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ORIGINAL CONTRIBUTION

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Endocardial versus epicardial differences of sarcoplasmic reticulum Ca^{2+}-ATPase gene expression in the canine failing myocardium

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Abstract It is unknown whether the transmural heterogeneity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase gene expression is present within the left ventricular (LV) wall. Moreover, the changes of transmural distribution have not been examined in the failing hearts. We thus quantified steady-state mRNA abundance of SR Ca^{2+} regulatory proteins by Northern blot analysis in both subendocardial and subepicardial LV layers from normal and rapid pacing-induced heart failure (HF) dog hearts. For normal LV, Ca^{2+}-ATPase mRNA abundance (normalized to glyceraldehyde-3-phosphate dehydrogenase [GAPDH] mRNA) was significantly reduced in the subendocardium, whereas calsequestrin mRNA abundance was comparable between the two layers. For HF LV, Ca^{2+}-ATPase mRNA abundance in the subendocardium was also reduced compared to the subepicardium. However, the endocardium to epicardium ratio was comparable between control and HF (0.62 ± 0.08 vs. 0.65 ± 0.07; p = NS). Therefore, the transmural gradient of this gene was constant in both control and HF. Even though the data on the transmural heterogeneity of protein level is not available, the subendocardium contained significantly less Ca^{2+}-ATPase mRNA, which might contribute, at least in part, to the transmural gradients of biochemical and mechanical function.

Key words Congestive heart failure – calcium – contractile function – sarcoplasmic reticulum – transmural heterogeneity

Introduction

Under normal physiological conditions, transmural heterogeneity has been demonstrated for myocardial metabolic (14) and mechanical function (20, 23). Even though the mechanisms underlying these phenomena have not been clarified, the transmural differences of intracellular calcium ($[Ca^{2+}]i$) homeostasis, an important regulator of myocyte metabolism and contractile function, might be involved. Figueredo et al. measured $[Ca^{2+}]i$ transients at the subendocardial and subepicardial layers of perfused rat hearts using indo-1 fluorescence and demonstrated that both diastolic and systolic levels of $[Ca^{2+}]i$ were elevated in the subendocardium compared with those in the subepicardium under normal conditions (4). Furthermore, Vatner et al. have shown that adenylate cyclase, which is involved in the phosphorylation of phospholamban, is decreased selectively in the subendocardium after ischemia/reperfusion and that reduced cAMP-activated phosphorylation of phospholamban and the resultant inhibition of Ca^{2+} uptake by the SR would contribute to the progression of heart failure (9, 30). Sarcoplasmic reticulum (SR) function, especially Ca^{2+} uptake via Ca^{2+}-ATPase, plays an important role in regulating $[Ca^{2+}]i$ homeostasis in cardiac myocytes. Therefore, it is conceivable that the transmural differences of $[Ca^{2+}]i$ transients may result from those of SR Ca^{2+}-ATPase activity.

Previous studies have demonstrated that Ca^{2+} uptake by SR is reduced in the failing myocardium, which is accompanied by a decrease in SR Ca^{2+}-ATPase activity, protein, and mRNA

levels (2, 6, 15, 16, 29). Chronic rapid ventricular pacing has been shown to cause dilated cardiomyopathy, which is characterized by an increase in left ventricular (LV) chamber dimension and a decrease of contractile function (8, 17, 25), along with the decrease of SR Ca^{2+}-ATPase mRNA abundance (8). In a recent preliminary report using failing human myocardium, SR Ca^{2+}-ATPase mRNA abundance and protein amount were lowered in the subendocardium compared to the subepicardium (21).

However, it is not known whether the transmural heterogeneity of steady-state abundance of SR Ca^{2+}-ATPase mRNA is present in the normal and failing myocardium.

Therefore, the goals of the present study were (1) to test the hypothesis that SR Ca^{2+}-ATPase mRNA abundance differ between the subendocardial and subepicardial layers of normal and failing myocardium and (2) to determine whether transmural heterogeneity of SR Ca^{2+}-ATPase mRNA abundance differs between normal and failing myocardium. In all studies on SR Ca^{2+}-ATPase mRNA published by now, it has been reported that mRNA levels are reduced in HF. However, findings have been controversial at the level of protein. Several studies indicated that SR Ca^{2+}-ATPase protein level was unchanged in HF (13, 18, 24).

The present study demonstrated that the subendocardium contained a significantly lower steady-state mRNA abundance of Ca^{2+}-ATPase, but the degree of its decrease at the subendocardium did not change in HF.

Methods

Preparation of animal models

Ten adult mongrel dogs with HF induced by rapid ventricular pacing (HF dogs) and 10 control dogs (12 to 24 kg body weight) were used in the present study (8). Under general anesthesia, a bipolar pacing lead (1236T; Pace Setter Inc.) was introduced into the external jugular vein and placed in the right ventricle under fluoroscopic guidance. After recovery from the surgery, rapid ventricular pacing at 240 beats/min was begun and maintained continuously for 4 weeks by connecting the lead to an external pulse generator (Nihon-Kohden). Proper pacing and 1:1 conduction were confirmed by cardiac auscultation daily. Control dogs were treated in an identical manner as HF dogs, in which a pacing lead was inserted without pacing.

In vivo LV function studies

On the day of the study, ventricular pacing was stopped and the echocardiographic studies were done in the conscious conditions in sinus rhythm after 15 minutes of stabilization (8). Two-dimensional and M-mode echocardiograms were recorded with the use of an ultrasonograph (SSH-65A; Toshiba Medical Inc.). LV short-axis (cross-sectional) views were recorded at the papillary muscle level, and the internal LV dimensions were measured. LV ejection fraction (percent) was then calculated by use of the formula [(LV End-Diastolic Dimension)^3 – (LV End-Systolic Dimension)^3] / [(LV End-Diastolic Dimension)^3] x 100.

After echocardiographic recordings, the animals were anesthetized, intubated, and ventilated with a respirator on a heating pad to maintain the body temperature at 37 °C. A catheter was inserted into the aortic arch via the left carotid artery for the measurement of systemic arterial pressure. After thoracotomy was performed, an externally calibrated 7F cathetertipped pressure transducer (PC 350; Millar Instruments) was inserted into the LV through the left atrium for the measurement of LV pressure. The protocols were approved by the Committee on the Ethics of Animal Experiments, Kyushu University and the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Northern blot analysis of steady-state mRNA abundance of SR Ca^{2+} regulatory proteins

Transmural samples from the LV free wall were divided into endocardial, midwall and epicardial thirds, and the steady-state mRNA abundance of SR Ca^{2+} regulatory proteins at each subendocardial and subepicardial layer was determined. We discarded the midwall samples. In brief, frozen tissue was homogenized with a Polytron homogenizer in a solution containing 4 mol/L guanidinium thiocyanate, and total RNA was isolated according to the methods of Chomczynski and Sacchi (3) with some modifications (8, 19). RNA was quantified by absorbance at 260 nm and the integrity was determined by examining the 28S and 18S rRNA bands in ethidium bromide-stained agarose gels visualized under ultraviolet (UV) light. RNA (20 µg) was denatured (65 °C, 15 min) and size-fractionated by electrophoresis on 1 % (wt/vol) agarose gels under denaturing conditions. RNA was transferred to nylon membranes (Hybond N^+; Amersham International plc.) and immobilized by UV irradiation. Hybridization with cDNA probes (2 x 10^6 cpm/ml) was performed overnight at 42 °C in buffer containing 50 % formamide, 5 x SSPE (0.9 mol/L NaCl, 0.05 mol/L sodium phosphate), 5 x Denhardt's solution (0.2 % polyvinylprolidone, 0.2 % bovine serum albumin, and 0.2 % Ficoll), 0.5 % SDS, and 50 µg/ml denatured salmon sperm DNA. The following cDNA clones were used: rabbit SR Ca^{2+}-ATPase, rabbit calsequestrin (kindly provided by Drs. Arai and Periasamy, University of Cincinnati College of Medicine),

and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA clones were radioactively labeled using a random-prime DNA labeling kit (Boehringer Mannheim). [^{32}]dCTP (DuPont / NEN) was included in the reaction mixture to obtain a specific activity of 5 to 20 x 10^8 cpm/µg DNA. Blots were washed in 2 x SSPE-0.4 % SDS (55 °C, 40 min) and 1 x SSPE-0.1 % SDS (55 °C, 20 min). All membranes were exposed at –80 °C for varying time periods to X-Omat x-ray film (Eastman Kodak Co.) using intensifier screens.

Quantification of Northern blots was performed by the integrated optical density increase over background density in a rectangular region of interest. In Northern blots, we are very careful in loading the gels and the transfer of RNA to be equal for each lane. In our preliminary Northern blot analysis of Ca^{2+}-ATPase mRNA and GAPDH mRNA, the reproducibility of our measurements of mRNA abundance was as high as 97 ± 5 % of initial value. However, we have to admit that there is unavoidable variability in RNA quantitation by UV spectrophotometric absorbance, gel loading, and transfer. To avoid the variations in sample and blotting efficiency of RNA, steady-state mRNA abundance for SR Ca^{2+}-ATPase and calsequestrin was expressed as the relative value to the consistently expressed GAPDH mRNA abundance. GAPDH mRNA abundance was proportional to the intensity of 28S and 18S ribosomal RNA on ethidium bromide staining.

Statistical analysis

All data are presented as mean ± SEM. A paired Student's t test was used to compare values between subendocardium and subepicardium obtained from single heart and an unpaired Student's t test was used to compare values between control and HF. Differences were considered statistically significant at $p < 0.05$.

Results

LV contractile function

Animal characteristics and LV contractile function at a spontaneous heart rate are summarized in Table 1. Body weight and LV weight did not differ significantly. Chronic rapid pacing caused an approximately 24 % increase in LV end-diastolic dimension, 71 % increase in end-systolic dimension and a 58% decrease in LV ejection fraction by echocardiography. For the HF dogs, LV peak +dP/dt was significantly depressed and LV end-diastolic pressure was significantly increased compared to control values. Thus, as reported previously by this laboratory (8) and by others (17, 25), chronic rapid pacing caused a hemodynamic and LV functional profile consistent with human dilated cardiomyopathy.

Table 1 Animal characteristics and LV function

	Control (n = 10)	HF (n = 10)
Body weight, kg	16.2 ± 0.8	20.5 ± 2.0
LV weight, g	77.7 ± 8.9	76.8 ± 3.4
LV weight / Body weight, g/kg	4.2 ± 0.4	4.1 ± 0.4
LV enddiastolic dimension, mm	36.1 ± 1.1	45.3 ± 2.5**
LV endsystolic dimension, mm	23.3 ± 0.9	39.8 ± 2.1**
LV ejection fraction, %	73.3 ± 2.2	30.9 ± 3.8**
LV peak +dP/dt, mmHg / s	2544 ± 350	1522 ± 133*
LV enddiastolic pressure, mmHg	6.0 ± 1.0	18.2 ± 3.9**

HF, heart failure; LV, left ventricular.
Values are mean ± SEM. *p < 0.05, **p < 0.01 vs. Control.

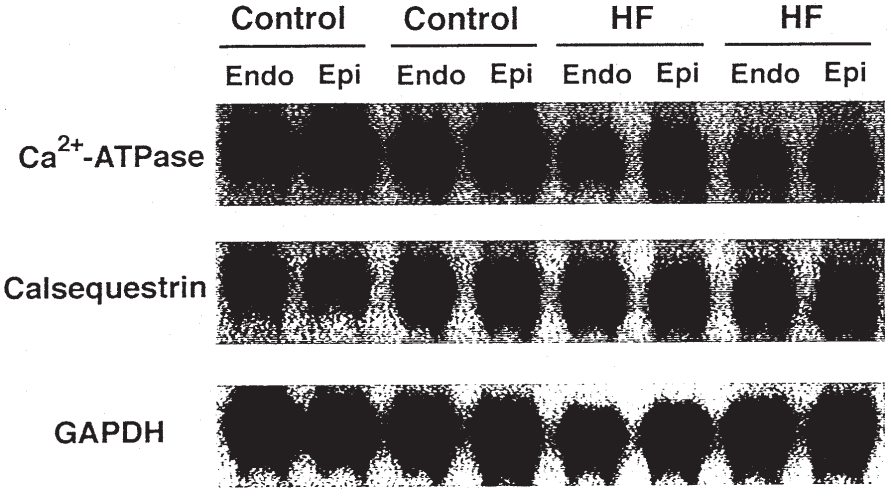

Fig. 1 An example of Northern blot analysis showing SR Ca^{2+}-ATPase (upper), calsequestrin (middle), and GAPDH mRNA (lower) at the subendocardial (ENDO) and subepicardial (EPI) layers from control and HF dog left ventricles.

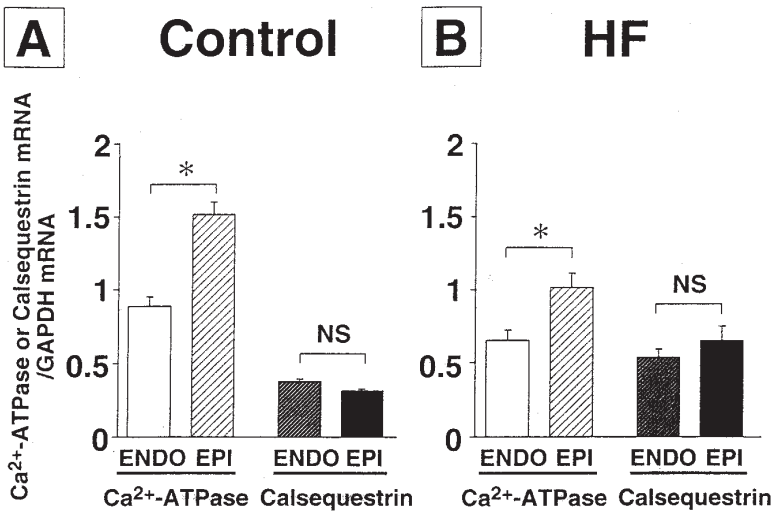

Fig. 2 A summary of the data for steady-state mRNA abundance for SR Ca^{2+}-ATPase and calsequestrin normalized to GAPDH mRNA for control (A; n = 10) and HF (B; n = 10) dogs. Ca^{2+}-ATPase mRNA levels were significantly depressed in the subendocardial layer (ENDO) compared to the subepicardial layer (EPI) whereas calsequestrin mRNA levels were comparable between these two layers. Data are the means ± SEM. *$p < 0.01$ denotes a statistically significant difference compared to ENDO. NS, not significant.

SR Ca^{2+}-ATPase and calsequestrin mRNA abundance

SR Ca^{2+}-ATPase mRNA abundance relative to GAPDH mRNA in the transmural LV wall specimens obtained from HF dogs was significantly reduced compared to that in control animals (0.79 ± 0.19 vs. 1.41 ± 0.26, $p < 0.01$). Figure 1 shows the representative Northern blot analysis, in which SR Ca^{2+}-ATPase, calsequestrin, and GAPDH mRNA were compared between the subendocardial and subepicardial myocardium from control and HF dogs. Figure 2 gives the summarized results of densitometric determination of the steady-state abundance of SR Ca^{2+}-ATPase and calsequestrin mRNA relative to GAPDH mRNA. GAPDH mRNA density values were comparable between the subendocardium and the subepicardium in both control (55.3 ± 2.4 vs. 56.2 ± 3.6, p = NS) and HF (56.7 ± 2.8 vs. 57.7 ± 3.1, p = NS). In both control and HF dogs, SR Ca^{2+}-ATPase mRNA abundance (normalized to GAPDH mRNA abundance) was lower at the subendocardium compared to that at the subepicardium in both control (0.89 ± 0.07 vs. 1.53 ± 0.09, $p < 0.01$) and HF (0.66 ± 0.08 vs. 1.03 ± 0.10, $p < 0.01$). In contrast, calsequestrin mRNA abundance was comparable between subendocardium and subepicardium in both groups.

Even when SR Ca^{2+}-ATPase mRNA abundance was normalized with calsequestrin mRNA in place of GAPDH mRNA, SR Ca^{2+}-ATPase mRNA abundance at the subendocardium was less than that at the subepicardium in control (2.54 ± 0.24 vs. 5.07 ± 0.58, $p < 0.01$) and HF (1.35 ± 0.21 vs. 2.09 ± 0.33, $p < 0.01$). Moreover, when SR Ca^{2+}-ATPase mRNA abundance was normalized with 18S rRNA in place of GAPDH mRNA, SR Ca^{2+}-ATPase mRNA abundance at the subendocardium was less than that at the subepicardium in control (0.58 ± 0.04 vs. 1.04 ± 0.04, $p < 0.01$) and HF (0.40 ± 0.04 vs. 0.72 ± 0.06, $p < 0.01$).

Despite the differences in SR Ca^{2+}-ATPase mRNA abundance between control and HF, the endocardial to epicardial ratios did not differ between these two groups of animals (0.62 ± 0.08 vs. 0.65 ± 0.07, p = NS).

Discussion

Even though a number of previous studies analyzed SR Ca^{2+}-ATPase mRNA abundance using myocardial tissue specimens (2, 6, 8, 15, 16, 29), this study has examined whether the transmural gradient exists in the steady-state abundance of SR Ca^{2+}-ATPase mRNA in normal hearts and whether the normal difference in the appearance may by altered in the failing heart. The most important finding of this study was that steady-state mRNA abundance of SR Ca^{2+}-ATPase was reduced in the subendocardial myocardium obtained from control as well as HF animals. However, there was no significant difference in the endocardial to epicardial ratio of SR Ca^{2+}-ATPase mRNA abundance between control and HF. In a recent preliminary report using failing human myocardium, SR Ca^{2+}-ATPase mRNA abundance and protein amount were lowered in the subendocardium compared to the subepicardium (21). Therefore, the present study is not the first that demonstrated a transmural heterogeneity of SR Ca^{2+}-ATPase gene expression. An important finding of this study is that the transmural heterogeneity of this gene is constant in the control and HF groups.

Under normal physiological conditions, SR Ca^{2+}-ATPase mRNA abundance was down-regulated in the subendocardium. These findings are in agreement with the previous observations of transmural gradients of mechanical and metabolic function (14, 20, 23). However, the exact causes responsible for these regional differences are not entirely clear. One

possibility might be relative ischemia at the subendocardium, which, however, seems not to be responsible for the transmural differences of SR Ca^{2+}-ATPase mRNA abundance since it has been demonstrated to be increased, not decreased, in the myocardium subjected to ischemia and reperfusion (5). Most importantly, the observed subendocardial versus subepicardial differences of SR Ca^{2+}-ATPase may provide a good explanation for the transmural heterogeneity of $[Ca^{2+}]i$ transient demonstrated by Figueredo et al. (4). The relative elevation of endocardial $[Ca^{2+}]i$ could be attributed to the decreased SR Ca^{2+}-ATPase mRNA abundance and the resultant reduction of Ca^{2+}-uptake by SR. Furthermore the relative elevation of endocardial $[Ca^{2+}]i$ could account for the transmural metabolic gradients for rates of substrate oxidation and glycolytic enzyme activities (14). It may also provide an explanation for the shorter sarcomere and myofiber lengths at the subendocardium (20).

The results that calsequestrin mRNA abundance was comparable between subendocardium and subepicardium indicate that SR Ca^{2+} regulatory proteins are independently regulated and the decrease of SR Ca^{2+}-ATPase mRNA abundance is not associated with the generalized depression of cardiac gene expression at the subendocardial layer.

In the failing myocardium, myocardial perfusion is demonstrated to be disturbed especially in the subendocardium (26). When the subendocardial layer is ischemic and dysfunctional in HF, a large reduction in SR Ca^{2+}-ATPase mRNA abundance would be expected to be evident at the subendocardial layer (even with normal Ca^{2+} uptake by SR in the subepicardial layer). Furthermore, Vatner et al. have shown that adenylate cyclase, which is involved in the phosphorylation of phospholamban, is decreased selectively in the subendocardium after ischemia/reperfusion and in heart failure (9, 30). As a result of decreased adenylate cyclase activity in association with β-adrenergic receptor down-regulation, cAMP levels are reduced in the failing myocardium. A decrease in cAMP generation would decrease cAMP-activated phosphorylation of phospholamban, which would result in a decreased affinity of Ca^{2+}-ATPase for Ca^{2+}. As a consequence, Ca^{2+} sequestration would be impaired and thereby would contribute to the contractile dysfunction. However, the present study demonstrated that SR Ca^{2+}-ATPase mRNA abundance in the subendocardium from HF heart was reduced to a similar extent as in control hearts. Even though the degree of decrease in SR Ca^{2+}-ATPase mRNA abundance at the subendocardial layer was comparable between control and HF, these results may well account for the substantial reduction of subendocardial contractile function in rapid-pacing induced HF (12). Moreover, a reduction of SR Ca^{2+}-ATPase in the subendocardium leads to the intracellular Ca^{2+} overload and a greater susceptibility of the myocardium to injury, which might be responsible for the predominant morphological alterations in the subendocardium such as the disruption of interstitial collagen network, lengthening of myocytes with the swelling of T-tubules and the poor alignment of myofibrils, and the abnormal swelling and distribution of mitochondria (7, 25).

Senescence has been shown to be associated with a decrease in SR Ca^{2+}-ATPase mRNA without any alterations of the ryanodine receptor (1), suggesting that the aged heart is comparable to the failing hearts. The mechanisms responsible for this decrease of SR Ca^{2+}-ATPase might be related to the hemodynamic load to the myocardium due to the increased aortic impedance and enhanced wall stiffness. In addition, impaired coronary circulation might be also involved since coronary blood flow is shown to be reduced in the aged heart. Taken together, the senescent heart resembles a subendocardial layer of LV in terms of hemodynamic characteristics, coronary blood flow, and modification of the SR Ca^{2+}-regulatory proteins.

This study has several potential limitations to be acknowledged. First, we measured only mRNA levels and did not examine the activity or protein level of SR Ca^{2+}-ATPase in this study. This should be a crucial issue since mRNA abundance may not always reflect the appearance of the specific associated protein. The potential reasons for the divergent results between mRNA and protein levels are as follows; first, steady-state mRNA abundance cannot necessarily be assumed to be representative of protein levels, in particular because protein levels are regulated by the complex mechanisms at the level of transcription, translation, and protein degradation (13, 24). Second, controversial results on the alterations of proteins might de due to the preparations studied. Because of the artifacts in preparing the sample fractions, differences between control and pathologic hearts may depend on whether crude homogenates or partially purified membrane preparations are studied. In fact, Schwinger et al. reported that Ca^{2+}-ATPase activity was decreased in crude membrane preparations but not in isolated sarcoplasmic reticulum vesicles from failing compared to non-failing hearts (24). Further, contractile function and $[Ca^{2+}]i$ transient were not assessed in myocytes isolated separately from the subendocardial and subepicardial layers. Therefore, the functional significance of reduced SR Ca^{2+}-ATPase mRNA in the subendocardial layer remains undetermined. However, our recent study demonstrated that there was a significant positive correlation between steady-state SR Ca^{2+}-ATPase mRNA abundance and Ca^{2+} uptake rate by SR ($r = 0.57$, $n = 14$, $p < 0.05$) (8). In addition, there was a significant positive relation between myocyte contractile function and SR Ca^{2+}-ATPase mRNA abundance (8), indicating that SR Ca^{2+}-ATPase could be a determinant of myocardial contractility. Therefore, these results could support, at least in part, the transmural heterogeneity in the electromechanical function of cardiac muscle. Second, the pathogenesis of rapid pacing-induced HF may not be directly related to that of clinical dilated cardiomyopathy. However, chronic rapid ventricular pacing for 4–6 weeks has been shown to cause the progressive

decrease of LV pump performance and the increase of chamber dimension (8, 17, 26). In addition, the development of LV dysfunction is associated with significant neurohumoral activation (22). These changes in LV function and neurohumoral systems that occur during chronic rapid pacing are similar to what is observed in HF patients with dilated cardiomyopathy. Rapid pacing also causes the reduction of interstitial collagen lattice (27), which might result in muscle slippage, fiber realignment, and wall thinning. These alterations of the interstitial collagen might be also involved in the further progression of LV dilatation. We thus consider that the results in this study could be applicable to HF in general. In fact, a recent preliminary report confirmed our findings using failing human myocardium (21). Third, the activity of SR Ca^{2+}-ATPase and Ca^{2+} uptake by the SR are tightly controlled by phospholamban and its phosphorylation state (10). It has been demonstrated that the rate of Ca^{2+}-uptake and the contractile function depend on the ratio of phospholamban to SR Ca^{2+}-ATPase rather than on expression of SR Ca^{2+}-ATPase alone (11). Nevertheless, the mRNA data of phospholamban are not available in the current animals. Further studies to determine the coordinate alterations of SR Ca^{2+}-ATPase and phospholamban mRNA abundance need to be performed in the failing hearts. Fourth, the present study did not provide the mechanisms responsible for the transmural heterogeneity of SR Ca^{2+}-ATPase. Thus, further studies to clarify this crucial question are obviously needed.

In conclusion, the present study demonstrates that the steady-state mRNA abundance of SR Ca^{2+}-ATPase is different between the subepicardium and subendocardium within the LV free wall in normal and failing hearts. These differences may partly account for the observations of transmural gradients of biochemical and mechanical function within the myocardium.

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