Role of SR Ca\(^{2+}\)-ATPase in contractile dysfunction of myocytes in tachycardia-induced heart failure

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Igarashi-Saito, Keiko, Hiroyuki Tsutsui, Shimako Yamamoto, Masaru Takahashi, Shintaro Kinugawa, Hirofumi Tagawa, Makoto Usui, Mitsutaka Yamamoto, Kensuke Egashira, and Akira Takeshita. Role of SR Ca\(^{2+}\)-ATPase in contractile dysfunction of myocytes in tachycardia-induced heart failure. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H31–H40, 1998.—Sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase gene expression is reduced in the failing myocardium. However, the functional relevance of these changes to myocardial contractility is not yet established. We assessed myocardial contractile function by analyzing sarcomere motion of isolated myocytes and also quantified SR Ca\(^{2+}\) regulatory protein gene expression by Northern blot analysis in the same hearts obtained from 10 dogs with pacing-induced heart failure (HF; 240 beats/min, 4 wk) and 7 control dogs. Sarcomere-shortening velocity was depressed in HF myocytes, accompanied by the prolongation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transient measured by indo 1 fluorescence ratio. SR Ca\(^{2+}\)-ATPase mRNA levels (normalized to glycerol-dehyde-3-phosphate dehydrogenase mRNA) were significantly depressed in HF, and calsequestrin mRNA was increased. For control and HF dogs, sarcomere-shortening velocity correlated positively with Ca\(^{2+}\)-ATPase mRNA levels (r = 0.73, n = 17, P < 0.01) but not with calsequestrin mRNA. Ca\(^{2+}\)-ATPase mRNA levels were correlated with 45Ca\(^{2+}\) uptake rate by SR, which was also reduced in HF. Moreover, the inhibition of SR Ca\(^{2+}\)-ATPase with thapsigargin or cyclopiazonic acid reproduced in normal myocytes the abnormalities observed in HF myocytes, such as depressed contractility and the prolonged [Ca\(^{2+}\)]\(_i\) transient duration. A downregulation of Ca\(^{2+}\)-ATPase gene expression and a resultant decrease in Ca\(^{2+}\) uptake by SR may be responsible for the contractile dysfunction and the alteration of [Ca\(^{2+}\)]\(_i\) transient in HF.

CONTRACTILE PERFORMANCE is impaired not only at the ventricular level but also at the isolated cardiac myocyte level in heart failure (HF) (29). Simultaneous measurement of force of contraction and Ca\(^{2+}\) signals with the use of aequorin in intact muscle preparations from failing human hearts exhibited abnormal Ca\(^{2+}\) handling, manifested as prolonged Ca\(^{2+}\) transients (16). Similar findings have also been demonstrated in the isolated myocyte obtained from failing human hearts (4). The abnormalities in excitation-contraction coupling and, in particular, altered sarcoplasmic reticulum (SR) Ca\(^{2+}\) handling have been suggested to be the major candidate mechanisms responsible for the contractile defects in failing hearts (16). SR function may be disturbed at the level of Ca\(^{2+}\) uptake by SR Ca\(^{2+}\)-ATPase or its regulatory protein, phospholamban, at the level of Ca\(^{2+}\) binding by calsequestrin, and at the level of Ca\(^{2+}\) release through ryanodine receptor (2). Previous studies on steady-state mRNA levels have consistently indicated that SR Ca\(^{2+}\)-ATPase mRNA levels are decreased, whereas the expression of the ryanodine receptor and calsequestrin is unchanged, in failing hearts (1, 2, 14, 25, 26). Reduced Ca\(^{2+}\)-ATPase expression in the failing myocardium was also suggested from several studies measuring protein levels (10, 15, 23).

Despite a number of consistent works showing the decrease of SR Ca\(^{2+}\)-ATPase in failing hearts, previous studies examining the functional relevance of these changes to the impairment of myocardial systolic contractile performance in HF have yielded conflicting results. Hasenfuss et al. (10) investigated the relation of myocardial contractile function and the expression of SR Ca\(^{2+}\)-ATPase in failing human hearts. They demonstrated that the protein levels of SR Ca\(^{2+}\)-ATPase were closely related to the force-frequency behavior of myocardium. In contrast, Mercadier et al. (14) did not find a significant correlation between SR Ca\(^{2+}\)-ATPase mRNA levels and left ventricular (LV) systolic function parameters obtained by echocardiographic studies in patients with end-stage HF. Thus the functional role of reduced SR Ca\(^{2+}\)-ATPase in the contractile dysfunction of failing hearts remained unanswered.

The failure of the previous studies to demonstrate a clear association between SR Ca\(^{2+}\)-ATPase and contractile function might have resulted from several potential limitations of studies using failing human hearts. First, myocardial tissue samples were obtained from patients with end-stage HF undergoing cardiac transplantation. The heterogeneity of the disease severity and the effects of prior pharmacological treatment could not be controlled in these patients. Furthermore, a significant delay (approximately several months) usually existed between hemodynamic studies and cardiac transplant surgery (25). Second, LV pump performance measurements may not indicate the intrinsic myocardial contractility, because in vivo LV function is influenced by the loading conditions and neurohumoral activation. In addition, the changes in myocardial architecture including myocyte loss, hypertrophy, and interstitial fibrosis, which is characteristic for failing human hearts, can also affect LV pump function. To circumvent such limitations, we employed the canine model of chronic pacing tachycardia-induced HF. In contrast to end-stage failing human hearts, this model is well characterized by LV dilatation and dysfunction before the development of significant fibrosis or hypertrophy (3).
Importantly, the contractile function is demonstrated to be depressed at the isolated myocyte level in this model (32). The use of isolated myocyte preparations has distinct advantages in the assessment of cardiac contractility independent of the effects of in vivo hemodynamic or neurohumoral influences and the extracellular matrix. Therefore, this animal model of HF allows the concomitant evaluation of the cellular physiology of myocardial contraction and its molecular basis using a single heart. It should also be noted that previous studies that examined the correlation between myocardial function and Ca²⁺-ATPase expression could not address the answer to the question of whether the abnormalities in SR Ca²⁺ uptake are pathognomonic of HF or simply a reflection of the presence of HF.

The present study aimed to determine the functional consequence of a decreased expression of SR Ca²⁺-ATPase. We examined the myocyte contractile function and the gene expression of SR Ca²⁺-ATPase in the same hearts obtained from dogs with rapid pacing-induced HF. In addition, we examined the effects of two specific and potent inhibitors of cardiac SR Ca²⁺-ATPase, thapsigargin and cyclopiazonic acid (CPA), on contractile function and intracellular free Ca²⁺ concentration ([Ca²⁺]i) transient in normal myocytes. The latter study was done to examine whether inhibition of SR Ca²⁺-ATPase affects the systolic contractile performance in normal myocytes. Thapsigargin and CPA are chemically unrelated, but both specifically inhibit SR Ca²⁺ uptake without affecting other sarcolemmal Ca²⁺- or myosin ATPases (12, 20, 21, 31).

**METHODS**

Preparation of animal models. Ten adult mongrel dogs with HF induced by rapid ventricular pacing (HF dogs) and seven control dogs (15–25 kg body wt) were used in the present study. Under general anesthesia, a bipolar pacing lead (1236T; Pace Setter) was introduced into the external jugular vein and placed in the right ventricle under fluoroscopic guidance. After recovery of dogs from the surgery, rapid ventricular pacing at 240 beats/min was begun and maintained continuously for 4 wk 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 a manner identical to that of HF dogs except that pacing leads were inserted without pacing.

Study of in vivo LV function. On the day of the study, ventricular pacing was stopped and the echocardiographic studies were done with dogs in the conscious condition in sinus rhythm after 15 min of stabilization. Two-dimensional and M-mode echocardiograms were recorded with the use of an ultrasonograph (SSH-65A; Toshiba Medical). 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 with the use of the formula: [(LV end-diastolic dimension)³ − (LV end-systolic dimension)³]/(LV end-diastolic dimension)³ × 100.

After echocardiographic recordings were done, the animals were generally anesthetized, intubated, and ventilated with a respirator while they rested on a heating pad that maintained their body temperatures 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 7-F catheter-tipped 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 were in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Evaluation of contractile function in isolated cardiac myocytes. The animals were killed with a lethal dose of a-chloralose, and their hearts were quickly excised. Cardiac myocytes were isolated from the LV free wall as described previously in detail (29). In brief, a wedge of LV free wall, perfused by a branch of the left circumflex coronary artery, was dissected free of the heart and perfused with nominally Ca²⁺-free buffer of the following composition (in mmol/l): 140.0 NaCl, 4.8 KCl, 2.4 MgSO4, 1.2 NaH2PO4, 2.5 NaHCO3, 12.0 HEPES, and 12.5 glucose, warmed to 37°C and gassed with 100% O2. The remaining myocardium was blotted dry, frozen in liquid N2, and stored at −80°C for the subsequent Northern blot analysis of SR Ca²⁺-regulatory proteins and the measurements of SR Ca²⁺ uptake. To dissociate the myocytes, the perfusate was changed to the buffer supplemented with type II collagenase (60 units/ml; Wako Pure Chemical), and the perfusion was terminated when the heart became flaccid. Mean perfusion pressure and pH were kept at ~80 mmHg and 7.4, respectively. After the completion of the perfusion, the LV myocardium was minced with scissors in fresh collagenase-containing buffer with 3% BSA and 300 µmol/l Ca²⁺ and was gently agitated for 5 min in the same buffer. The myocytes were harvested by drawing off the supernatant in which they were suspended and filtering it through 210-µm nylon mesh. Myocytes were kept in collagenase-free buffer supplemented with 1.25 mmol/l Ca²⁺ for 1 h at room temperature before the contractile function was assessed.

Sarcomere motion in isolated myocytes was measured using the laser diffraction techniques described previously (11, 28). Briefly, an aliquot of isolated myocytes was placed in a 10-ml plastic chamber and then allowed to settle down through the buffer onto the bottom of the dish, which was placed on the stage of an inverted microscope (Nikon). The buffer was kept at 37.0 ± 0.1°C with the use of a thermostated heating stage. The myocytes were stimulated to contract between a pair of platinum wire electrodes by 0.255-Hz, 100-µA direct-current pulses of alternating polarity. Changes in sarcomere length were measured from the movement of the first-order diffraction pattern cast by a substrate laser light passing through the sarcomeres of a given myocyte onto diometrically opposed optical sensors situated above the microprobe stage. Each sensor was composed of a linear array of 512 photo diodes, and the distance between the first-order diffraction patterns was calculated and stored in a computer. After the extent of sarcomere shortening became stable, 10 contractions were sampled and averaged to yield a final profile of sarcomere length and velocity versus time during contraction.

Only myocytes with the following characteristics were analyzed: single rod-shaped cells, unattached to either adjacent cells or debris, that contracted with each stimulus and were quiescent between stimuli. Sarcomere mechanics were analyzed by using 15–20 myocytes from each animal, and their parameters were averaged to obtain the extent and velocity of sarcomere shortening, which were correlated to mRNA levels determined in the same hearts.
Measurements of [Ca\textsuperscript{2+}] transient. In parallel experiments, the changes in [Ca\textsuperscript{2+}] were measured by using the fluorescent indicator dye indo 1. Briefly, cardiac myocytes, isolated as described in Evaluation of contractile function in isolated cardiac myocytes, were loaded with 5 µmol/l indo 1 acetoxy-methyl ester (indo 1-AM; Molecular Probes) in the buffer supplemented with fatty acid-free 0.5% BSA and 0.03% Pluronic F-127 (Molecular Probes) at room temperature. Cells were subsequently washed with buffer containing 1 mM Ca\textsuperscript{2+} for 30 min before being used for experiments. Indo 1-loaded myocytes were placed in a chamber on the stage of an epifluorescence microscope (Olympus) and stimulated to contract by a pair of platinum wire electrodes. A single myocyte was then excited at 350 nm by epi-illumination, and indo 1 fluorescence emission light, split by a 455-nm dichroic mirror and selected using rectangular band-pass interference filters in the wavelength ranges of 380–430 nm (405-nm channel) and 455–505 nm (480-nm channel), was directed to a pair of photomultiplier tubes. The photocurrent from each tube was integrated at 1-ms intervals, and the ratio of indo 1 emission at the two wavelengths was calculated as an index of [Ca\textsuperscript{2+}] by a computer. The results were expressed as a fluorescence ratio rather than as absolute [Ca\textsuperscript{2+}], values because of the difficulties in obtaining quantitative calibration due to significant variety of the degree of compartmentalization of this indicator from cell to cell (22). Five successive [Ca\textsuperscript{2+}] transients were recorded and averaged to improve the signal-to-noise ratio and to yield a final profile of [Ca\textsuperscript{2+}] transient.

Northern blot analysis of SR Ca\textsuperscript{2+} regulatory proteins. One subset of investigators (K. Igarashi-Saito and M. Takahashi), who were not informed of the results of the evaluation of myocyte contractile function performed by another subset of investigators (S. Yamamoto and S. Kinugawa), isolated RNA and evaluated mRNA expression in the same LV. In brief, frozen LV myocardial tissue (200 mg) 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 (5) with some modifications (18). 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+) and immobilized by UV irradiation. Hybridization with cDNA probes (2 × 10\textsuperscript{6} counts·min\textsuperscript{-1}·ml\textsuperscript{-1}) was performed overnight at 42°C in buffer containing 50% formamide, 5× SSPE (0.9 mol/l NaCl, 0.05 mol/l sodium phosphate), 5× Denhardt's solution (0.2% polyvinyl pyrrolidone, 0.2% BSA, and 0.2% Ficoll), 0.5% SDS, and 50 µg/ml denatured salmon sperm DNA. The following cDNA clones were used: rabbit SR Ca\textsuperscript{2+}-ATPase, rabbit calsequestrin (kindly provided by Drs. Arai and Pitasiasny, University of Cincinnati College of Medicine, Cincinnati, OH), and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA clones were radioactively labeled using a random-prime DNA labeling kit (Boehringer Mannheim). 32P-labeled dCTP (NE) was included in the reaction mixture to obtain a specific activity of 5–20 × 10\textsuperscript{6} counts·min\textsuperscript{-1}·µg DNA\textsuperscript{-1}. Blots were washed in 2× SSPE-0.4% SDS (55°C, 40 min) and 1× 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) using intensifier screens. Quantification of Northern blots was performed by the integrated optical density increase over background density in a rectangular region of interest. Data were expressed as the densitometric intensity of the hybridization signals for SR Ca\textsuperscript{2+}-ATPase and calsequestrin mRNA levels relative to the consistently expressed GAPDH mRNA to avoid the variations in sample loading and blotting efficiency of RNA, because the GAPDH mRNA level was proportional to the intensity of 28S and 18S ribosomal RNA on ethidium bromide staining.

Measurement of Ca\textsuperscript{2+} uptake into SR. To prepare crude homogenates for measurement of Ca\textsuperscript{2+} uptake by SR, myocardial tissues (200 mg) were homogenized with a Polytron homogenizer in 3 ml of isolation medium containing 30% glycerol, 5 mmol/l sodium azide, and 20 mmol/l Tris·HCl (pH 7.4) according to the method of de la Bastie et al. (6). The homogenate was centrifuged for 10 min at 1,100 g, and the supernatant was collected and kept frozen at −70°C. All procedures were performed at 0°C. Protein concentration was determined by biinchoninic acid assay (Pierce) using BSA as standard. The rate of Ca\textsuperscript{2+} uptake was measured at 37°C in 1 ml of medium containing (in mmol/l) 100 KCl, 20 Tris·HCl buffer (pH 6.8), 5 MgCl\textsubscript{2}, 5 K-oxalate, 5 ATP 5, and 0.05 mmol/l Ca\textsuperscript{2+} (NEN). Sodium azide (5 mmol/l) was added to inhibit mitochondrial Ca\textsuperscript{2+} uptake. The reaction was started by adding 50 µg of total protein. After incubation for 5 min in 37°C, aliquots were filtered (HAWP 0.45 µm, Millipore). The filters were washed in 15 ml of a cold solution containing (in mmol/l) 100 KCl, 1 EGTA, and 10 histidine (pH 6.4) to stop the reaction. Radioactivity was determined by liquid scintillation spectroscopy. The rate of Ca\textsuperscript{2+} uptake was calculated using the linear regression analysis from the slope of the relationship between Ca\textsuperscript{2+} uptake and reaction time, which was linear for at least 5 min, and was expressed as nanomoles of Ca\textsuperscript{2+} per minute per milligram of protein. Each uptake rate was calculated as a mean of three independent determinations.

Effects of SR, Ca\textsuperscript{2+}-ATPase inhibitors on myocyte contractile function and [Ca\textsuperscript{2+}] transient. To further characterize the functional consequences of reduced SR Ca\textsuperscript{2+}-ATPase expression in the systolic contractile abnormalities, thapsigargin and CPA (Sigma Chemical) were added to the chamber containing myocytes and the contractile function and [Ca\textsuperscript{2+}] transient of the same single cell were followed over time.

Statistical analysis. All data are presented as means ± SE. An unpaired Student's t-test was used to compare values between control and HF myocytes. Correlations were examined by linear regression analysis using the least-squares method. Differences were considered statistically significant at P < 0.05.

RESULTS

LV and myocyte contractile function. Animal characteristics and LV contractile function at a spontaneous heart rate are summarized in Table 1. Body weight and

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Values are means ± SE. HF, heart failure; LV, left ventricular. *P < 0.01 vs. control.
LV weight did not differ significantly. Chronic rapid pacing caused a 39% increase in end-systolic dimension and a 58% decrease in LV ejection fraction as measured by echocardiography. For the HF dogs, LV peak rate of rise in pressure was significantly depressed and LV end-diastolic pressure was significantly increased compared with control values. Thus, as previously reported by others (3), chronic pacing-induced tachycardia caused a hemodynamic and LV functional profile consistent with human dilated cardiomyopathy. As shown in Fig. 1A, sarcomere shortening of HF myocytes was decreased compared with that in control myocytes. Figure 1, B and C, are the summarized data of the mechanical properties of myocytes. The average yield of rod-shaped myocytes was comparable between control and HF dogs. The extent of sarcomere shortening (initial sarcomere length minus minimum sarcomere length) and the maximum velocity of sarcomere shortening were significantly depressed in the HF myocytes. There were no significant differences in resting sarcomere length between control and HF myocytes [1.98 ± 0.01 vs. 1.97 ± 0.02 µm, P = not significant (NS)]. The sarcomere mechanics of nonfailing and failing hearts in the present study are in good agreement with the results of the studies by our group (32) and others (27).

[Ca^{2+}] transient. The duration of [Ca^{2+}] transient was significantly prolonged in myocytes from HF dogs, especially in the Ca^{2+} uptake phase (Fig. 2).

Fig. 1. Representative sarcomere-shortening mechanics (A) and summary data (B and C) in left ventricular (LV) myocytes from control (n = 7) and pacing-induced heart failure (HF; n = 10) dogs. A, top: sarcomere shortening; bottom: sarcomere-shortening velocity. Data were accumulated from 10–15 myocytes in each animal studied. Both extent of sarcomere shortening (B) and maximum velocity of sarcomere shortening (C) were significantly depressed for HF myocytes. Data are means ± SE; n, no. of individual animals studied. *P < 0.05 compared with control.
myocytes exclusively \((r = 0.68, n = 10 \text{ dogs}, P < 0.05 \text{ for sarcomere shortening}; r = 0.84, n = 10 \text{ dogs}, P < 0.01 \text{ for sarcomere-shortening velocity})\). No significant correlation was found between sarcomere shortening or sarcomere-shortening velocity and calsequestrin mRNA expression \((r = 0.47, n = 17 \text{ dogs}; P = \text{NS} \text{ for extent of sarcomere shortening}; r = 0.46, n = 17 \text{ dogs}, P = \text{NS} \text{ for velocity of sarcomere shortening})\).

Fig. 2. Representative intracellular \(\text{Ca}^{2+}\) concentration \([\text{Ca}^{2+}]_i\) transients (A) and summary data for time to peak fluorescence (B), time to 50% decline from peak fluorescence (C), and time to 90% decline from peak fluorescence (D) in \([\text{Ca}^{2+}]_i\) transients obtained from myocytes from control and HF dogs measured using indo 1 fluorescence ratio. Duration of \([\text{Ca}^{2+}]_i\) transient was markedly prolonged in HF myocytes. Data are means \(\pm\) SE. *\(P < 0.05\) compared with control.

Fig. 3. A: example of Northern blot analysis showing sarcoplasmic reticulum (SR) \(\text{Ca}^{2+}\)-ATPase mRNA, calsequestrin mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from control and HF dog left ventricles. Summary data show results of Northern blot analysis for SR \(\text{Ca}^{2+}\)-ATPase (B) and calsequestrin (C) mRNA levels normalized to GAPDH mRNA. \(\text{Ca}^{2+}\)-ATPase mRNA levels were significantly depressed in HF compared with control, whereas calsequestrin mRNA levels were elevated. Data are means \(\pm\) SE. *\(P < 0.05\) compared with control.
Relationship between SR Ca\textsuperscript{2+}-ATPase mRNA levels and Ca\textsuperscript{2+} uptake by SR. Ca\textsuperscript{2+} uptake rate by SR was decreased in HF compared with control myocytes (3.48 ± 0.44 vs. 2.22 ± 0.37 nmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}, P < 0.05). When Ca\textsuperscript{2+}-ATPase mRNA levels and Ca\textsuperscript{2+} uptake rate measured in the same hearts were plotted, there was a significantly positive correlation between these two variables (r = 0.57, n = 14, P < 0.05).

Effects of SR Ca\textsuperscript{2+}-ATPase inhibitors on myocyte contractile function and [Ca\textsuperscript{2+}]\textsubscript{i} transient. Figure 5A presents an example of time-dependent action of thapsigargin (100 nmol/l) on sarcomere mechanics in normal myocytes. Thapsigargin prolonged the duration of contraction and also decreased the extent and velocity of sarcomere shortening in a time-dependent manner, but it did not affect the resting sarcomere length. These abnormalities of sarcomere mechanics induced by thapsigargin were similar to those seen in HF myocytes (Fig. 1). The effects of thapsigargin and CPA on the velocity of sarcomere shortening were dose dependent, and the half-maximal effects were obtained at ~50–100 nmol/l and 3 µmol/l, respectively, both of which were in accordance with the results of \textsuperscript{45}Ca\textsuperscript{2+} uptake studies (Fig. 5, B and C). Importantly, thapsigargin decreased the amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transient in control myocytes in a dose-dependent manner without altering the resting fluorescence levels (Figs. 6 and 7).

We also examined the effects of thapsigargin and CPA on myocyte contraction and [Ca\textsuperscript{2+}]\textsubscript{i} transient in HF myocytes. In a manner similar to that in control cells, these drugs also reduced the magnitude of cell shortening and [Ca\textsuperscript{2+}]\textsubscript{i} transient amplitude in HF myocytes. Despite the differences in prethapsigargin baseline shortening magnitudes, the relative effects of thapsigargin on cell shortening were similar in both groups of myocytes (Fig. 6A). Moreover, there were no significant differences with respect to the sensitivity to this drug. Thapsigargin also decreased the amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transient to a comparable degree in control and HF myocytes (42 ± 4 vs. 46 ± 4% of baseline, P = NS) (Fig. 6B). In addition, CPA reduced the amplitude of twitches or [Ca\textsuperscript{2+}]\textsubscript{i} transient to the same degree in control and HF myocytes (data not shown).

Thapsigargin prolonged not only the time from the peak to the resting level of Ca\textsuperscript{2+} fluorescence but also the time from the resting level to peak value in control myocytes.
myocytes (Fig. 7A). The prolongation of [Ca\textsuperscript{2+}] transient in normal myocytes produced by thapsigargin was characteristic for HF myocytes (Fig. 2). Thus SR Ca\textsuperscript{2+}-ATPase inhibitors reproduced in normal myocytes the abnormalities of [Ca\textsuperscript{2+}] transient and contractility that were similar to those found in HF myocytes (Figs. 5 and 7).

We further analyzed the decline kinetics of [Ca\textsuperscript{2+}] transient under the inhibition of SR Ca\textsuperscript{2+}-ATPase in control and HF myocytes. To emphasize the effects of thapsigargin on [Ca\textsuperscript{2+}] transient decline, indo 1 fluorescence values at baseline and in the presence of thapsigargin (100 nmol/l) were normalized to the same peak amplitude of fluorescence (Fig. 7B).

In control myocytes, thapsigargin prolonged the time to 50% decline from peak fluorescence (322 ± 48 ms at baseline to 795 ± 172 ms after thapsigargin, P < 0.05) and the time to 90% decline (901 ± 104 ms at baseline to 1,988 ± 328 ms after thapsigargin, P < 0.001). Similar results were obtained in HF cells, in which thapsigargin caused a significant slowing of [Ca\textsuperscript{2+}] transient (568 ± 90 to 1,426 ± 196 ms in the time to 50% decline and 1,196 ± 255 to 2,533 ± 584 ms in the time to 90% decline, P < 0.05 and P < 0.001, respectively). The degree of thapsigargin-induced slowing of [Ca\textsuperscript{2+}] transient was comparable between control and HF myocytes (260 ± 66 vs. 253 ± 19% increase in the time to 50% decline by thapsigargin, P = NS).

**DISCUSSION**

In this study, we have shown that 1) myocyte contractility was depressed and the duration of [Ca\textsuperscript{2+}] transient was prolonged in tachycardia-induced HF, which was in accordance with the previous studies (27, 32); 2) SR Ca\textsuperscript{2+}-ATPase gene expression was decreased in HF; 3) there was a significantly positive relationship between SR Ca\textsuperscript{2+}-ATPase mRNA levels and myocyte contractile function; and 4) thapsigargin and CPA inhibited Ca\textsuperscript{2+} uptake by SR and also depressed myocyte contractile function. It is also noteworthy that the dose responses of the effects of thapsigargin and CPA on myocyte shortening were quite similar to those on Ca\textsuperscript{2+} uptake by SR. Thus these results provide evidence that a downregulation of SR Ca\textsuperscript{2+}-ATPase expression is responsible for the contractile dysfunction in HF.
To determine the relationship between myocyte function and SR Ca\(^{2+}\)-ATPase, we correlated the parameters of sarcomere-shortening mechanics to the levels of SR Ca\(^{2+}\)-ATPase expression in single hearts obtained from control and HF dogs. There was a close correlation between myocyte function and SR Ca\(^{2+}\)-ATPase mRNA levels (Fig. 4), suggesting that the expression levels of SR Ca\(^{2+}\)-ATPase could be a determinant of myocardial contractility. Most importantly, this correlation was also highly significant when the analysis was performed in failing myocardium exclusively. In other words, the variation in myocyte contractility within the group of HF dogs matched closely with the variation seen in mRNA levels of SR Ca\(^{2+}\)-ATPase. Furthermore, we confirmed that our measurements carried out at mRNA levels of SR Ca\(^{2+}\)-ATPase could directly reflect the Ca\(^{2+}\) uptake function by SR. Even though we did not measure the activity or protein level of SR Ca\(^{2+}\)-ATPase in this study, steady-state mRNA levels could be assumed to be predictive of Ca\(^{2+}\) uptake by SR in comparisons of nonfailing and failing myocardium. Therefore, the results suggest that the decrease in SR Ca\(^{2+}\) uptake is responsible for the contractile dysfunction in failing hearts. However, we have to admit that the close correlation between myocyte function and Ca\(^{2+}\)-ATPase does not prove a causal relationship. It cannot be excluded that reduced SR Ca\(^{2+}\)-ATPase levels might reflect a response to another process primarily causing the abnormal myocyte function.

It should be noted that Williams et al. (30) did not detect any significant alterations in mRNA levels of SR Ca\(^{2+}\)-ATPase using the same animal model. The disparity between the present data and those of Williams et al. (30) might be related to the differences in the severity of LV contractile dysfunction and the methods to quantify the steady-state mRNA levels (polymerase chain reaction vs. Northern blot analysis). Our finding that calsequestrin mRNA levels were increased in the failing myocardium in contrast to the previous results wherein calsequestrin mRNA or protein levels were unaltered (1, 17, 19, 26). Even though the specific reasons for this discrepancy are unclear, the increase in calsequestrin mRNA levels in HF could be a compensatory mechanism for a decreased uptake of Ca\(^{2+}\) into the SR. However, the calsequestrin gene expression level did not correlate with myocyte contractile function. More importantly, the divergent pattern in the gene expression of SR Ca\(^{2+}\)-ATPase and calsequestrin, which is consistent with that in previous studies (15), indicates that SR Ca\(^{2+}\)-regulatory proteins are independently regulated among SR Ca\(^{2+}\)-regulatory proteins, and the decrease of Ca\(^{2+}\)-ATPase is not caused by the disturbances of generalized depression of cardiac gene expression in HF.

Thapsigargin and CPA have been well characterized to be specific for SR Ca\(^{2+}\)-ATPase and not to affect sarcolemmal Ca\(^{2+}\) pump, Na\(^+\)-K\(^-\)-ATPase, and myosin ATPase activity, all of which could regulate myocardial contractility (12, 20, 21, 31). Moreover, these drugs do not affect passive permeability of SR membrane to Ca\(^{2+}\) and the properties of Ca\(^{2+}\) release channels of SR (31). Our preliminary experiments using cardiac skinned myocytes demonstrated that neither thapsigargin nor CPA modified the myofilibrillar sensitivity to Ca\(^{2+}\) (unpublished observation). Thus, of all major intracellular systems involved in the regulation of Ca\(^{2+}\) homeostasis and of myocyte contraction, these drugs could specifically inhibit Ca\(^{2+}\) pumping activity of the SR. Our present results demonstrated that thapsigargin and CPA decreased myocyte contraction and also prolonged the duration of [Ca\(^{2+}\)]\(_i\) transients, which indicates that the inhibition of Ca\(^{2+}\) uptake by SR reproduced in normal myocytes the abnormalities well documented in HF myocytes. Importantly, thapsigargin decreased the amplitude (maximal levels minus resting levels) of [Ca\(^{2+}\)]\(_i\) transients, which indicates that a selective inhibition of the Ca\(^{2+}\) pump by this drug induces a gradual emptying of the SR reservoir, which is likely to account directly for the decrease in contractility of myocytes. Therefore, the functional experiments clearly demonstrated that the decreased expression of SR Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) uptake could result in the contractile performance defects. Only recently, Hajjar et al. (9) demonstrated that adenovirus-mediated gene transfer of SR Ca\(^{2+}\)-ATPase increased the peak [Ca\(^{2+}\)]\(_i\) transient and myocyte shortening in neonatal rat myocytes, which provided direct evidence that SR Ca\(^{2+}\)-ATPase could modify the myocardial contraction by controlling the rate and amount of Ca\(^{2+}\) sequestered by SR.

The present study demonstrated that the sensitivity of Ca\(^{2+}\)-ATPase to the specific inhibitors is preserved to be normal even in HF myocytes (Fig. 6), which may indicate that the reduction of Ca\(^{2+}\) uptake in HF myocytes is not due to the functional derangement of SR Ca\(^{2+}\)-ATPase but to the decreased density of this enzyme. In other words, there may be normal Ca\(^{2+}\) uptake function for each pump, but simply because of a decrease in SR Ca\(^{2+}\)-ATPase density, Ca\(^{2+}\) uptake by SR is diminished in HF myocytes. These results are also consistent with mRNA data in this study. This study also demonstrated that the thapsigargin-induced slowing of [Ca\(^{2+}\)]\(_i\) transient decline was identical in both groups of myocytes (Fig. 7), which could suggest that the removal of Ca\(^{2+}\) from the cytosol depends mainly on Ca\(^{2+}\) uptake through SR Ca\(^{2+}\)-ATPase rather than on the Na\(^+\)/Ca\(^{2+}\) exchange. Therefore, the functional significance of Na\(^+\)/Ca\(^{2+}\) exchanger in the efflux of Ca\(^{2+}\) from the cytosol might be minor in this model of HF, even though the mRNA levels of Na\(^+\)/Ca\(^{2+}\) exchanger have been reported to be upregulated in end-stage human failing hearts (23).

There are several potential limitations that should be acknowledged in this study. First, we did not investigate the changes in other Ca\(^{2+}\)-regulatory proteins including Ca\(^{2+}\) release channel, phospholamban, or sarcolemmal Ca\(^{2+}\) channel and could not exclude the possibility that these proteins may also contribute to the contractile defects in HF (1, 8). Second, we could not exclude the possibility of a relative loss of myocytes to nonmyocytes in LV tissue and no change in the expression of SR Ca\(^{2+}\)-ATPase mRNA within myocytes. However, we believe that this is unlikely for the following
reasons. The level of myosin heavy chain mRNA is not altered in this model of tachycardia-induced HF (7), which is consistent with human failing hearts (8, 14). Furthermore, Mercadier et al. (14) demonstrated that the level of Ca\(^{2+}\)-ATPase mRNA is reduced in failing hearts, even when corrected by myosin heavy chain mRNA in place of GAPDH mRNA. To “dilute” a muscle-specific transcript by 50%, a magnitude of decline that we observed here, the cellular mass of nonmyocytes should occupy at least one-half of the failing myocardium, which was not the case according to the histological examinations of these hearts. Third, the microsomal preparations we used in this study may not represent the total SR function in normal and failing hearts. However, in our preliminary data, Ca\(^{2+}\) uptake measured using the whole homogenates in HF myocytes was ~40% less than that in control myocytes, which was in agreement with the results obtained using microsomal fractions. Furthermore, previous studies demonstrated that Ca\(^{2+}\) transport as well as Ca\(^{2+}\)-ATPase activity was decreased in hypertrophied/failing hearts, even with the use of the microsomal preparations we used in this study may not represent the total SR function in normal and failing hearts. However, in our preliminary data, Ca\(^{2+}\) uptake measured using the whole homogenates in HF myocytes was ~40% less than that in control myocytes, which was in agreement with the results obtained using microsomal fractions. Furthermore, previous studies demonstrated that Ca\(^{2+}\) transport as well as Ca\(^{2+}\)-ATPase activity was decreased in hypertrophied/failing hearts, even with the use of the microsomal preparations (13, 24). Fourth, the present study examined the myocyte contractility and Ca\(^{2+}\)-ATPase gene expression with long-term rapid pacing at only one point in time. Thus serial changes in these measurements during the progression of HF were not addressed.

In conclusion, rapid pacing caused the depressed LV and myocyte contractility in dogs, associated with the decreased gene expression of SR Ca\(^{2+}\)-ATPase. The close, positive correlation between Ca\(^{2+}\)-ATPase and contractility based on the parallel measurements of these two indexes and the reproduction of contractile abnormalities in normal myocytes with SR Ca\(^{2+}\)-ATPase inhibitors indicates that SR Ca\(^{2+}\)-ATPase is a significant determinant of systolic contractile function of the myocytes and that the abnormalities of the transcriptional level of this protein gene may be responsible for the contractile defects in congestive HF. These results suggest that SR Ca\(^{2+}\)-ATPase may be a target for pharmacological or molecular interventions in the therapy of HF in the future.

We thank Erina Tazima for technical assistance.

This study was supported in part by grants (nos. 07266220, 08258221) from the Ministry of Education, Science, and Culture, a J A n heart Foundation–Pfizer Pharmaceuticals Grant for Research on Coronary Artery Disease; and the Foundation for Advancement of International Science.

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Received 12 September 1997; accepted in final form 16 March 1998.

REFERENCES


