Gene Expression of SERCA2a and L- and T-type Ca Channels during Human Heart Development

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ABSTRACT

In this study we report, for the first time, on the gene expression of human cardiac SERCA2, L-type (α_{1C}) and T-type (α_{1H}) Ca channels during development, using RNase protection assay, relative quantitative RT-PCR and Western blot. Human hearts during early gestation (8- to 20-wk gestation), neonatal (1- to 4-d-old) and adult (18- to 48-year-old) stages were used. The results show that T-type Ca channel α_{1H} subunit mRNA decreased and that L-type Ca channel α_{1C} subunit mRNA increased with development. While the levels of sarcoplasmic reticulum ATPase (SERCA2a) mRNA did not significantly change with development, its protein levels increased with development. In conclusion, SERCA2a, L-type and T-type Ca channel transcripts were detected as early as 8-wk gestation. Defining the profile of Ca handling proteins during development is important to the understanding of excitation-contraction (EC)-coupling of the developing human heart. (*Pediatr Res* **50: 569–574, 2001**)

Abbreviations

ADL, adult
Bp, base pair
Ca, calcium
NCX, Na/Ca exchanger
NEO, neonatal
PAGE, polyacrylamide-gel electrophoresis
PCR, polymerase chain reaction
PMSF, phenylmethyl sulfonyl fluoride
RPA, RNase protection assays
RT-PCR, reverse transcription-PCR
RyR, ryanodine receptor
SDS, sodium dodecyl sulfate
SERCA, sarcoplasmic reticulum Ca-ATPase
SR, sarcoplasmic reticulum
W, week

In the cardiovascular system, Ca channels play a vital role in the control of processes that are responsible for Ca homeostasis and signaling (1, 2). They include high- and low-voltage activated channels (1). L-type Channels are characterized by long-lasting, high voltage activated currents. They are well characterized, contribute to the excitation contraction (EC) coupling and contain the site of action for the existing clinically available Ca channel antagonists such as nifedipine, verapamil and diltiazem. Transient low voltage-activated currents characterize T-type Ca channels. They are predominantly found in the fetal and not adult myocardium. The exact physiologic function(s) of T-type Ca channels are not yet well defined. However, recently currents through T-type Ca channels have been implicated in triggering Ca release from the sarcoplasmic reticulum (SR) (3), and in EC-coupling of Purkinje cells (4).

The mechanisms of fetal and adult EC-coupling in the heart are distinct. In the adult heart, it is generally accepted that contraction is elicited by a primary entry of Ca ions through L-type Ca channels and secondary release of Ca from the SR (1, 2). In the immature heart, previous studies suggested that SR function is diminished in neonatal compared with adult rabbit cardiac ventricular myocyte (5). However, it is often assumed but not proven that SR is less abundant in early fetal than adult ventricular muscle.

We recently demonstrated that both NCX mRNA and NCX protein levels were higher in human fetal than in adult hearts (6). To date, no data are available concerning the gene expression of SERCA2a (sarcoplasmic reticulum Ca-ATPase), T-type and L-type Ca channel during human heart development, all of which could contribute to the EC-coupling of cardiac myocytes. This study is the first to report on the gene expression of α_{1C} subunit of L-type and α_{1H} subunit of T- type Ca channels, which are the pore forming subunits of these channels, and SERCA2a during human heart development with emphasis on early ontogeny.

MATERIALS AND METHODS

Hearts. The Institutional Review Board under document #1456 and protocol # 96–149 has approved this study and informed consent from the mothers has been obtained. Human

fetal hearts (ages 8- to 23-wk gestation) were obtained after elective termination of normal pregnancy. Hearts were immediately snap frozen in liquid nitrogen and transported to the laboratory on dry ice. Normal adult (18- to 40-year-old) and neonate (1 to 4 d after birth) heart tissues were obtained frozen from a tissue bank in Baltimore, MD.

RNA preparation. Total cellular RNA was isolated from frozen ventricles using an RNAzol[TM] B kit (TEL-TEST, Inc) based on the method developed by Chomczynski and Sacchi (7) and as previously described (6). RNA was quantified by spectrophotometry at 260 nm, and the ratio of absorbance at 260nm to that of 280nm 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. Aliquots of RNA were stored in RNase free water at $-80°C$.

Protein preparation. Total proteins were prepared by homogenizing frozen ventricular tissues in 1.0% SDS (SDS) containing 1mM phenylmethyl sulfonyl fluoride (PMSF). Briefly, 100 mg of frozen heart tissue trimmed of atria and fat were homogenized using a polytron homogenizer, centrifuged at 1500g for 10 min at 4°C to remove cell debris. The protein concentration was determined in triplicate by a Bio-Rad D_C protein assay Kit at absorbance of 750nm using BSA as a standard according to Lowry (8). Aliquots were stored at -80°C until used.

Western blot analysis. The same amount of total proteins from each age group (30–100 μg) was analyzed by SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) on 8% Tris-glycine gel as previously described (6). Proteins were transferred to a PVDF membrane. The transfer was checked by staining the PVDF membrane with Ponceau S. The blot was blocked two hours in 5% nonfat milk and 0.3% Tween-20[TM] and probed with a 1:1000 diluted monoclonal mouse anti-SERCA2a antibody (Affinity Bioreagent, Inc.) for 2 h. Immunodetection of the primary antibody against SERCA2a was carried out with a 1:5000 diluted peroxidase -conjugated anti-mouse IgG for 60 min and detected with enhanced chemiluminescence (ECL). Quantitative evaluation was carried out using scanning densitometric analysis of the corresponding bands.

RNase protection assays. RPAs were performed through concomitant measurement of cyclophilin mRNA (internal control) as previously described (6, 9). DNA templates of human cardiac SERCA2a, α_{1C} subunit of L-type Ca channel, and α_{1H} of T-type Ca channel were prepared by subcloning of cDNA fragments into pCRII (Invitrogen) vectors as previously described (6). cDNA fragments were prepared by reverse transcription and PCR amplification of total cellular RNA isolated from normal human hearts. The constructs were confirmed by sequencing and were used to prepare ^{32}P-UTP radiolabeled antisense cRNA probes (MAXIscript[TM], Ambion). The DNA template of human cyclophilin was purchased from Ambion Inc. All cRNA probes were gel purified before using a 5% denatured polyacrylamide gel. Hybridization of the probes using 10μg total RNA from each age group was carried out at 48°C for 18 h followed by digestion with RNAses A and T1 at 37°C for 30 min. The reaction was terminated by addition of SDS and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. The protected fragments were visualized by autoradiography after electrophoresis on a 5% denatured polyacrylamide gel. Ten μg of yeast RNA was used as a negative control to test for the presence of probe self-protection bands. Because of the low amount of Ca channels in human fetal hearts, especially the α_{1H} subunit of T-type Ca channels, we increased the exposure time to 20 d as previously recommended (10) for T-type Ca channel α_{1H} subunit mRNA. The exposure time for SERCA2a was 24 h. Quantitative evaluation was carried out using scanning densitometric analysis of the protected fragments.

Relative quantitative RT-PCR. Relative quantitative RT-PCR was also performed for quantification of L-, T-type Ca channels and SERCA2a mRNA levels using QuantumRNA kit from Ambion. Multiplex PCR was performed using 18S rRNA primer and gene specific primers to compensate for variations in RNA quality, initial quantification error and random tube-to-tube variations. Same amount of total RNAs were used for cDNA preparation. To use the 18S rRNA internal control, random primers were used in the reverse transcription. Four hundred and eighty-nine bp classic 18S rRNA internal standard primer pair and 18S rRNA PCR competimers were purchased from Ambion. SERCA2a forward primer was: CGAAAAC-CAGTCCTTGCTGAGGAT; SERCA2a reverse primer was: TACTCCAGTATTGGCATGCCGAGA. The predicted fragment length was 296bp. L-type Ca channel α_{1C} subunit forward primer was: TGGAAGCTCAGCTCCAACAG, L-type Ca channel α_{1C} subunit reverse primer was: TCCTGGTAG-GAGAGCATCTC. The predicted fragment length was 270bp. T-type Ca channel α_{1H} subunit forward primer was: GAA-GACCATGGACAACGTGG, T-type Ca channel α_{1H} subunit reverse primer was: TTGAAGAGCACATAGTTGCCG. The predicted fragment length was 297bp. Pilot experiments were performed to determine the linear range of reaction and optimal ratio of 18S primers/competimers for L-, T-type Ca channels and SERCA2a genes. Final PCR products were evaluated on ethidium bromide stained 1.2% agrose gel. Quantitative evaluation was carried out using scanning densitometric analysis of the corresponding bands. The product levels from the genes of interest were normalized against the products from the internal control.

Data analysis. Significant differences between the groups were determined by ANOVA. When the F ratio exceeded the critical value ($p < 0.05$), Bonferronic p-values were determined for identifying significant group-to-group differences.

RESULTS

SERCA2a mRNA levels. To examine the developmental changes in SERCA2a gene expression, the levels of the SERCA2a mRNA were determined by RNase protection assay and relative quantitative RT-PCR. Figure 1*A* illustrates a representative RNase protection experiment using total RNA isolated from ventricles aged 8-, 11- 12-, 14-, 15-, and 19-wk gestation, neonate (1 d after birth), and adult (18-year-old). Densitometric analysis was used to measure the abundance of SERCA2a transcripts expressed in arbitrary units relative to

cyclophilin for each age group. Averaged data from four hearts at each stage showed no significant developmental changes in SERCA2a mRNA levels from fetal to the adult stage (Fig. 1A). In addition to the use of cyclophilin as an internal control, additional experiments (n = 4 for each age) were performed using 18S rRNA as another internal control. Figure 1B shows that when normalized to 18S rRNA, SERCA2a mRNA also did not significantly change during development. Normalized SERCA2a mRNA data to cyclophilin and 18S rRNA is summarized in Table 1.

SERCA2a protein levels. To examine whether the observed SERCA2a transcript patterns are accompanied by changes at the protein level, the immunoreactive SERCA2a protein was quantified by Western blot using a specific monoclonal mouse anti-SERCA2a antibody (MA3–919, Affinity Bioreagents Inc).

A. SERCA2a mRNA (RPA)
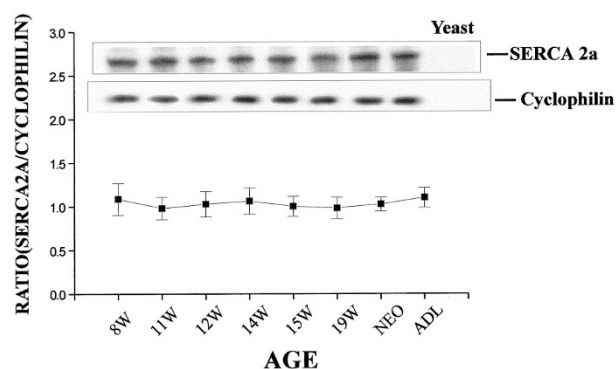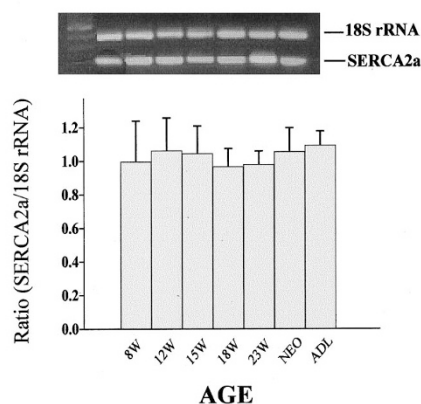

B. SERCA2a mRNA (RT-PCR)

Figure 1. Developmental expression of SERCA2a mRNA in human hearts assessed by RNase Protection Assay (RPA) and relative quantitative RT-PCR. (*A*) RNase protection assay of SERCA2a mRNA using cyclophilin as an internal control. The upper panel represents the protected fragments corresponding to SERCA2a mRNA and the lower panel corresponds to cyclophilin mRNA. The graph shows the average data of the ratio of SERCA2a to cyclophilin density at each age group. (*B*) Relative quantitative RT-PCR analysis of SERCA2a mRNA using 18S rRNA as an internal control. The upper panel represents 18S rRNA fragments and the lower panel represents the fragments corresponding to SERCA2a. The graph shows average data of the ratio of SERCA2a to 18S rRNA density at each age group. Values are mean±SE and *n* = 4 at each stage. NEO = neonatal, W = week, ADL = adult.

Figure 2 illustrates a representative Western blot experiment using hearts aged 8-, 10-, 12-, 15-, 18-, 20-wk gestation, neonate (2 d after birth), and adult (40-year-old). The mouse monoclonal anti-SERCA2a antibody cross-reacted with the protein preparations of fetal, neonatal and adult human hearts. The protein levels of SERCA2a increased with development to the adult stage. Densitometric analysis of average data from five hearts of each stage is shown in Fig. 2. Normalization of SERCA2a protein against the earliest gestation age (8-wk) showed that SERCA2a protein levels increased from 1 at 8-wk gestation to 2.5 at neonate ($p < 0.05$) and reached 3.2 at the adult stage ($p < 0.05$). Normalized SERCA2a protein data are summarized in Table 1.

α_{1C} Subunit of L- type and α_{1H} subunit of T-type Ca channel mRNA levels. Because of the low amounts of Ca channel protein in fetal human cardiac tissue, and because of the limited availability of large amount of human fetal heart tissue for partial purification to get detectable bands by Western blot, only mRNA levels but not protein levels were determined for α_{1C} subunit of L-type and α_{1H} subunit of T-type Ca channels (also to date there are no available specific antibodies for T-type α_{1H} subunit). L-type Ca channel mRNA increased with development to reach a maximum at the adult stage. L-type Ca channel α_{1C} subunit mRNA levels normalized to cyclophilin significantly increase from 1 at 8-wk gestation to 6 at neonate stages ($p < 0.05$), and further increased to 9 at the adult stage ($p < 0.05$). Figure 3A illustrates such an example.

L-type Ca channel α_{1C} subunit mRNA levels were also normalized to 18S rRNA and showed an increase from 1 at 8-wk gestation to 2.5 at neonate ($p < 0.05$) and to 4 at the adult stage ($p < 0.05$, Fig. 3B). In contrast to L-type Ca channel α_{1C} subunits, T-type Ca channel α_{1H} subunit mRNA decreased with development to reach the lowest level at the adult stage. T-type Ca channel α_{1H} subunit mRNA levels normalized to cyclophilin decreased from 1 at 8-wk gestation to 0.65 ($p < 0.05$) at neonate stage and to the lowest level of 0.60 ($p < 0.05$) at the adult stage (Fig. 4A). T-type Ca channel α_{1H} subunit mRNA levels were also normalized to 18S rRNA and demonstrated a decrease from 1 at 8-wk gestation to 0.45 ($p < 0.05$) at neonate stage and to the lowest level of 0.26 ($p < 0.05$) at the adult stage (Fig. 4B). Normalized L-type and T-type Ca channel mRNA levels to cyclophilin and 18S rRNA are summarized in Table 1.

DISCUSSION

The present data showed that 1) mRNA level for SERCA2a, L-type α_{1C} subunit and T-type α_{1H} subunit of Ca channels could be detected as early as 8-wk gestation, 2) T-type Ca channel α_{1H} subunit mRNA levels were more abundant at fetal stages than adult stages, 3) L-type Ca channels α_{1C} subunit mRNA levels increased with development, and 4) SERCA2a protein levels increased with development but SERCA2a mRNA levels did not significantly vary during development.

SERCA2 gene expression. The expression of the cardiac SERCA2 gene during development has been studied in several mammalians species (animals, not human) (11–18). SERCA2 mRNA and protein content were reported to be lower in the

Table 1. *Developmental changes in SERC2a protein per microgram of total protein, SERCA2a mRNA, L-type Ca channel α_{1C} subunit mRNA and T-type Ca channel α_{1H} subunit mRNA levels normalized to cyclophilin and 18s rRNA, respectively*

Age	SERCA2a mRNA		SERCA2a protein	L-type Ca channel α_{1C} subunit mRNA		T-type Ca channel α_{1H} subunit mRNA	
	Per cyclophilin	Per 18S rRNA	Per µg of total protein	Per cyclophilin	Per 18S rRNA	Per cyclophilin	Per 18S rRNA
8 wk	1	1	1	1	1	1	1
10 wk	ND	ND	0.86	1.2	ND	0.80	ND
11 wk	0.91	ND	ND	ND	ND	ND	ND
12 wk	0.95	1.07	0.89	1.35	1.1	0.68	0.95
14 wk	0.98	ND	ND	ND	ND	ND	ND
15 wk	0.93	1.04	1.05	1.3	1.15	0.66	0.8
18 wk	ND	1.0	1.75	2.0	1.3	0.69	0.82
19 wk	0.95	ND	ND	ND	ND	ND	ND
20 wk	ND	ND	1.86	2.5	ND	0.68	ND
23 wk	ND	1.02	ND	ND	2.01	ND	0.4
Neonatal	0.94	1.06	2.5	6.0	2.5	0.65	0.29
Adult	1.01	1.11	3.25	9.0	4	0.60	0.26

The values of 8 wk gestation were set to 1 for easy comparison. ND, not determined.

fetal rat, mouse, and rabbit heart compared with the adult heart (14, 16, 19–24). The present study showed increased protein levels of SERCA2a in the adult compared with the fetal stages, consistent with previous findings in animal studies (14, 16, 19–24). However, we found that SERCA2a mRNA levels did not show any significant changes during human heart development when normalized to either cyclophilin or to 18S rRNA. The exact mechanisms responsible for the unparalleled expression of SERCA2a mRNA and protein are not known. One possible explanation is that mRNA lifetime may be longer in adult compared with fetal stages and/or that there is highly effective translation machinery in the adult myocytes. Although most of the animal studies on SERCA2 were limited to neonatal stages and at the most to only one fetal stage (11, 14, 16, 20, 22, 24), they reported an increase in SERCA2a mRNA (11, 14, 16, 20, 22, 24). This apparent discrepancy may result

SERCA2a PROTEIN

Figure 2. Developmental expression of SERCA2a protein in human hearts assessed by Western blot. Thirty µg total proteins were separated on 8% SDS-PAGE gels. A 110 KD band was observed in all age groups. The graph shows quantitative analysis of SERCA2a protein levels in human hearts at different ages. Values are mean ± SE and $n = 5$ at each stage, NEO = neonatal, W = week, ADL = adult. *$p < 0.05$ compared with 8-wk gestation.

from the normalization method used and/or differences between species. Thus, caution must be taken when data obtained from animal studies are inferred to humans.

Another finding in our study relates to the robust expression of both SERCA2a mRNA and protein during fetal stages. Moorman *et al.* (21), reported that SERCA2 transcript is already abundant in the cardiogenic plate at nine embryonic days of rat development. Whether the existence of SEARCA2a transcript and protein during development translates into functional Ca-ATPase remains debatable. On one hand ryanodine has been shown to reduce heart contractility in the chicken embryo at day 5 in ove, which corresponds to E13 in the rat. Handock *et al.* (25), showed a functional ryanodine receptor 2 (RyR2) in newborn rabbit myocytes. In a recent study, Chen F *et al.* (24) showed that significant quantities of SERCA2a were present early in the immature rabbit heart and SERCA2a protein function *in situ* was found to be comparable between immature and adult myocytes in maintaining SR Ca stores (24). On the other hand, functional studies (5, 26–29) showed that immature rabbit heart does not critically dependent upon the release of SR Ca stores for cell contraction. Specifically, blockade of Ca release from the SR with ryanodine or depleting SR Ca stores with thapsigargin markedly inhibits cell contraction in adult myocytes but has little effect on neonatal intracellular Ca transient and cell contraction (5, 26–29). Taken all together, the relative abundance of SERCA2a protein in human fetal cardiac myocytes should not be taken as an indicator for the levels of the SR abundance. Further functional and biochemical experiments of ryanodine receptors/SR levels during development in human heart are thus warranted.

L-type and T-type Ca channel expression. Patch-clamp and biochemical studies in mature heart have demonstrated the existence of both long-lasting, dihydropyridine-sensitive, L-type Ca channels and transient, dihydropyridine-insensitive, T-type Ca channels (3, 4, 10, 30–36). Their expressions are highly species, tissue and age dependent. L-type Ca channel density in rabbit heart increases with development (31–33, 37–39). The current density increased 5-fold between gestational day 21 and adulthood (25, 31), and the mRNA coding for
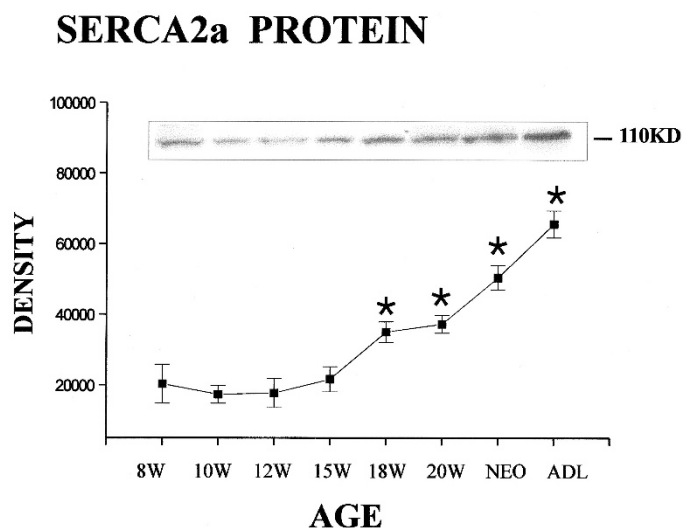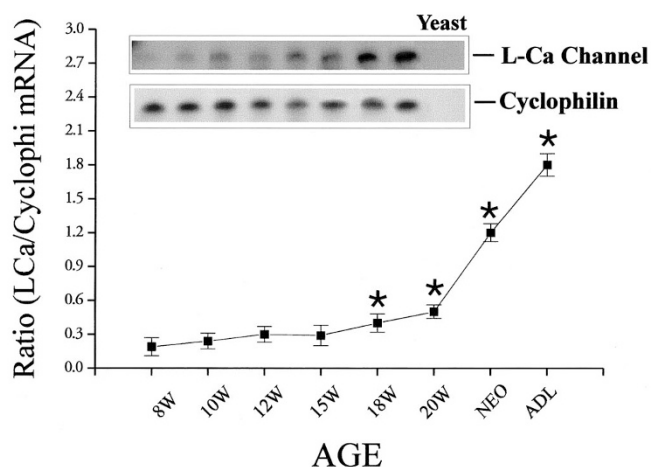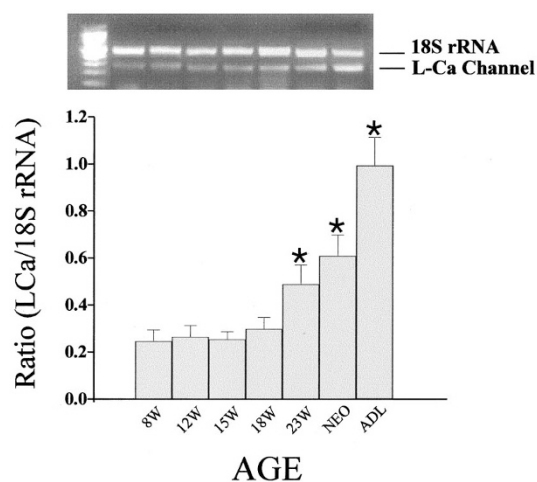
A. L-TYPE Ca CHANNEL mRNA (RPA)

A. T-TYPE Ca CHANNEL mRNA (RPA)

B. L-TYPE Ca CHANNEL mRNA (RT-PCR)

B. T-TYPE Ca CHANNEL mRNA (RT-PCR)

Figure 3. Developmental expression of α_{1C} subunit of L-type Ca channel mRNA in human hearts assessed by RNase protection assay (RPA) and relative quatitative RT-PCR. (*A*) RPA using cyclophilin as an internal control. The upper protected fragments correspond to α_{1C} subunit of L-type Ca channel and the lower bands represent cyclophilin mRNA. The graph illustrates average ratio of α_{1C} subunit of L-type Ca channels to cyclophilin density at different developmental stages. (*B*) Relative quantitative RT-PCR using 18S rRNA as an internal control. The upper fragments correspond to 18S rRNA and the lower bands represent α_{1C} subunit of L-type Ca channel. The graph shows average ratio of α_{1C} subunit of L-type Ca channels to 18S rRNA density at different developmental stages. Values are mean $\pm$ SE and $n = 4$ at each stage. *$p < 0.05$ compared with 8-wk gestation, W = week, NEO = neonatal, ADL = adult.

Figure 4. Developmental expression of T-type Ca channel α_{1H} subunit mRNA in human hearts assessed by RNase protection assay (RPA) and relative quantitative RT-PCR. (*A*) RPA using cyclophilin as an internal control. The upper protected fragments correspond to T-type Ca channel α_{1H} subunit mRNA, and the lower bands represent cyclophilin mRNA. The graph shows average ratio of T-type Ca channel α_{1H} subunit to cyclophilin mRNA levels at different stages. (*B*) Relative quantitative RT-PCR using 18S rRNA, as an internal control. The upper fragments correspond to 18S rRNA and the lower bands represent α_{1H} subunit of T-type Ca channel. The graph shows the average ratio of α_{1H} subunit of T-type Ca channels to 18S rRNA density at different developmental stages. Values are mean $\pm$ SE and $n = 4$ at each stage. *$p < 0.05$ compared with adult stage. NEO = neonate, W = week. ADL = adult.

the α_{1C} subunit increased by 2–3 fold between the perinatal period and adulthood (33). In contrast to rabbit, Ca current density in cultured rat neonatal myocytes is much greater than in acutely isolated rat adult myocytes (34). Although L-type Ca channels have been shown to play a central role in cardiac EC-coupling, little is known about the role of T-type Ca channels in this process (35). T-type Ca current has been
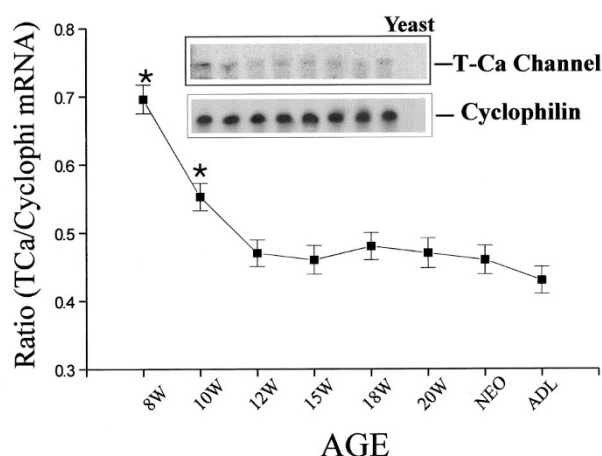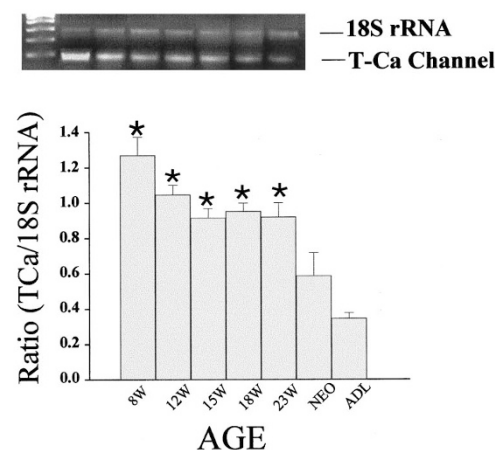
involved in pacemaker and low threshold Ca spikes (36). Interestingly, Zhou *et al.* (4) showed that in cardiac Purkinje cells, Ca entry through the T-type Ca channel could activate cell contraction. Also T-type Ca current has been implicated in triggering Ca release from the SR in guinea-pig ventricular myocytes (3). De Paula Brotto *et al.* (40), found that as much as one–fourth of the Ca entering *via* sarcolemmal Ca channels

is due to T-type Ca channels that are present in day 11 embryonic chick cardiac myocytes. The age and species dependent expression of Ca channels during development underlies the necessity of characterizing this changing pattern during human heart development.

The present data showed that there are less L-type Ca channel α_{1C} subunit mRNA levels in fetal stages than in the adult stage. If these mRNA profiles reflect protein levels, it further supports the idea that in fetal and neonatal hearts, sarcolemmal Ca handling proteins may play an important role in the sarcolemmal Ca entry. This is supported by our previous study (6), which showed that there is more NCX expression in human fetal stages than in adult stages and from animal studies in immature hearts (29). More T-type Ca channel α_{1H} subunit mRNA levels in fetal stages than in adult stages suggests that T-type Ca channel might provide an additional route of Ca entry through the sarcolemma and compensate for the relative low abundance of L-type Ca channels.

Physiologic significance. The present data showed temporal changes of gene expression of SERCA2a, α_{1C} subunit of L- and α_{1H} subunit of T-type Ca channels and are consistent with our previous findings on NCX expression during human heart development (6). The potential consequences of increased expression of T-type Ca channel, NCX and decreased expression of L-type Ca channel and SERCA2a mRNA during fetal stages, is that fetal EC-coupling may rely more on transsarcolemmal Ca influx. Inhibition of this Ca influx would be expected to adversely affect the fetal heart. It has been suggested that during fetal and neonatal stages, when expression of L-type Ca channels involved in EC-coupling is low, small doses of Ca channel blockers could have adverse effects on the myocardium (33). A Ca channel blocking effect could result in diminished cardiac contractility. If future functional studies confirm the present gene expression patterns in the human fetal cardiac myocytes, then caution must be taken in the therapeutic use of sarcolemmal Ca fluxes blockers in the human fetal heart during pregnancy.

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