^{6}\) cpm/mL) for the hybridization. After the high-stringency wash, the blots were exposed to XRP film using double-intensifying screens at \(-70^\circ\)C. Autoradiograms were analyzed using laser densitometry, and several timed exposures were made to ensure that the densitometric scans were within the linear response range.

**Normalization of the Amount of Steady-State Exchanger mRNA**

RNA slot blots were stripped in a solution containing 0.5% sodium pyrophosphate, 0.2 mmol/L EDTA, 0.1× Denhardt’s solution, and 5 mmol/L Tris-Cl, pH 7.5, for 1 to 2 hours at 65°C and exposed to XRP film 12 to 24 hours to assess dissociation of the probe from the blots. The blots were then reprobed with excess (150 pmol/mL) \(^{32}\)P 5′-end-labeled oligo(dT)\(_{18}\) according to the method of Harley\(^{23}\) with 1% SDS included in the prehybridization and hybridization solutions. After a 2- to 4-hour hybridization at 30°C, the blots were washed with 2× SSC at room temperature and exposed to XRP film at room temperature. Experiments were performed in duplicate for each age group. Autoradiograms of several timed exposures were analyzed by laser densitometry. The amount of exchanger probe was normalized to the amount of hybridized oligo(dT)\(_{18}\) on the basis of densitometric measurements of the Northern slot blots. Exchanger steady-state mRNA levels were quantitated relative to the adult levels observed for each species.

**Results**

**Qualitative Analysis of Exchanger mRNA**

Northern analysis of the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger mRNA using poly(A\(^+\)) RNA isolated from adult rat, rabbit, and dog hearts reveals a predominant 7-kb transcript with only slight variations in size (Fig 1). A minor 4-kb transcript was also observed in the rabbit. Nicoll et
al found that a 7-kb transcript corresponded to the exchanger mRNA in canine heart. The relative abundance of the cardiac exchanger mRNA in mature myocardium appears to be species dependent (dog > rabbit > rat). With longer exposures (data not shown), only the rabbit was clearly observed to contain the minor band at 4 kb with no other bands detected.

Northern analysis using poly(A') RNA from developing rabbit (Fig 2) and rat (Fig 3) hearts reveals the presence of the same 7-kb transcript from fetal stages through the adult age groups. No other bands were detected in the rat heart; however, the minor 4-kb transcript persisted for all stages of development in the rabbit (Fig 2). In both Figs 2 and 3, the abundance of the exchanger mRNA appears to be greatest in the late fetal or early newborn and declines postnataally to the lowest levels in the adults. The 4-kb transcript observed for the rabbit does not interfere with quantitations of the exchanger mRNA by Northern slot-blot analysis, since the abundance of the 4-kb transcript is quite minor and the relative ratio of the 7-kb and 4-kb transcripts appears to remain constant throughout development.

Quantitative Analysis of Exchanger Steady-State mRNA Levels

Northern slot-blot analysis of poly(A') RNA from developing rabbit (Fig 4) and rat (Fig 5) hearts confirms a more abundant exchanger message level (top panels) in the late fetal and early newborn with a subsequent postnatal decline in both species. The same blots were stripped and reprobed with [32P] 5'-end-labeled oligo(dT) (lower panels). Variations were minimal in the total amount of message present, as demonstrated by the relative amounts of bound oligo(dT) probe.

Densitometric quantitation of the hybridized exchanger probe was normalized to the amount of hybridized oligo(dT) probe. Use of the hybridized oligo(dT) represents the total amount of poly(A') RNA immobilized in each slot. Therefore, normalization of the
amount of hybridized exchanger with that of hybridized oligo(dT) expresses the content of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger mRNA relative to the total pool of all mRNA species present. The abundance of exchanger mRNA was then expressed relative to the adult levels for each species (Fig 6). Values presented in Fig 6 represent the average of duplicate experimental determinations. For both rabbit and rat, the exchanger steady-state mRNA levels were greatest during the late fetal and early newborn periods and declined postnatally to the lowest levels observed for the adults. Steady-state exchanger mRNA levels were approximately sixfold higher in fetal rabbits and eightfold higher in newborn rats compared with adults of the respective species.

**Discussion**

These data provide strong evidence supporting the hypothesis that expression of the SL Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger in the developing heart is maximal near the time of birth and declines postnatally as other Ca\textsuperscript{2+}-regulatory mechanisms gain functional maturity. Considering previous studies demonstrating maximal exchanger activity and protein levels in fetal and newborn hearts,\textsuperscript{15-17} our present results suggest that transcriptional/posttranscriptional rather than translational mechanisms regulate Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression during normal myocardial development.

In examining the exchanger message size, Northern analysis of newborn and adult rabbit heart RNA reveals a 7-kb transcript, similar to the transcript size first shown to correspond to a canine cardiac exchanger mRNA by Nicoll et al.\textsuperscript{24} Northern analysis of RNA from a variety of species and tissues studied to date has shown the same hybridizing transcript of \(\approx 7\) kb, corresponding to a similar exchanger mRNA.\textsuperscript{25-30} Several minor bands were also detected by Northern analysis using total RNA from these species. This has been a common observation with no clear explanation.\textsuperscript{25-30} It has been suggested that the minor bands are artifacts of ribosomal rRNAs,\textsuperscript{26,28} but this may be only a partial explanation, since the minor band of \(\approx 4\) kb found in the rabbit persists when poly(A\textsuperscript{+}) RNA is used. The poly(A\textsuperscript{+}) RNA used for these experiments was determined to be relatively free of contaminating rRNAs, on the basis of the absence of 28S or 18S rRNAs in an ethidium bromide-stained agarose gel containing 6 \(\mu\)g poly(A\textsuperscript{+}) RNA (data not shown). In comparing poly(A\textsuperscript{+}) RNA from adult rat, rabbit, and dog hearts, we found the minor 4-kb transcript only in the rabbit heart. In contrast to our results, a minor 4-kb transcript has previously been reported in poly(A\textsuperscript{+}) RNA from rat heart.\textsuperscript{30} The reason for this discrepancy is not clear, but it may be due to differences in the purity of the poly(A\textsuperscript{+}) RNA preparations.

In rabbit heart, the 4-kb transcript remained after higher stringency washes of 0.1 \(\times\) SSC at 65°C (data not shown). On the basis of the guanosine and cytosine content of the exchanger cDNA probe, the calculated
melting temperature would predict a 94% to 100% complementation of the probe and hybridized transcript under these conditions. Although this 4-kb transcript may share at least one region of high homology with the 7-kb exchanger mRNA, it remains unclear whether the 4-kb transcript is a processed exchanger mRNA or an isoform. By use of poly(A+) RNA prepared from the developing hearts of rabbit and rat, Northern analysis clearly shows that the 7-kb transcript is most abundant early in development and declines postnatally, with no detectable changes observed in the message size. For the rabbit, the minor 4-kb transcript persisted throughout development and appeared to remain in constant proportion compared with the 7-kb message. This might argue against the 4-kb transcript being an exchanger isoform, since expression of most isoforms shift during development.

Quantitation of steady-state mRNA levels was performed using Northern slot-blot analysis with the same hybridization and washing conditions as used for Northern analysis. When the steady-state exchanger mRNA levels are compared at various developmental stages in both the rabbit and rat, it is evident that the abundance of exchanger is greatest during the late fetal and early newborn periods. These findings support earlier observations that the highest levels of SL exchanger activity occur during perinatal periods of myocardial development in chick,15 rat,16 and rabbit17 hearts. In contrast, Kumoro et al28 examined exchanger steady-state mRNA levels by Northern analysis in 17- and 19-week fetal human hearts (term, 40 weeks) and found that the fetal hearts contained less exchanger mRNA than did adult hearts. However, on the basis of the sparsity of myofibril content at 17 to 19 weeks of gestation in humans, these fetal hearts may represent much earlier stages of myocardial development compared with late fetal rabbit and rat hearts used in the present study.13,15,16 It may be that the exchanger is expressed at relatively low levels in very early myocardial development, but as the fetus matures and cardiac demand increases, expression of the exchanger increases to meet these requirements.

We speculate that the Na+-Ca2+ exchanger assumes a relatively greater functional role in regulating cytosolic free Ca2+ in the late fetal and early newborn heart. In mature cardiomyocytes, the exchanger operates predominately in a Ca2+ efflux mode during relaxation, but the exchanger is reversible and may contribute to Ca2+ influx during contraction in some species or under certain conditions (eg, elevated intracellular Na+).30-35 Nábauer and Morad36 have shown that Ca2+ influx through the Na+-Ca2+ exchanger and Ca2+ channels is sufficient to activate contraction in adult shark ventricular myocytes, which lack functionally relevant intracellular Ca2+-release pools. This situation may be analogous to immature mammalian myocytes with an underdeveloped SR.2,4,25 Additionally, Conforti et al37 have shown that the fetal rat heart exhibits a prolonged plateau phase during the action potential because of a sustained Na+ current. Conceivably, the electrogenic exchanger may operate in a Ca2+ influx mode during the prolonged action potential to provide Ca2+ at the contractile proteins for contraction in fetal and newborn rabbit and rat hearts. The exchanger presumably operates in a Ca2+ efflux mode during relaxation (as in the adult), but the relative magnitude of Ca2+ removed by the exchanger would be expected to be greater in immature myocytes.

In summary, SL Na+-Ca2+ exchanger steady-state mRNA levels are maximal near the time of birth in rabbit and rat hearts and decline postnatally. A greater abundance of the Na+-Ca2+ exchanger implies that Ca2+ regulation is relatively more dependent on Na+-Ca2+ exchange in the developing heart than in the adult heart. Since previous studies have shown that Na+-Ca2+ exchange activity and protein levels are highest in fetal and newborn hearts, it appears that a pretranslational event regulates exchanger expression during early myocardial development. The postnatal decline in Na+-Ca2+ exchanger expression coincides temporally with maturation of Ca2+ regulation by the SR, supporting the concept of a postnatal transition from the SL alone to the SL and SR functioning together as the major membrane components regulating intracellular Ca2+ during contraction and relaxation. Thus, some of the fundamental concepts of excitation-contraction coupling and relaxation that have been defined for mature cardiac myocytes may not be accurate in the fetal or newborn heart.
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