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Changes in the Expressional Levels of Sarcoplasmic Reticulum Ca^{2+}-regulatory Proteins in the Postnatal Developing Rat Heart

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In the present study, the postnatal developmental changes in the expressional levels of cardiac sarcoplasmic reticulum (SR) Ca^{2+} regulatory proteins, i.e. Ca^{2+}-ATPase, phospholamban, and Ca^{2+} release channel, were investigated. Both SR Ca^{2+}-ATPase and phospholamban mRNA levels were about 35% of adult levels at birth and gradually increased to adult levels. Protein levels of both SR Ca^{2+}-ATPase and phospholamban, which were measured by quantitative immunoblotting, were closely correlated with the mRNA levels. The initial rates of Ca^{2+} uptake at birth were about 40% of adult rates and also increased gradually during the myocardial development. Consequently, the relative phospholamban/Ca^{2+}-ATPase ratio was 1 in developmental hearts. Ca^{2+} release channel (ryanodine receptor) mRNA was about 50~60% at birth and increased gradually to adult level throughout the postnatal rat heart development. ^{3}[H]ryanodine binding increased gradually during postnatal myocardial development, which was closely correlated with ryanodine mRNA expression levels during the development except the ryanodine mRNA level at birth. These findings indicate that cardiac SR Ca^{2+}-ATPase, phospholamban, and Ca^{2+} release channel are expressed coordinately, which may be necessary for intracellular Ca^{2+} regulation during the rat heart development.

Key Words: Heart, Phospholamban, Ca^{2+}-ATPase, Ca^{2+} release channel, Development

INTRODUCTION

Fetal and newborn hearts from variety of species exhibit profound differences in contraction and relaxation processes compared with adults (Nakanishi et al, 1988; Artman, 1992; Kojima et al, 1992). Many developmental changes in cardiac contractile function have been attributed to alterations in Ca^{2+} regulation by the cardiomyocyte (Nakanishi et al, 1988; Kojima et al, 1992). In addition to sarcolemma, sarcoplasmic reticulum (SR) is also an important determinant in the regulation of intracellular Ca^{2+} fluxes (Luo et al, 1994). Ultrastructural and biochemical analyses indicate that Ca^{2+} regulation by the SR of the immature heart is deficient compared with that of the adult heart (Pegg & Michalak, 1987; Arai et al, 1992; Kojima et al, 1992). To compensate for an underdeveloped SR, immature cardiomyocytes appear to be relatively more dependent on transsarcolemmal Ca^{2+} fluxes for directly regulating Ca^{2+} concentrations at the contractile proteins during excitation-contraction coupling (Hoerter et al, 1981; Klitzner & Friedman, 1988; Nakanishi et al, 1988; Chin et al, 1990). Futhermore, developmental studies on Na^{+}-Ca^{2+} exchanger, one of the major proteins of the sarcolemma (SL), have been actively carried out. These studies show that during normal cardiac development, Na^{+}-Ca^{2+} exchanger expression is maximal near the time of birth and then declines postnatally, and Ca^{2+} regulation by the sarcoplasmic reticulum reaches functional maturity (Boerth et al, 1994). Furthermore, Reed et al. (2000) reported that the expression of SR Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger are antithetically regulated during mouse cardiac development.

Previous study has shown that Ca^{2+} uptake and Ca^{2+}-ATPase activity were low in fetal SR compared to adult control membranes, although the apparent affinities of the enzyme for Ca^{2+} were similar (Michalak, 1987). These findings indicate that developmental changes in cardiac function have been attributed to changes in SR protein expression levels (Nakanishi & Jarmakani, 1984; Mahony & Jones, 1986; Pegg & Michalak, 1987). Several studies have shown that SR Ca^{2+}-ATPase mRNA and protein are lower in neonatal animals than in adult animals (Mahoney, 1988; Kaufman et al, 1990; Fisher et al, 1992). However, there are a few conflicting findings on the developmental changes of phospholamban in cardiac muscle (Pegg & Michalak, 1987; Mahoney & Jones, 1988; Arai et al, 1992; Szymanska et al, 1995). Arai et al. (1992) reported that the steady-state mRNA levels of phospholamban were not changed in the postnatal developing rabbit heart, although the SR Ca^{2+}-ATPase increased throughout development. Another group reported that both phospholamban and SR Ca^{2+}-ATPase mRNA and protein levels were lower in neonatal rabbits compared with adults (Szymanska et al, 1995). These conflicting findings could be explained that transcript expression levels are not always indicative of protein levels (Godwin et al, 1992; Mearow et al, 1993). Harrer et al. (1997) also reported that both phospholamban and Ca^{2+}-ATPase

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ABBREVIATIONS: SR, sarcoplasmic reticulum; PLB, phospholamban; SERCA, SR Ca^{2+}-ATPase; RyR, ryanodine receptor

mRNAs were ~40% of adult levels at birth and gradually increased to approach adult levels (100%) by day 15 of postnatal development. These changes in transcript levels were indicative of changes at the protein level for both phospholamban and Ca^{2+}-ATPase.

Ca^{2+} release channel is also an important Ca^{2+} transport protein located in SR membranes, which releases the Ca^{2+} from the SR, resulting in muscle contraction. However, there are currently no extensive studies available on the developmental changes of SR Ca^{2+} release channel in rat hearts. Therefore, in this study we examined changes in the expressional levels of phospholamban, SR Ca^{2+}-ATPase, and Ca^{2+} release channel throughout rat heart development.

METHODS

cDNA expression and isolation

E. coli DH5 cells (Pharmacia Biotec.) transfected with the pCRII (vector inserted 389bp ryanodine receptor cDNA, Pharmacia Biotech.) constructs were grown in 5~10 ml of Luria Bertani (LB) medium supplemented with 100 μg of ampicillin/ml, to an A_{600}=1.0−1.5 at 37°C, then treated with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated 12~16 h at 20°C with constant shaking. The cells were harvested by centrifugation (10,000×g, 5 min) and the pellet was used as the DNA purification source. The cardiac-specific ryanodine receptor cDNA was a generous gift from Dr. Do Han Kim in Kwang-Ju Institute for Science and Technology. DNA was isolated using DNA purification system (Promega) according to the procedure described by the manufacturer.

RNA isolation and dot blot analysis

Total RNA was isolated from whole rat hearts, by the guanidium isothiocyanate method (Chomczynski & Sacchi, 1987), on the following days during development: 0.5, 3, 4, 5, 7, 15, 25 and 30 days after birth, and adult (100 days postnatal). For dot blots, RNA samples were denatured by heating at 55°C for 5 min, cooled on ice, diluted with 2 vol of 4% formaldehyde in 20× SSC, and further diluted with 8 vol of 20× SSC. Serial dilutions (5, 2.5, 1.25, 0.625, 0.312, and 0.156 μg) were spotted onto Gene Screen Plus membranes using a dot blot manifold (Bio-Rad, Hercules, CA). The filters were dried, baked at 80°C for 2h under vacuum, and then processed for hybridization according to the manufacture's instructions. Dot blots were hybridized with an excess of either the ^{32}P end-labeled oligonucleotide (60-mer), corresponding to the phospholamban-coding region or the ^{32}P end-labeled 60-mer specific for rat cardiac SR Ca^{2+}-ATPase, or the 389 bp cardiac-specific ryanodine receptor cDNA probe. The specificity of the ryanodine receptor probe was tested in preliminary studies by Northern blot analysis (Data not shown). Autoradiography was done with Kodak X-Omat AR film at −70°C for 1 day. The degree of labeling for dot blots was determined using PhosphorImager analysis and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To normalize for RNA loading onto the blotted membranes, the hybridized blots were subsequently rehybridized with a ^{32}P end-labeled 60-base oligonucleotide antisense to a portion of the rat 18S rRNA probe.

Oligonucleotides

Three kinds of synthetic 60-mer oligonucleotides were used in the hybridization; one is specific for phospholamban (5' TGA CGT GCT TGC TGA GGC ATT TCA ATA GTG GAG GCT CTC CTG ATA GCC GAG CGA GTG AGG 3'), one for Ca^{2+}-ATPase (5' AGG TGT GTT GCT AAC AAC GCA GAT GCA CGC ACC CGA ACA CCC TTA TAT TTC TGC AAA TGG 3'), and one for 18S rRNA (5' GTA TCT GAT CGT CTT CGA ACC TCC GAC TTT CGT TCT TGA TTA ATG AAA ACA TTC TTG GCA 3'). The oligonucleotides were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and unincorporated nucleotides were removed by C18 Sep-Pak cartridge (Millipore, Bedford, USA).

Quantitative immunoblots

Whole hearts from 0.5, 4, 7, 15 days postnatal and adult (100 days postnatal) rat were homogenized in 10 mM imidazole, pH 7.0, 300 mM sucrose, 1 mM dithiothreitol, and 1 mM sodium metabisulfite. Protein concentrations were determined by the Bio-Rad standard method using bovine gamma globulin for the standard. The relative protein levels of phospholamban and the SR Ca^{2+}-ATPase in cardiac homogenates from adult and developing rat were estimated with quantitative immunoblotting (Harrer et al, 1995). For the study of ryanodine receptor, purified heavy SR protein was used instead of cardiac homogenate. Cardiac homogenate or heavy SR proteins were separated by SDS-polyacrylamide gel electrophoresis (10~18% gradient gel for homogenate, 4% gel for ryanodine receptor), and were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, USA). Transblots were reacted with a monoclonal antibody specific for phospholamban (PLB-Ab, 1 : 1000 dilution) or specific for cardiac SR Ca^{2+}-ATPase (SERCA-Ab, 1 : 1000 dilution), or specific for ryanodine receptor (RyR-Ab, 1 : 1000 dilution). PLB-Ab was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA) and SERCA-Ab and RyR-Ab were from Affinity Bioreagents Inc. (Golden, Co, USA). The antibody-antigen reactions were done using the horseradish peroxidase (HRP)-linked anti-rat whole antibody (from goat) from Amersham International (Little Chalfont, UK) at 1 : 2000 for 2 h. Antibody binding was detected using enhanced chemiluminescence (ECL)-HRP developing agents from Amersham International (Little Chalfont, UK), and membranes were exposed to a sheet of autoradiography film. The developed bands were quantitated by the scanned image using ImageQuant software, with data reported as integrated density units.

Determination of Ca^{2+} uptake

Frozen hearts from adult (100-day) and 0.5-day-old postnatal rat were homogenized, as described above (Luo et al, 1994). Ca^{2+} uptake into SR vesicles in cardiac homogenates (0.1 mg/ml) was determined at 37°C by using $^{45}CaCl_2$ and Millipore filters with a pore size of 0.45 mm (type GS) with a modification of the Millipore filtration technique described by Martonosi & Feretos (1964). Homogenates were incubated in 40 mM imidazole, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 5 mM Na^+ oxalate, 0.5 mM EGTA, 5 μM ruthenium red, and various amounts

of $^{45}CaCl_2$ to yield 0.05~5 μM free Ca^{2+}. The reaction was initiated by the addition of 5 mM ATP, and the rates of Ca^{2+} uptake were calculated by least squares linear regression analysis of the 20-, 40-, and 60-s values of Ca^{2+} uptake.

Preparation of heavy sarcoplasmic reticulum vesicles

Heavy sarcoplasmic reticulum (HSR) vesicles were prepared by the method previously described (Lee et al, 2001). Briefly, rats were killed and the heart muscle was quickly removed, minced, and homogenized in 6 vol. of cold 20 mM Tris-HCl, pH 7.0, 0.1 M KCl, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 50 U/ml aprotinin, and 10 μM benzetonium chloride. Homogenates were centrifuged at 500×g for 10 minutes, and supernatants were centrifuged at 12,000×g for 30 min. The pellets were suspended and homogenized in 10 mM histidine, pH 7.0, 0.6 M KCl to extract contractile protein. The suspension was centrifuged at 40,000×g for 15 minutes. The pellets were then resuspended and centrifuged at 120,000×g for 60 minutes. The resultant precipitates were resuspended in 10% sucrose solution and subjected to a discontinuous sucrose gradient centrifugation for 5 h at 100,000×g. Microsomal fractions were recovered from the interfaces of 20, 30, 35, 40% of sucrose layers and HSR vesicles were obtained from the interface between 30% and 35% sucrose layers. The subfraction was diluted with 10% sucrose solution and centrifuged for 90 min at 100,000×g. The pellet was resuspended in a solution containing 0.3 M sucrose, 0.1 M KCl, and 5 mM Na-PIPES, pH 6.8, frozen in liquid nitrogen, and stored at −80°C before use.

[^{3}H]ryanodine binding assay

[^{3}H]ryanodine binding was carried out for 90 min at 37°C in 0.1 ml of 0.2 M KCl, 1 mM Na_2EGTA, 0.995 mM $CaCl_2$, and 10 mM Na-PIPES, pH 7.2. Free Ca^{2+} concentration was 10 μM. [^{3}H]ryanodine (60 mCi/mmol) was purchased from Du Pont-New England Nuclear and was diluted directly in the incubation medium to achieve concentrations in the saturable range of 1~30 nM. During incubation, cardiac SR (0.3~0.5 mg/ml) were the last reagent to be added to the medium. Aliquots of the samples were placed on Whatman GF/B glass fiber filters, using a Brandel (Gaithersburg, Maryland, U.S.A.) cell harvester. Filters were washed three times with 5-ml of ice-cold 0.1 M KCl, 1 mM K-PIPES, pH 7.0. Nonspecific [^{3}H]ryanodine binding was determined in the presence of 10 μM unlabeled ryanodine and was subtracted from each sample. In order to establish whether [^{3}H]ryanodine binding was affected by proteolytic degradation that may have occurred during the binding assay, control incubations were carried out in the presence or absence of the protease inhibitors pepstatin A (1 μM), iodoacetamide (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (1 μM), and benzamidine (1 mM). Addition of the protease inhibitor mixture did not result in a significant change in the site density or in the affinity of [^{3}H]ryanodine binding. Radioactivity was measured by scintillation counting.

RESULTS

Quantitation of phospholamban and SR Ca^{2+}-ATPase mRNA expression levels during postnatal myocardial development

Phospholamban and SR Ca^{2+}-ATPase expression in postnatal developing rat hearts has not been clearly defined. In the present study, quantitative analyses of expression levels for these two SR proteins was performed using RNA dot blot analysis. Total RNA was isolated from rat hearts at various ages during the postnatal (0.5~25 days) development, and the mRNA expression levels were compared with those expressed in adult (100 days) hearts. Phospholamban mRNA was ~40% at birth and gradually increased to ~80% of adult levels at 25 days of development (Fig. 1). SR Ca^{2+}-ATPase mRNA was ~35% at birth and increased gradually to adult levels (Fig. 3), which were closely correlated with phospholamban mRNA expression levels during the development.

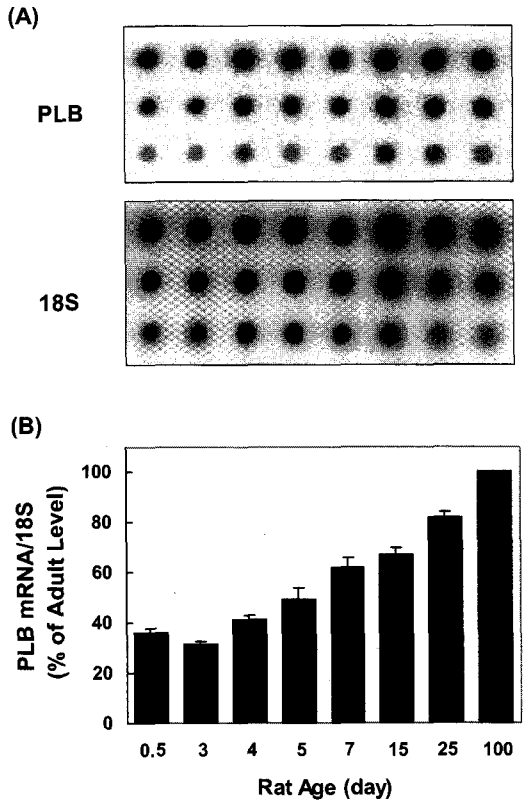

Fig. 1. RNA dot blot analysis of phospholamban during postnatal myocardial development. (A) Representative RNA dot blots of phospholamban. (B) Quantitative representation of percent changes in phospholamban mRNA levels. RNA was isolated form rat hearts of 0.5, 3, 4, 5, 7, 15, 25 days after birth and adult (100 days). mRNA levels of phospholamban corrected by 18S rRNA level in the same membrane were expressed as percentage of adult mRNA levels (100%). Values in each experiment were expressed as mean±SE. Each assayed in triplicates of 5 different preparations. PLB; phospholamban.

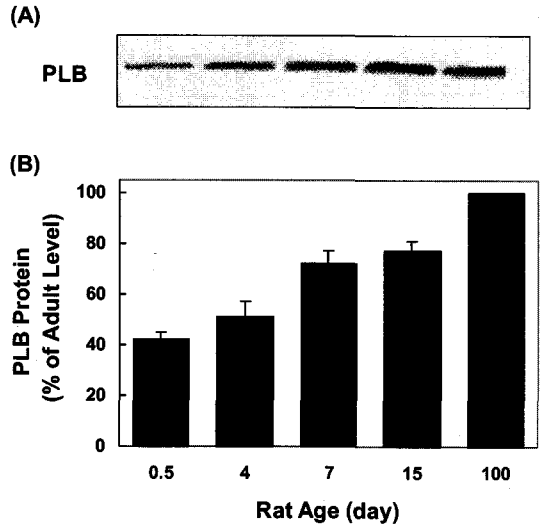

Fig. 2. Western blot analysis of phospholamban during postnatal myocardial development. (A) Representative Western blots of phospholamban. (B) Quantitative representation of percent changes in phospholamban protein levels. Protein levels of phospholamban in 0.5, 4, 7, 15-day old rat hearts were expressed as a percentage of levels (100%) in adult (100-day) hearts. Protein concentrations were within the linear range of detection. Values in each experiment were expressed as mean±SE. Each assayed in triplicates of 5 different preparations. PLB; phospholamban.

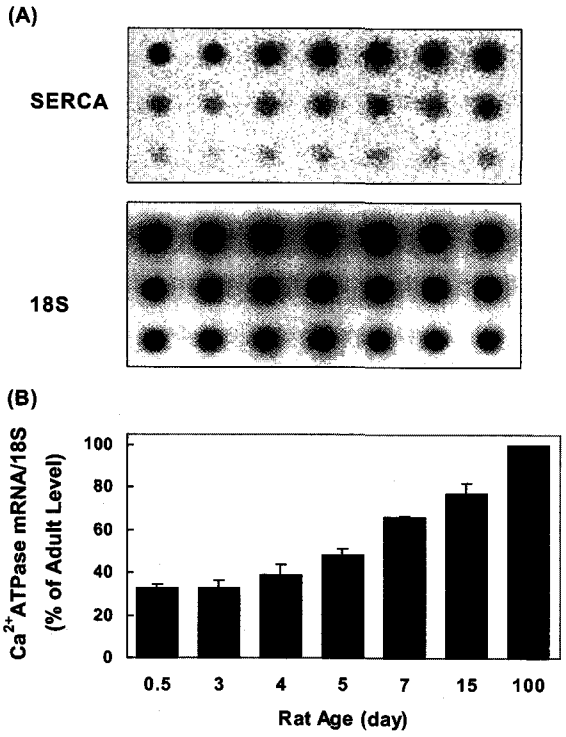

Fig. 3. RNA dot blot analysis of SR Ca^{2+}-ATPase during postnatal myocardial development. (A) Representative RNA dot blots of SR Ca^{2+}-ATPase. (B) Quantitative representation of percent changes in SR Ca^{2+}-ATPase mRNA levels. RNA was isolated form rat hearts of 0.5, 3, 4, 5, 7, 15, 25 days after birth and adult (100 days). mRNA levels of SR Ca^{2+}-ATPase corrected by 18S rRNA level in the same membrane were expressed as percentage of adult mRNA levels (100%). Values in each experiment were expressed as the mean± SE. Each assayed in triplicates of 5 different preparations. SERCA; SR Ca^{2+}-ATPase.

Quantitation of phospholamban and SR Ca^{2+}-ATPase protein levels during postnatal myocardial development

To determine whether the observed changes in mRNA levels in the developing hearts reflected closely those changes in the protein levels, Western blot analyses of phospholamban and SR Ca^{2+}-ATPase were performed. The expression levels of phospholamban and SR Ca^{2+}-ATPase in hearts of 0.5, 4, 7, and 15 days of postnatal development were compared with the levels expressed in adult rat heart (Fig. 2, 4). After SDS-PAGE, gel was cut in half (top and bottom), and phospholamban and SR Ca^{2+}-ATPase proteins on the gel were transferred to a separate sheet of PVDF membrane. On the day of birth (0.5 day), phospholamban protein levels were ~40% (Fig. 2) and SR Ca^{2+}-ATPase protein levels were ~30% (Fig. 4) of the protein levels observed in the adult hearts (100%). These levels increased gradually. By the day 15, the relative levels of both proteins were ~80% of adult levels (Fig. 2 and 4).

SR Ca^{2+} uptake of rat hearts during postnatal development

To determine whether developmental changes in the expression levels of phospholamban and SR Ca^{2+}-ATPase affected SR Ca^{2+} uptake, initial rates of Ca^{2+} uptake were studied in cardiac homogenates prepared from 0.5- and 100-day-old rat. At pCa of 6.0, maximal rate of Ca^{2+} uptake was 50±5 at birth (0.5 day) and the rate observed in the adult hearts was 120±12 nmol Ca^{2+}/mg/min (Fig. 5). The increase in the maximal rates of Ca^{2+} uptake correlated with the SR Ca^{2+}-ATPase expression levels of neonatal and

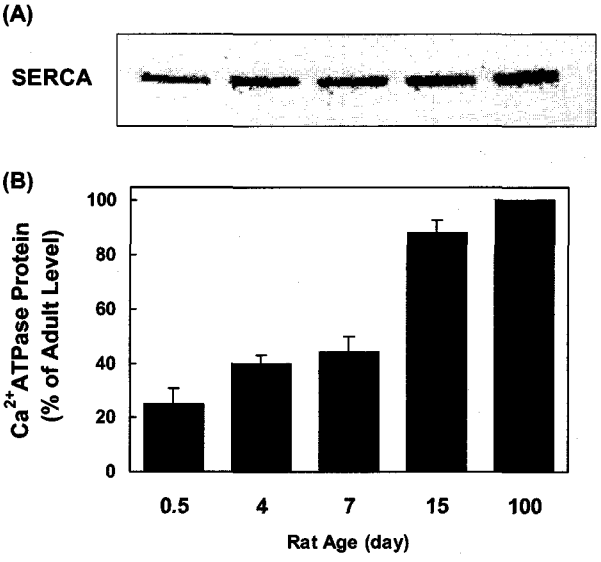

Fig. 4. Western blot analysis of SR Ca^{2+}-ATPase during postnatal myocardial development. (A) Representative Western blots of SR Ca^{2+}-ATPase. (B) Quantitative representation of percent changes in SR Ca^{2+}-ATPase protein levels. Protein levels of SR Ca^{2+}-ATPase in 0.5, 4, 7, 15-day old rat hearts were expressed as a percentage of levels (100%) in adult (100-day) hearts. Protein concentrations were within the linear range of detection. Values in each experiment were expressed as mean±SE. Each assayed in triplicates of 5 different preparations. SERCA; SR Ca^{2+}-ATPase.

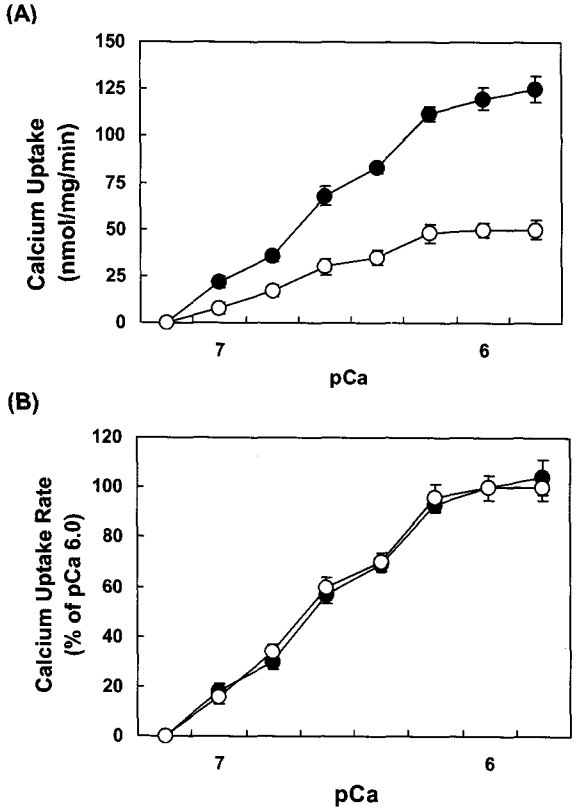

Fig. 5. Ca^{2+} uptake rates in neonatal and adult rat hearts. Initial rates of Ca^{2+} uptake were determined in cardiac homogenates from 0.5, and 100-day-old rat. Ca^{2+} dependency of Ca^{2+} uptake was determined in the presence of 5 μM ruthenium red. (A) Data expressed as nmol Ca^{2+}/mg/min in neonatal (○) and adult (●) hearts. (B) Data expressed as percentage of Ca^{2+} uptake rates at pCa 6.0 for neonatal (○) and adult (●) hearts, respectively. Values in each experiment were expressed as mean±SE. Each assayed in triplicates of 5 different preparations. A minimum pool of six hearts was used for each experiment.

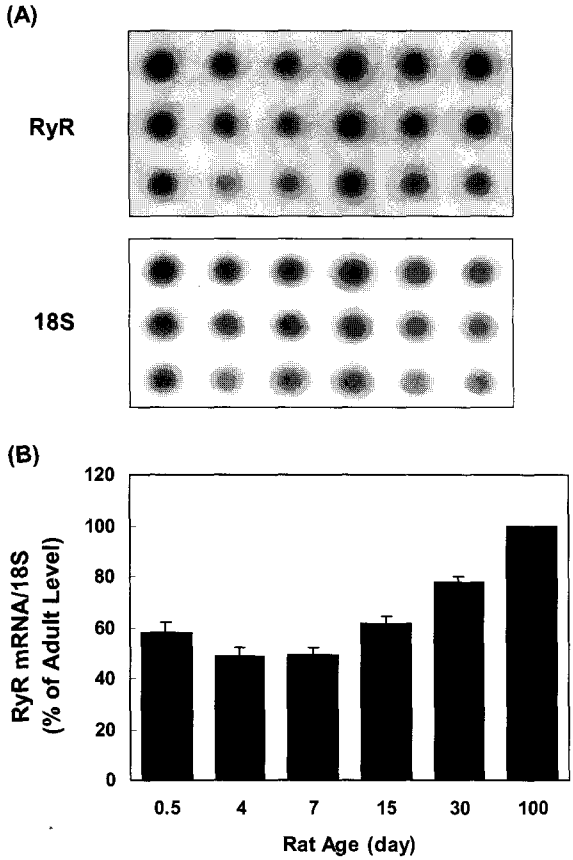

Fig. 6. RNA dot blot analysis of SR ryanodine receptor during postnatal myocardial development. (A) Representative RNA dot blots of SR ryanodine receptor. (B) Quantitative representation of percent changes in cardiac ryanodine receptor mRNA levels. RNA was isolated form rat hearts of 0.5, 4, 7, 15, 30 days after birth and adult (100 days). mRNA levels of cardiac ryanodine receptor corrected by 18S rRNA level in the same membrane were expressed as percentage of adult mRNA levels (100%). Values in each experiment were expressed as mean±SE. Each assayed in triplicates of 5 different preparations. RyR, ryanodine receptor.

adult hearts (Fig. 3~5). To determine the affinity of the SR Ca^{2+}-ATPase, we expressed the data as percentage of the maximal uptake rates obtained at pCa 6.0 for 0.5-day and adult rat hearts. However, the affinity of the SR Ca^{2+}-ATPase did not change significantly between 0.5-day and adult rat hearts (Fig. 5). Data indicated that the relative ratio of phospholamban to the SR Ca^{2+}-ATPase was maintained constant in the developing rat heart.

Expression of SR Ca^{2+} release channel and ^{3}[H]ryanodine binding during postnatal myocardial development

To study possible correlation between the developmental changes in the expression of SR Ca^{2+} release channel and those of phospholamban and SR Ca^{2+}-ATPase, we analyzed the expression level of mRNA for cardiac ryanodine receptor during postnatal myocardial development. mRNA levels of SR Ca^{2+} release channel in rat hearts of 0.5-, 4-, 7-, 15-, 30-days of postnatal and the adult (100-day) were examined by the RNA dot blot method. Ryanodine receptor mRNA was ~60% at birth, which was higher than mRNA levels of phospholamban and SR Ca^{2+}-ATPase at birth. mRNA

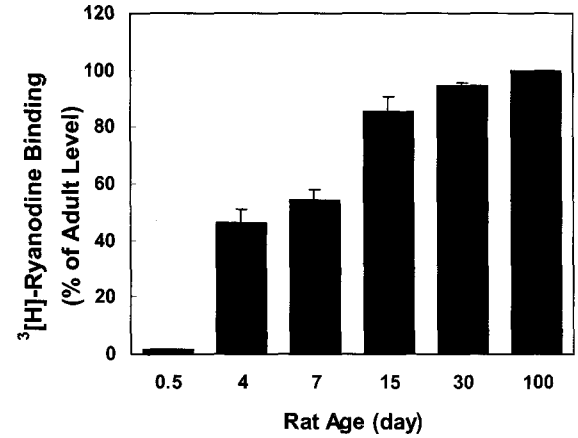

Fig. 7. ^{3}[H]ryanodine binding of rat hearts during postnatal myocardial development. [^{3}H]ryanodine binding was carried out for 90 min at 37 °C in 0.1 ml of 0.2 mM Na_2EGTA, 0.995 mM $CaCl_2$, and 10 mM Na-PIPES, pH 7.2. The calculated free Ca^{2+} was 10 μM. Values are mean±SE of 5 different preparations, each assayed in triplicate.

levels of ryanodine receptor at 4-, and 7-day postnatal hearts seemed to be slightly decreased, but gradually increased to the adult level (Fig. 6). ^{3}[H]ryanodine binding increased gradually during postnatal myocardial development, which was closely correlated with ryanodine mRNA expression levels during the development, except the mRNA level at birth (Fig. 7).

DISCUSSION

Our present study demonstrates that there is a close correlation between the increasing expression levels of SR Ca^{2+}-ATPase and phospholamban in the developing rat heart. Previous studies on the developmental regulation of the SR Ca^{2+}-ATPase revealed that mRNA levels were lower in neonatal rabbit hearts than in adult hearts (Arai et al, 1992; Fisher et al, 1992). To determine the developmental changes in the expression levels of phospholamban and SR Ca^{2+}-ATPase in the rat heart, both SR Ca^{2+}-ATPase and phospholamban mRNA and protein levels were measured during the heart development. Both SR Ca^{2+}-ATPase and phospholamban mRNA levels in newborn rat hearts were ~40% of adult levels and subsequently increased to adult levels. The increase of SR Ca^{2+}-ATPase mRNA levels during heart development in rat was similar to the increase during heart development in rabbit (Arai et al, 1992). Harrer et al. (1997) also reported that the increase of phospholamban mRNA levels was occurred during heart development in mouse, and expression levels of SR Ca^{2+}-ATPase and phospholamban were also closely coordinated during the heart development of mouse. Recently, Reed et al. (2000) also demonstrated that the expression of SERCA2 (SR Ca^{2+}-ATPase) and phospholamban mRNA in murine hearts increased eight-fold from fetal to adult stages. However, the increase of phospholamban mRNA levels during mouse heart development was different from previous observations by Arai et al. (1992) who reported that the phospholamban mRNA levels during rabbit heart development were similar between neonatal and adult levels. However, it has been shown that alterations at the transcriptional levels might not necessarily correlate with alterations at the translational level (Mearow et al, 1993). Our data showed that alterations of mRNA levels were closely correlated with alterations of the protein level for both phospholamban and SR Ca^{2+}-ATPase during rat cardiac development. Furthermore, Ca^{2+} uptake rates of neonatal rat hearts were lower than those of the adult hearts. Our data were in support of previous findings of SR Ca^{2+} uptake rates in the neonatal and adult rabbit (Fisher et al, 1992; Szymanska et al, 1995), mouse (Harrer et al, 1997), and rat (Vetler et al, 1995) hearts. The coordinated developmental expression of the SR Ca^{2+}-ATPase and phospholamban suggests that the phospholamban/Ca^{2+}-ATPase ratio may be important for regulation of cardiac SR function. Our result on the Ca^{2+} uptake indicated that the affinity of the SR Ca^{2+}-ATPase for Ca^{2+} was maintained in neonatal or adult rat hearts. Such regulated coordination between the SR Ca^{2+}-ATPase and phospholamban may be important in maintaining SR function in normal physiological hearts.

Our previous studies showed that in pathologic conditions such as diabetic cardiomyopathy and hypothyroid hearts, there existed an inverse relationship between SR Ca^{2+}-ATPase and phospholamban expression. Both mRNA and protein levels of SR Ca^{2+}-ATPase were markedly decreased in diabetic cardiomyopathy (Kim et al, 1999a; Kim et al, 2001) and hypothyroid hearts (Kim et al, 1999b), whereas they were significantly increased in hyperthyroid hearts (Kim et al, 1999b; Kim et al, 2001). Phospholamban expression, however, showed an opposite trend. Reed et al. (2000) also reported that SERCA2 mRNA/protein levels were downregulated in hypothyroid hearts, whereas an opposite response was observed in hyperthyroid hearts.

In addition to SR Ca^{2+}-ATPase, another protein termed SR Ca^{2+} release channel also contributes to intracellular Ca^{2+} homeostasis by releasing Ca^{2+} to the cytosol, which results in muscle contraction. However, the contributions of SR Ca^{2+} release channel during postnatal rat heart development have not been studied extensively yet. In the present study, expression levels of mRNA coding for cardiac SR Ca^{2+} release channel were low at birth and increased gradually to the adult level. In contrast to the mRNA levels of SR Ca^{2+}-ATPase and phospholamban (~35% of the adult) at birth, the mRNA level of the SR Ca^{2+} release channel was relatively higher (~60% of the adult) at birth. Levels of [^{3}H]-labeled ryanodine binding throughout the postnatal rat heart development were relatively well correlated with levels of the SR Ca^{2+} release channel mRNA, except the level of SR Ca^{2+} release channel mRNA at birth. Since [^{3}H]ryanodine binds to the open state of SR Ca^{2+} release channel, our data of low [^{3}H]ryanodine binding to the SR Ca^{2+} release channel in the neonatal rat heart may indicate that the SR Ca^{2+} release channel exists but dwells more in a closed state. However, further evaluation by electrophysiological studies is in need. In summary, SR Ca^{2+}-ATPase/phospholamban/Ca^{2+} release channel expressions were low at birth and increased gradually to adult levels during postnatal rat heart development. It is, therefore, apparent that relative contribution of these SR Ca^{2+} regulating proteins is important for cardiac muscle function during postnatal rat heart development.

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REFERENCES

Arai M, Otsu K, MacLennan DH, Periasamy M. Regulation of sarcoplasmic reticulum gene expression during cardiac and skeletal muscle development. *Am J Physiol* 262 (*Cell physiol.* 31): C614–620, 1992

Artman M. Developmental changes in myocardial contractile responses to inotropic agents. *Cardiovasc Res* 26: 3–13, 1992

Boerth SR, Zimmer DB, Artman M. Steady-state mRNA levels of the sarcolemmal Na^{+}-Ca^{2+} exchanger peak near birth in developing rabbit and rat hearts. *Circ Res* 74: 354–359, 1994

Chin TK, Friedman WF, Kiltzner TS. Developmental changes in cardiac myocyte calcium regulation. *Circ Res* 67: 574–579, 1990

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987

Fisher DJ, Tate CA, Phillips S. Developmental regulation of the sarcoplasmic reticulum calcium pump in the rabbit heart. *Pediatr Res* 31: 474–479, 1992

Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME. High resistance to in cisplatin in human ovarian cancer cell lines is associated with marked increase of gluta-

thione synthesis. *Proc Natl Acad Sci* 89: 3070−3074, 1992
Harrer JM, Haghighi K, Kim HW, Ferguson DG, Kranias EG. Coordinate regulation of SR Ca^{2+}-ATPase and phospholamban expression in developing murine heart. *Am J Physiol* 41: H57−66, 1997
Harrer JM, Kiss E, Kranias EG. Application of the immunoblot technique for quantitation of protein levels in cardiac homogenates. *Biotechniques* 18: 995−997, 1995
Hoerter J, Mazet F, Vassort G. Perinatal growth of the rabbit cardiac cell: possible implications for the mechanism of relaxation. *J Mol Cell Cardiol* 13: 725−740, 1981
Kaufman TM, Horton JW, White DJ, Mahony L. Age-related changes in myocardial relaxation and sarcoplasmic reticulum function. *Am J Physiol* 259 (*Heart Circ. Physiol.* 28): H309−316, 1990
Kim HW, Cho YS, Lee HR, Park SY, Kim Y-H. Diabetic alterations in cardiac sarcoplasmic reticulum Ca^{2+}-ATPase and phospholamban protein expression. *Life Sci* 70: 367−379, 2001
Kim HW, Cho YS, Lee YS, Lee EH, Lee HR. Minimal amount of insulin can reverse diabetic heart function: Sarcoplasmic reticulum Ca^{2+} transport and phospholamban protein expression. *Korean J Physiol Pharmacol* 3: 175−182, 1999a
Kim HW, Noh KM, Park M, Lee HR, Lee EH. Thyroid hormone-induced alterations of Ca^{2+}-ATPase and phospholamban protein expression in cardiac sarcoplasmic reticulum. *Korean J Physiol Pharmacol* 3: 223−230, 1999b
Klitzner T, Friedman WF. Excitation-contraction coupling in developing mammalian myocardium: evidence from voltage clamp studies. *Pediatr Res* 23: 428−432, 1988
Kojima M, Sperelakis N, Sada H. Ontogenesis of transmembrane signaling systems for control of cardiac Ca^{2+} channels. *J Dev Physiol* 14: 181−219, 1992
Lee EH, Seo YJ, Kim Y-H, Kim HW. Unchanged protein level of ryanodine receptor but reduced [^{3}H]ryanodine binding of cardiac sarcoplasmic reticulum from diabetic cardiomyopathy rats. *Korean J Physiol Pharmacol* 5: 397−405, 2001
Luo W, Grupp IL, Harrer JM, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced mycocardial contractility and loss of b-agonist stimulation. *Circ Res* 75: 401−409, 1994
Mahoney L. Maturation of calcium transport in cardiac sarcoplasmic reticulum. *Pediatr Res* 24: 639−643, 1988
Mahony L, Jones LR. Developmental changes in cardiac sarcoplasmic reticulum reticulum in sheep. *J Biol Chem* 261: 15257−15265, 1986
Martonosi A, Feretos R. The uptake of Ca^{2+} by sarcoplasmic reticulum fragment. *J Biol Chem* 239: 648−658, 1964
Mearow KM, Thilander BG, Khan I, Garfield RE, Grover AK. In situ hybridization and immunocytochemical localization of SERCA2 encoded Ca^{2+}pump in rabbit heart and stomach. *Mol Cell Biochem* 121: 155−165, 1993
Michalak M. Sarcoplasmic reticulum membrane and heart development. *Can J Cardiol* 3: 251−260, 1987
Nakanishi T, Jarmakani JM. Developmental changes in myocardial mechanical function and subcellular organelles. *Am J Physiol* 246 (*Heart Circ. Physiol.* 15): H615−625, 1984
Nakanishi T, Seguchi M, Takao A. Development of the myocardial contractile system. *Experimentia* 44: 936−944, 1988
Pegg W, Michalak M. Differentiation of Sarcoplasmic reticulum during cardiac myogenesis. *Am J Physiol* 252: H22−H31, 1987
Reed TD, Babu GP, Ji Y, Zilberman A, Heyen MV, Wuytack F, Periasamy M. The expression of SR calcium transport ATPase and the Na^+/Ca^{2+} exchanger are antithetically regulated during mouse cardiac development and in hypo/hyperthyroidism. *J Mol Cell Cardiol* 32: 453−464, 2000
Szymanska G, Grupp IL, Slack JP, Harrer JM, Kranias EG. Alterations in sarcoplasmic reticulum calcium uptake, relaxation parameters and their responses to -adrenergic agonists in the developing rabbit hearts. *J Mol Cell Cardiol* 27: 1819−1829, 1995
Vetter R, Studer R, Reinecke H, Kolar F, Ostadalova I, Drexler H. Reciprocal changes in post-natal expression of sarcolemmal Na^+-Ca^{2+} exchanger and SERCA2 in rat heart. *J Mol Cell Cardiol* 27: 1670−1689, 1995