Developmental and Tissue-Specific Regulation of Rabbit Skeletal and Cardiac Muscle Calcium Channels Involved in Excitation-Contraction Coupling

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Abstract Two types of calcium channels signal excitation-contraction (E-C) coupling in striated muscle: dihydropyridine receptors (DHPRs, voltage-gated L-type calcium channels on the transverse tubule) and ryanodine receptors (RYRs, calcium release channels on the sarcoplasmic reticulum). Sarcolemmal depolarization activates the DHPR; subsequently, the RyR is activated and releases calcium that activates muscle contraction. We show in the present study that expression of the E-C coupling calcium channels is upregulated during myogenic development in the rabbit. Skeletal and cardiac muscle isoforms of the following genes were examined: the DHPR $\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$ subunits and the RyR. Distinct cardiac and skeletal muscle-specific cDNAs were isolated, encoding each of the DHPR subunits and the RyR. The skeletal muscle DHPR $\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$ subunits and the cardiac DHPR $\alpha_1$ subunit mRNA levels increased on the day of birth and at the adult stage compared with fetal levels. The skeletal and cardiac RyR mRNA levels increased on the day of birth and at adult stages compared with fetal levels. Ryanodine binding sites increased in both skeletal and cardiac muscle. We now provide a molecular explanation for the physiological "maturation" of the E-C coupling apparatus observed at the day of birth and during early postnatal development in both skeletal and cardiac muscles. Low levels of calcium channel expression in fetal cardiac and skeletal muscle make these tissues more sensitive to pharmacological therapy with calcium channel blockers, a phenomenon that has been reported in human neonates. (Circ Res. 1994;75:503-510.)

Key Words • ryanodine receptor • dihydropyridine receptor • sarcoplasmic reticulum • muscle development

Calcium signaling during striated muscle contraction is mediated by the cooperative activation of both voltage-sensitive calcium channels and intracellular calcium release channels. The voltage-sensitive calcium channels (dihydropyridine receptors [DHPRs]) in both cardiac and skeletal muscles comprise five subunits encoded by four separate genes. Similarly, skeletal and cardiac muscle contain distinct isoforms of homotetrameric ryanodine receptor (RyR)/intracellular calcium release channels encoded by separate genes. Thus, in each type of striated muscle, at least five separate genes encode the major channels forming the pathways for calcium signaling during muscle contraction. The primary structures of these calcium channels have been deduced from cDNA cloning, and their critical roles in excitation-contraction (E-C) coupling are well established. The present study demonstrates that expression of both classes of calcium channels, the voltage-gated calcium channel and the intracellular calcium release channel, is coordinately regulated during late myogenic development in the rabbit. Moreover, we show that the four genes encoding the five subunits of the DHPR are also coordinately regulated during late myogenic development.

We have chosen to focus on late events during development because the functional consequences of calcium channel gene expression on striated muscle contraction are more important during these later stages. Indeed, it has been shown that fetal development in utero and myogenic differentiation are abnormal in the dysgenic (mdg) mouse, which does not express the $\alpha_1$ subunit of the DHPR. Moreover, this mutation is lethal at birth. Thus, regulation of calcium channel gene expression in early stages of development is of interest in terms of the effects of altering calcium homeostasis on gene expression, whereas in the later developmental stages, the quality and quantity of muscle contraction are directly influenced by channel expression.

The five subunits of the DHPR have been termed $\alpha_1$, $\alpha_2$, $\beta$, $\delta$, and $\gamma$ (the $\alpha_2$ and $\delta$ subunits are encoded by a single gene and are linked by disulfide bonds). The DHPR subunits have been characterized structurally and recent studies have begun to define their functional roles. The $\alpha_2$ subunit modulates activation of the $\alpha_1$ subunit and the $\beta$ subunit substantially accelerates the activation and inactivation kinetics of the calcium channel current. The sarcoplasmic reticulum calcium release channel is localized to the junctional face membrane of the terminal cisternae and includes a large cytoplasmic foot structure and a transmembrane baseplate. Purification and biochemical characterization of both the skeletal and cardiac forms of the RyR have been achieved. Ultrastructural studies suggest that the cal-

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ium release channels are homotetramers comprising four RyR protomers, each of 565 KD, forming a 2 300 000-D structure. The skeletal 18-20 and cardiac 21-23 RyRs have been cloned, and their primary structures have been determined. Models based on hydrophatic analyses 19-21 and surface topographic mapping 24 indicate that 90% of the linear sequence encodes the cytoplasmic “foot structure” spanning the gap between the junctional face membrane of the sarcoplasmic reticulum and the transverse tubule, whereas 4 to 10 putative transmembrane regions are encoded by =10% of the 3’ terminal sequence.

Previously, regulation of the skeletal muscle DHPR and RyR genes by growth factors during myogenic differentiation in vitro was demonstrated. 25 Recently studies have reported the developmental regulation of expression of the α1 and α2 subunits of the DHPR during skeletal muscle development 26-28 and of sarcoplasmic reticulum proteins. 29 Numerous studies have demonstrated functional differences between fetal and mature hearts in terms of calcium handling. 30-35 Moreover, it has been shown that newborn hearts require higher extracellular calcium to optimize contractility, and a “deficiency” in sarcoplasmic reticulum development has been proposed. 36-39 Furthermore, it has been shown that the neonatal heart is highly sensitive to calcium channel blockers. 40

In the present study, we have directly examined the developmental regulation of the expression of the two calcium channels (and their subunits) required for E-C coupling in both cardiac and skeletal muscle. We have demonstrated that mRNAs encoding these calcium channels are coordinately upregulated at critical time points during myogenic development in rabbit skeletal and cardiac muscles. The most dramatic induction of calcium channel mRNA occurred on the day of birth. These data provide a molecular basis for understanding the previously observed functional differences between neonatal and mature sarcoplasmic reticulum calcium handling.

Materials and Methods

Animals

New Zealand White rabbits were used for the present studies. The gestational period in these animals is 30 days. Animals were cared for according to the standard IACUC protocols in the Mount Sinai animal facility.

cDNA Probes

The skeletal muscle RyR cDNA probe, CRC105, corresponding to nucleotides 13546-15361 (plus 11 adenine residues at the 3’ end), was isolated from a rabbit skeletal muscle library as previously described. 18 The cardiac RyR cDNA probe, HCR1, isolated by reverse-transcriptase/polymerase chain reaction amplification of rabbit cardiac total RNA, corresponds to nucleotides 5027-5647 of the cDNA encoding the rabbit cardiac RyR. 41 The DHPR α1 subunit cDNA, DHPSKE1L, 25 is a skeletal muscle–specific 550-bp cDNA, corresponding to nucleotides 3046-3596 of the rabbit skeletal DHPR cDNA sequence (isolated by reverse-transcriptase/polymerase chain reaction amplification of rabbit skeletal muscle total RNA). The cardiac DHPR probe, DHPCARD1, 42 corresponds to nucleotides 3402-3971 of the rabbit cardiac DHPR cDNA sequence (isolated by reverse-transcriptase/polymerase chain reaction amplification of rabbit cardiac muscle total RNA). The α2 subunit cDNA, RSKM-A2, corresponding to nucleotides 1992-2519, the β subunit cDNA, RSKM-B, corresponding to nucleotides 1025-1493, and the γ subunit cDNA, RSKM-G, corresponding to nucleotides 43-726, 43 were all isolated by reverse-transcriptase/polymerase chain reaction amplification of rabbit skeletal muscle total RNA as previously described. 44 Briefly, sense and antisense primers were designed on the basis of the published cDNA sequences for each of the calcium channel subunits. First-strand cDNA was synthesized using 1 μg of total rabbit skeletal muscle RNA and 2 pmol of random primers with avian myeloblastosis virus reverse transcriptase (Life Sciences) at 42°C for 1 hour. Thirty-five cycles of polymerase chain reaction with Taq DNA polymerase (Perkin-Elmer Cetus) were then performed on 0.1% of the products of first-strand cDNA synthesis with 20 pmol of each primer by use of the following parameters: 1 minute denaturation at 94°C, 1-minute annealing at 55°C, and 1-minute extension at 72°C. The polymerase chain reaction products were separated from primers on low-melt agarose gels, purified, and subcloned into pBluescript (Stratagene). The cDNA clones were sequenced with an ABI 373 automated sequenator; the M13 forward and reverse primers were used. The 28S ribosomal RNA probe was a synthetic antisense oligonucleotide based on the cloned human 28S cDNA with the following sequence: CATTCCCAAGCAACCCGACTCCGGGAACAC. Oligonucleotide probes were labeled by using T4 kinase and [γ-32P]ATP to a specific activity of >109 cpm/μg. All cDNA probes were uniformly labeled with random primers by using Klenow and [α-32P]dCTP to a specific activity of >109 cpm/μg.

RNA Preparation and Analysis

Total cellular RNA was purified from rabbit tissue samples (whole heart and hind-limb skeletal muscle) by using the guanidinium isothiocyanate/cesium chloride centrifugation method essentially as previously described. 45 The cellular composition of the whole-heart samples at each stage of development consisted primarily of cardiac myocytes and fibroblasts, 44-46 whereas the skeletal muscle samples were predominantly skeletal myocytes. 47-49 RNA was quantified by spectrophotometry at 260 nm, and the ratio of absorbance at 260 nm to that at 280 nm was >1.8 for all samples. Degradation of RNA samples was monitored by the observation of appropriate 28S-to-18S ribosomal RNA ratios as determined by ethidium bromide staining of the agarose gels. Northern blot analyses were carried out as previously described. 18 Total cellular RNA (20 μg) was denatured in formamide agarose gels run at 30 mA overnight to provide resolution of high-molecular-weight mRNAs. RNA transfer onto nitrocellulose filters was conducted overnight with 10X standard saline citrate (SSC) transfer buffer. Filters were then baked at 80°C in a vacuum oven for 2 hours. Slot blot hybridization analyses were also performed. RNA samples were added to a solution of 50% formamide, 7% formaldehyde, and 1× SSC and denatured by heating at 65°C for 15 minutes and cooled on ice. Two volumes of 20× SSC was added to each sample, and serial dilutions (2.5, 5, and 10 μg) were spotted onto nitrocellulose filters by using a slot blot manifold (Bio-Rad). After all of the samples had passed through the filter, 10× SSC was passed through each slot. Filters were air-dried and then baked at 80°C in a vacuum oven for 2 hours. Northern and slot blot filters were prehybridized overnight in buffer containing 1× Denhardt’s solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 5× SSC (1× SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0), 0.025 mol/L sodium phosphate (pH 7.4), sonicated calf thymus DNA (50 μg/ml), 0.1% sodium dodecyl sulfate (SDS), and 5% (vol/vol) formaldehyde. Blots were hybridized with cDNA probes in the same buffer mixture overnight at 42°C. Hybridization to oligonucleotide probes was carried out in the same hybridization buffers using 10% formamide. Blots were then washed at a final stringency of 0.2× SSC/0.1% SDS at 65°C for 15 minutes.
Filters were exposed at −80°C on x-ray films (X-OMAT, AR, Eastman Kodak) with a single intensifying screen or at room temperature on a storage phosphor screen (Molecular Dynamics). The yield of total RNA per gram tissue from skeletal and cardiac muscles did not vary significantly with developmental stage (all of the yields in terms of micrograms RNA per gram tissue were within ±20%, and the variations did not correlate with development).

**Quantification of Calcium Channel mRNA**

All filters were hybridized separately to a calcium channel-specific cDNA probe and a 28S probe to control for total RNA bound to the filters and washed under conditions used to achieve specific hybridization during Northern hybridization analyses. After each hybridization, a single exposure time was used for densitometric quantification on a phosphorimager using IMAGE QUANT software. For phosphorimager analyses, values for each mRNA were normalized to 36S ribosomal RNA, and the relative mRNA value of this ratio for the fetal time point was set at 1.0. To quantify changes in mRNA levels, phosphorimager data at each time point were obtained from three or more individual animals.

**Membrane Preparations**

Cellular membranes used for ryanodine binding assays were prepared essentially as previously described. Fresh rabbit skeletal muscle and rabbit whole heart were flash-frozen in liquid nitrogen and stored at −80°C until use. Frozen tissue was minced with scissors and homogenized with an OMNI homogenizer in 10 vol of 10 mMol/L HEPES, pH 7.4, 20 mMol/L KCl, 0.5% CHAPS, 100 mMol/L phenylmethylsulfonyl fluoride, and 0.5% (w/v) leupeptin. After centrifugation at 3000g for 10 minutes with a Beckman 60-Ti rotor, pellets were reextracted twice by homogenizing in the same media and recentrifuging at the same speed. The resulting pellet (pellet 1) was resuspended in storage buffer containing 0.3 mol/L sucrose, 10 mmol/L imidazole (pH 7.4), and 0.5 μmol/mL leupeptin. The low-speed supernatants were pooled and centrifuged at 120000g for 90 minutes with a Beckman 45-Ti rotor to obtain pellet 2, which was resuspended in storage buffer.

[3H]Ryanodine Binding Assay

Binding of [3H]ryanodine to membrane samples was determined as previously described. Membrane samples (pellet 2; see above) were incubated for 90 minutes at 37°C in 80 μL of solution containing 50 mmol/L Tris (pH 7.4), 0.5 mol/L KCl, 10 mmol/L ATP, 1.35 mmol/L CaCl2, and 10 mmol/L [3H]ryanodine. After incubation, 0.125 mg gamma globulin and 12 mg polyethylene glycol 8000 were added, and the suspension was mixed. After an additional 10 minutes, samples were centrifuged for 10 minutes at 12000g in an Eppendorf centrifuge. The supernatants were aspirated, and the pellets were washed with buffer and solubilized in soluene. After an addition of 4 mL Ecolscint scintillation cocktail (NEN), radioactivity was determined in a liquid scintillation counter. Non-specific binding was measured in the presence of 6 μmol/L unlabeled ryanodine. Each data point was obtained from three or more animals.

**Results**

**Specificity of cDNA Probes**

Northern blot hybridization analyses were performed by using each cDNA probe to demonstrate the specificity of the probes and the tissue distribution of each mRNA. As shown in Fig 1, cDNAs encoding the skeletal and cardiac muscle DHPRs and their subunits each hybridized to single specific bands in all tissue RNA samples, with the exception of the cardiac αi subunit and the skeletal β subunit (see below). The skeletal DHPR αi subunit cDNA probe hybridized to a single 6.5-kb mRNA in mixed skeletal muscle and in both fast-twitch tibialis anterior muscle and slow-twitch soleus muscle (Fig 1a). The cardiac DHPR αi subunit cDNA hybridized to three specific mRNA species, 7.5, 9, and 22 kb. In addition, the cardiac DHPR αi subunit cDNA recognized the 6.5-kb skeletal mRNA species, which was consistent with both skeletal muscle and heart mRNA species. The specific mRNA species of the cardiac DHPR αi subunit cDNA were also consistent with the specific mRNA species of the skeletal muscle DHPR αi subunit cDNA.
DHPR mRNA (Fig 1b). The αβ subunit cDNA detected an ≈8 kb mRNA species in increasing relative abundance in heart, brain, and skeletal muscle but not in uterus or kidney (Fig 1c). The β subunit cDNA probe detected an ≈3.4 kb mRNA in brain. In skeletal muscle, the β subunit cDNA probe detected a major ≈ 1.3 kb skeletal muscle isoform and a less abundant, larger ≈3.4 kb mRNA corresponding to the size of the brain β subunit isoform (Fig 1d). By use of this same probe, no signals were detected in heart, kidney, or uterus. The γ subunit cDNA, which included the entire coding region of the rabbit skeletal muscle mRNA, hybridized to an ≈1.2 kb transcript in skeletal muscle; minor signals were detected migrating at higher molecular weights, which may represent nonspecific hybridizations (Fig 1e).

The skeletal RyR cDNA probe hybridized to an ≈16 kb mRNA in mixed, fast-twitch, and slow-twitch skeletal muscle (Fig 2a), as previously reported.18 There was no detectable signal when using the skeletal muscle RyR in heart, liver, or kidney. After longer exposure, a 2.4 kb mRNA species was detected in brain, which hybridized to the skeletal muscle-specific RyR cDNA (Fig 2a, right). Indeed, it was recently reported that the 3′ portion (2.4 kb) of the skeletal RyR is expressed in brain.51 Two higher-molecular-weight bands were also detected (Fig 2a, right). One of the larger bands comigrated with the skeletal RyR mRNA and could represent cross hybridization with the brain form of the RyR.52 A second high-molecular-weight band was observed, which was distinctly smaller (≈10 kb) and could represent cross hybridization to the inositol-1,4,5-trisphosphate (IP3) receptor, which shares some homology to the RyR in the 3′ portion of the mRNA (the putative transmembrane domain).53,55 To test this possibility, we hybridized this same Northern blot to a cDNA encoding the IP3 receptor54 and found that the ≈10 kb band (Fig 2a, right) did indeed correspond to the IP3 receptor (data not shown). The cardiac RyR cDNA hybridized to a single ≈16 kb mRNA in cardiac
tissue and also detected the less abundant ~16 kb brain RyR mRNA (Fig 2b).

Skeletal Muscle Calcium Channel mRNA Levels

To examine relative mRNA levels of the slow calcium channel subunits and the ryanodine receptor during muscle development, Northern and slot blot analyses were performed by using total RNA purified from 26-day fetal, 0-, 1-, 3-, 5-, and 7-day postnatal, and adult rabbit skeletal muscle. Northern and slot blots were hybridized separately to each calcium channel cDNA, and a 28S ribosomal RNA oligonucleotide probe was hybridized to control for total RNA loading. Developmental regulation of the skeletal muscle α1 subunit of the DHPR (Fig 3a) and the RyR (Fig 3b) is shown by using representative Northern hybridizations. The mRNAs encoding both of these calcium channels demonstrated consistent increases during late myogenic development (Fig 3a and 3b). Both DHPR and RyR mRNA levels increased by ~2-fold on the day of birth, continued to increase during postnatal development, and reached a final adult level 16-fold greater than fetal levels for the DHPR and 5-fold greater than fetal levels for the RyR mRNA (Fig 3a and 3b). There was a reproducible decrease in skeletal muscle RyR mRNA at postnatal day 5 (Fig 3b).

The same Northern and slot blots were hybridized to cDNAs encoding the α2, β, and γ subunits of the skeletal muscle DHPR (Fig 4a through 4c). Northern blot analyses showed an upregulation of all three of these mRNAs in skeletal muscle (Fig 4a through 4c). As for the DHPR α1 subunit and the RyR, the α2, β, and γ subunits of the skeletal muscle DHPR all increased ~2-fold on the day of birth and reached final adult levels ~17-fold, ~16-fold, and ~8-fold higher than the fetal levels for each of the subunits, respectively (Fig 4a through 4c).

Cardiac Muscle Calcium Channel mRNA Levels

Similar studies were performed to characterize the developmental expression of the cardiac E-C coupling calcium channels. Total RNA was isolated from 26-day fetal, 0-, 1-, 3-, 5-, 7-, 21-, and 28-day postnatal, and adult rabbit cardiac muscle and hybridized on Northern and slot blots to cDNAs encoding the cardiac DHPR α1 subunit and the cardiac RyR cDNA (Fig 5a and 5b). All three cardiac DHPR α1 subunit mRNAs increased ~2.5-fold from fetal to adult levels (Fig 5a). The cardiac RyR mRNA increased on the day of birth and for 3 days after birth; however, there was a consistent decrease on postnatal day 5 (similar to that observed for the skeletal muscle RyR, Fig 3b), followed by an increase to an adult level ~9-fold higher than the fetal level (Fig 5b).

Ryanodine Binding to Skeletal and Cardiac Muscle RyR

[3H]Ryanodine binding was performed to determine whether increases in RyR mRNA were reflected in increased protein levels. RyR density (determined as picomoles [3H]ryanodine per gram of tissue) was increased ~5-fold in adult skeletal muscle compared with fetal samples (45 versus 8 pmol/L per gram) and ~2-fold in heart muscle (15 versus 7 pmol/L per gram).

Discussion

In the present study, we demonstrate that in both skeletal and cardiac muscle the expression of the calcium channels required for E-C coupling is developmentally upregulated in the rabbit. In all cases, the day of birth is marked by a consistent 2-fold increase in
Three DHPR mRNA time points. Each time point represents Northern blot. Hybridization of the 28S ribosomal RNA is shown below each Northern blot. Quantification of normalized mRNA levels is shown in the graph below each representative Northern blot. Each time point represents values determined from RNA samples from three or more animals. Error bars represent SEM.

calcium channel mRNAs. The signal for this increased expression has not been identified but could be related to increased physiological stresses on both the skeletal and cardiac muscle systems experienced during birth, including the conversion from fetal to neonatal circulation in the cardiovascular system.

All four calcium channels in both forms of striated muscle exhibited a steady upregulation of expression (measured in terms of mRNA levels) during development. There were corresponding increases in specific [3H]ryanodine binding in both cardiac and skeletal muscles. These increases in cardiac and skeletal muscle calcium channel expression support the findings of earlier functional studies that reported a “maturation” of sarcoplasmic reticulum during development. However, previous functional studies did not identify specific molecular events associated with developmental changes in sarcoplasmic reticulum function. The present study establishes that the calcium channels required for E-C coupling are in fact upregulated at important developmental stages and provides a basis for understanding alterations in calcium fluxes observed, particularly when comparing fetal with adult muscles. The present study is in agreement with a previous study that examined the expression of sarcoplasmic reticulum proteins during development and extends that study to now include the other calcium channel responsible for E-C coupling, the DHPR and its subunits.

The expression of each of the skeletal muscle DHPR-subunit mRNAs was coordinately regulated during development (Fig 4a through 4c). This finding suggests that the relative levels of each of the DHPR subunits remain comparable throughout development, although the increases in γ subunit expression are less pronounced than those observed for both the α1 and β subunits. It has been speculated that alterations in calcium channel function observed during development could be due to changes in subunit expression or in the expression of different isoforms of the DHPR subunits. In this regard, it is interesting to note that the predominant form of the DHPR β subunit identified by Northern analysis in the brain is a 3.4 kb species (Fig 1d). This larger mRNA is detected in skeletal muscle, but the major form is the 1.3 kb mRNA (Fig 1d). During development, the brain form is expressed in fetal tissue, but its levels decrease during the early postnatal period and subsequently rise again in the adult (Fig 4b). It has not been established whether these two mRNAs encode functionally distinct β subunits. If so, they could interact with the same DHPR α1 subunit(s) but invoke different effects, which might be developmentally regulated.

On the day of birth, when the heart is first exposed to increased pressures associated with switching from fetal to neonatal circulation, calcium channel mRNA levels increased by ~2-fold. Growth factors could be involved in regulating these alterations in ion channel expression. For example, transforming growth factor-β has been shown to upregulate RyR expression, whereas other growth factors, most notably fibroblast growth factor, downregulate calcium channel gene expression. The pattern of cardiac calcium channel gene regulation during development is reminiscent of that observed for cardiac-specific genes during heart failure. It has been reported that during heart failure fetal isoforms of cardiac-specific genes are expressed. This reversion to the fetal phenotype has been shown to be mediated by growth factors. We have previously reported that DHPR and RyR expression is decreased in end-stage human heart failure. The decreased expression of calcium channels in human heart failure simulates the fetal pattern of expression of calcium channel mRNA observed in the rabbit.

The present study provides a molecular basis for understanding the reported sensitivity of neonates to calcium channel blockers. Our data would suggest that at fetal and neonatal stages, when expression of calcium channels involved in E-C coupling is low, small doses of channel blockers could have adverse effects on the myocardium and on pulmonary performance. Treatment with calcium channel blockers could result in diminished cardiac contractility, vascular tone, and diaphragmatic activation. This heightened sensitivity to calcium channel blockers in fetal and neonatal tissues is offset by the protective effect of reduced cellular calcium influx during hypoxia and acidosis.

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