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Altered intracellular Ca^{2+} regulation in chronic rat heart failure

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Abstract Altered intracellular Ca^{2+} handling by the sarcoplasmic reticulum (SR) plays a crucial role in the pathogenesis of heart failure (HF). Despite extensive effort, the underlying causes of abnormal SR Ca^{2+} handling in HF have not been clarified. To determine whether the diastolic SR Ca^{2+} leak along with reduced Ca^{2+} reuptake is required for decreased contractility, we investigated the cytosolic Ca^{2+} transients and SR Ca^{2+} content and assessed the expression of ryanodine receptor (RyR2), FK506 binding protein (FKBP12.6), SR-Ca^{2+} ATPase (SERCA2a), and L-type Ca^{2+} channel (LTCC) using an SD-rat model of chronic HF. We found that the cytosolic Ca^{2+} transients were markedly reduced in amplitude in HF myocytes ($\Delta F/F_0 = 12.3 \pm 0.8$) compared with control myocytes ($\Delta F/F_0 = 17.7 \pm 1.2$, $P < 0.01$), changes paralleled by a significant reduction in the SR Ca^{2+} content (HF: $\Delta F/F_0 = 12.4 \pm 1.1$, control: $\Delta F/F_0 = 32.4 \pm 1.9$, $P < 0.01$). Moreover, we demonstrated that the expression of FKBP12.6 associated with RyR2, SERCA2a, and LTCC was significantly reduced in rat HF. These results provide evidence for phosphorylation-induced detachment of FKBP12.6 from RyRs and down-regulation of SERCA2a and LTCC in HF. We conclude that diastolic SR Ca^{2+} leak (due to dissociation of FKBP12.6 from RyR2) along with reduced SR Ca^{2+} uptake (due to down-regulation of SERCA2a) and defective E-C coupling (due to down-regulation of LTCC) could contribute to HF.

Keywords Heart failure · Sarcoplasmic reticulum · Ryanodine receptor · Ca^{2+} ATPase · FK506 binding protein

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Abbreviations

ACT	Action potential-induced Ca^{2+} transients
CCT	Caffeine-induced Ca^{2+} transients
CICR	Ca^{2+}-induced Ca^{2+} release
E-C coupling	Excitation-contraction coupling
FKBP12.6	12.6 kDa FK506 binding protein
HF	Heart failure
LTCC	L-type Ca^{2+} channel
NCX	Na$^+$-Ca^{2+} exchanger
PKA	Protein kinase A
RyR	Ryanodine receptor
SERCA2a	SR-Ca^{2+}ATPase
SR	Sarcoplasmic reticulum

Introduction

Heart failure (HF) and sudden cardiac death are escalating major health problems worldwide. HF is a chronic syndrome characterized by fatigue, peripheral edema, pulmonary congestion, and shortness of breath, caused by a reduction in the ability of the heart to provide adequate blood flow to match the metabolic requirements of the organs [1]. Among a variety of factors, abnormal intracellular Ca^{2+} handling by the sarcoplasmic reticulum (SR) plays a crucial role in the pathogenesis of HF [2].

In the normal heart, intracellular Ca^{2+} movements critically regulate subsequent mechanical contractions. It is clear that activation of the contractile apparatus by calcium ions involves a molecular rearrangement of contractile proteins leading to altered protein-protein interactions in the thin filament. These events ultimately lead to strong cross-bridge binding, ATP hydrolysis by myosin, force development, and mechanical work generation. In cardiac excitation-contraction (E-C) coupling, a small amount of Ca^{2+} first enters through the L-type Ca^{2+} channel (LTCC) during membrane depolarization. This Ca^{2+} influx triggers a large-scale Ca^{2+} release through the Ca^{2+} release channel of the SR, referred to as the ryanodine receptor (RyR). This process is known as Ca^{2+}-induced Ca^{2+} release (CICR). The released Ca^{2+} then binds to the troponin C within the myofilaments, which induces activation of the myofilaments and a consequent muscle contraction [3, 4]. Relaxation is initiated by dissociation of Ca^{2+} from troponin C, followed by its reuptake into the SR through phospholamban-regulated (PLB-regulated) Ca^{2+} ATPase (SERCA2a) and subsequent trans-sarcolemmal Ca^{2+} removal through the Na^{+}/Ca^{2+} exchanger (NCX) operating in its forward mode [5]. The whole process of Ca^{2+} movement is characterized by a transient increase in intracellular $[Ca^{2+}]$ from 100 nM to about 1 μM.

Heart failure occurs when there is a reduction in cardiac output that is inadequate to meet the metabolic demands of the body. Although there are many etiologies of HF, it is generally agreed that the reduced amplitude and prolonged duration of the systolic Ca^{2+} transient account for, or contribute to, the reduced contractile force generated by the failing heart [6, 7]. The SR Ca^{2+} content reflects the balance between Ca^{2+} uptake and Ca^{2+} efflux. Some reports suggest that the reduction of SR Ca^{2+} content has been attributed to depressed SERCA2a function and/or enhanced NCX activity during HF [8, 9]. These changes are expected to facilitate Ca^{2+} removal from the cell at the expense of its uptake into the SR and result in under-filled SR Ca^{2+} stores in HF. Another potential cause of reduced SR Ca^{2+} content is enhanced diastolic leak of Ca^{2+} via the RyRs [10, 11]. Although extensive investigations of both mechanisms

have been reported, the results from these studies are not always in agreement. Furthermore, direct experiments concerning the other Ca^{2+} regulatory proteins is lacking. Therefore, further studies are needed to determine the mechanisms of altered Ca^{2+} handling in HF.

In this study, a rat model of chronic HF was used to examine intracellular Ca^{2+} cycling and the expression of the major SR Ca^{2+} handling proteins at the subcellular and molecular levels, respectively. The results show that enhanced SR Ca^{2+} leak via RyR2s and reduced SR Ca^{2+} uptake are primary factors in abnormal intracellular Ca^{2+} handling during HF. At the same time, defective E-C coupling (due to down-regulation of LTCC) may also be involved in the progression of HF.

Materials and methods

Animal model preparation

Adult male SD rats weighing 200–250 g were housed in an accredited laboratory animal facility, and all procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Second Military Medical University. Rats were randomized to receive either myocardial infarction [12] or a sham procedure. Rats were deeply anesthetized with intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg). Following intubation and placement on a respirator, a left lateral thoracotomy was performed in the fourth intercostal space, and a ligature (5.0 nylon suture) was placed around the left anterior descending coronary artery 2 mm below its origin. Ischemia was verified by visual inspection, the chest was closed, and the rat was extubated and returned to its cage. Sham-operated animals underwent the same procedure without ligation of the left anterior descending coronary artery.

Assessment of hemodynamics and heart weight

Twenty-eight days after myocardial infarction, the rats were anesthetized with 10% chloral hydrate and placed on a pad. A micromanometer catheter was introduced into the right carotid artery and advanced across the aortic valve into the left ventricular cavity to record left ventricular pressures. Left ventricular end-diastolic pressure (LVEDP) and the maximum rates of contraction (dp/dt_{max}) and relaxation (dp/dt_{min}) were derived from the left ventricular pressure pulse. Each hemodynamic evaluation was completed within 20–30 min. At the termination of the functional study, the heart was removed and weight was obtained.

Cell dissociation and patch-clamp study

Single cardiomyocytes of the rat heart were isolated by an enzymatic dissociation method [13]. The heart was removed from the open chests of rats anaesthetized with 10% chloral hydrate and mounted on a modified Langendorff perfusion system for retrograde perfusion with Ca^{2+}-free Tyrode's solution; then the heart was perfused with the solution containing collagenase II and bovine serum albumin for about 8–10 min. After digestion, ventricular myocardium tissue was cut into small pieces in the modified KB solution [14] and gently shaken to dissociate cells. All experiments were conducted at room temperature (20–22°C).

Whole-cell patch clamp recordings of transmembrane ionic currents were performed with an EPC10 amplifier (HEKA Instruments, Germany). Cardiomyocytes were placed in a perfusion chamber. Patch electrodes were fabricated from borosilicate glass with a micropipette puller. Its resistance was 2–4 MΩ when the electrode was filled with the pipette solution. The potential of the electrode was adjusted to zero current between the pipette solution and the bath solution immediately before seal formation. After obtaining a gigaseal, a suction pulse was applied to establish the whole-cell mode. Command pulses were delivered and data were acquired with a patch-clamp amplifier controlled by the PULSE software connected to a computer. In the end, data analysis was performed.

Dosing system

Sarcoplasmic reticulum Ca^{2+} release was measured by the method of caffeine perfusion. In the experiment, caffeine (20 mM) perfused the cardiomyocytes with the extracellular solution. The perfusion device used was the dosing system, whose dosing head was put to target cardiomyocyte at lower reach about 200 μm. Before perfusion, the dosing head rapidly moved to target cardiomyocyte at lower reach 100 μm. When caffeine triggered the release of SR Ca^{2+}, the record of the most significant range of Ca^{2+} transients was SR Ca^{2+} content.

Laser scanning confocal microscope analysis

Intracellular Ca^{2+} imaging was performed using a Leica TCS SP2 confocal microscope (Leica, Germany) in line-scan mode. To monitor the cytosolic Ca^{2+} levels, cardiomyocytes were loaded with 10 μM Fluo-3 for 30 min. Fluo-3 was excited by the 488 nm line of an argon-ion laser, and the fluorescence was acquired at wavelengths of 500–560 nm.

Patch-clamp and laser scanning confocal microscope synchronous recording system

Patch-clamp and confocal microscope synchronous recording system software (researched and developed by our department) was used to record transmembrane Ca^{2+} currents ($I_{Ca\cdot L}$) and intracellular Ca^{2+} sparks simultaneously.

For quantitative studies, the temporal dynamics in fluorescence were expressed as $\Delta F/F_0 = (F - F_0)/F_0$, where F represents fluorescence at time t and F_0 stands for baseline fluorescence.

Real-time quantitative RT-PCR analysis

The mRNA levels of RyR2, FKBP12.6, SERCA2a, and LCTT were measured using real-time quantitative RT-PCR. The total RNA was extracted from the homogenate using TRIzol reagent and reversely transcribed to cDNA by using reverse transcriptase according to previous publications and the manufacturer's instructions [15]. Reverse transcription was performed in a 30 μl reaction mixture at 40°C for 60 min and 70°C for 10 min. The total volume of the PCR reaction was 50 μl and was composed of the following: 1 μl cDNA, 2 mmol/L $MgCl_2$, 20 mmol/L each dNTP, 0.2 nmol/L each primer, 2 units DNA Taq polymerase, and the appropriate buffer.

Real-time PCR was carried out on a real-time quantitative PCR system (FTC2000, Canada) for the detection of PCR products. The temperature profile was as follows: 93°C for 4 min, followed by 40 cycles of 93°C for 20 s, 60°C for 30 s, and 72°C for 45 s. At the end of each extension step, the fluorescence intensity was read on the light cycler. Melting curve analyses were created following the final PCR cycle to confirm the presence of a single PCR product. In the experiment, GAPDH mRNA was used as the internal control for each sample since it was consistently expressed in all the cells. The specific primers were as follows: for RyR2, sense 5′-TAACCTACCAGGCCG TGGAT-3′, antisense 5′-GCTGCGATCTGGATAAGTTC AA-3′; for FKBPl2.6, sense 5′-TGCAGGTGGAGACCAT CTCTT-3′, antisense 5′-TCTTCAAGCATCCCCGTGTA G-3′; for SERCA2a, sense 5′-CTGGCCGACGACAACT TCTC-3′, antisense 5′-TGAGGTAGCGGATGAACTGC TT-3′; for LTCC, sense 5′-CATCTTTGGATCCTTTTTC GTTCT-3′, antisense 5′-TCCTCGAGCTTTGGCTTTCT C-3′; and for GAPDH, sense 5′-TGGAGTCTACTGGCG TCTT-3′, antisense 5′-TGTCATATTTCTCGTGGTTCA-3′.

Coimmunoprecipitation and Western blotting analysis

Cell lysates (RyR2–FKBP12.6) were incubated with Protein G PLUS-Agarose that was pre-bound with 1 μl of

anti-RyR antibody overnight at 4°C. Then the beads were washed three times with ice-cold buffer, each time for 10 min. The proteins bound to the Sepharose beads were then solubilized by the addition of Laemmli's sample buffer plus 5% (v/v) β-mercaptoethanol and boiled for 5 min. The samples were then separated on sodium dode-cyl sulphate gel electrophoresis (SDS/PAGE) (6% separation gel for RyR2, 15% separation gel for FKBP12.6). The SDS/PAGE-resolved proteins were transferred to nitrocel-lulose membranes at 30 mA for 16.5 h at 4°C. The nitro-cellulose membranes containing the transferred proteins were blocked for 1 h with PBS containing 0.5% Tween-20 and 5% (w/v) skimmed milk. The blocked membranes were then incubated with anti-RyR or anti-FKBP anti-bodies (both 1:1,000) for 1 h and washed three times for 5 min in PBS containing 0.5% Tween-20. The membrane was then incubated with the appropriate horseradish per-oxidase-conjugated secondary antibody (1:5,000) for 1 h. After washing three times for 5 min each in PBS con-taining 0.5% Tween-20, the RyR2 or FKBP12.6 proteins were detected by enhanced chemiluminescence (ECL). Band densities were quantified by using LabWorks4.6 software (PerkinElmer). The level of SERCA2a protein was also determined by immunoblot analysis. Cell lysate proteins were subjected to SDS 8% PAGE, blotted onto nitrocellulose membranes, and probed with anti-SERCA2a.

Drugs and solutions

Fluo-3/AM was purchased from Biotium; TRIzol reagent was purchased from Invitrogen Life Technologies (USA); Protein G PLUS-Agarose (lot number #B2009) and Normal Mouse IgG (lot number #I2208) were purchased from Santa Cruz; RyR antibody (lot number 660277), FKBP12 antibody (lot number 584394), and SERCA2a antibody (lot number 629766) were purchased from Abcam; and colla-genase II was purchased from Worthington (USA). All other reagents were purchased from Sigma (USA). The composition of the Ca^{2+}-free Tyrode's solution was (in mM) NaCl 135, KCl 5.4, $MgCl_2$ 1.0, NaH_2PO_4 0.33, glucose 5.0, and HEPES 5.0 (pH 7.3). The composition of the digestion solution was Ca^{2+}-free Tyrode's solution 25 ml, collagenase II 15 mg, BSA 50 mg, and 72 mmol/L $CaCl_2$ 20 μl. The composition of the modified KB solution was (in mM) KOH 80, L-glutamic acid 50, KCl 30, taurine 20, KH_2PO_4 30, $MgCl_2$ 3, glucose 10, EGTA 0.5, and HEPES 10 (pH 7.3). The external solution contained (in mM) NaCl 133.5, CsCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 10, and glucose 11.1 (pH 7.3). Patch pipettes were filled with a solution that contained (in mM) CsCl 120, TEA–Cl 10, Na_2ATP 5, $MgCl_2$ 6.5, Tris GTP 0.1, and HEPES 10 (pH 7.2).

Statistical analysis

All data are presented as mean $\pm$ SEM, and t-test was used for the statistical analyses. P-values of <0.05 were con-sidered significant.

Results

Characterization of cardiac function

Hemodynamic responses were assessed in control rats and HF rats (Table 1). There was a significant decrease in the maximum change in systolic pressure over time (dp/dt_{max}) in HF rats (2,001.54 $\pm$ 210.02 mmHg/s, $n = 15$) com-pared with control rats (4,554.79 $\pm$ 203.26 mmHg/s, $n = 10$, $P < 0.01$). Furthermore, there was a significant increase in heart weight (HW) divided by body weight (BW) in HF rats (6.87 $\pm$ 0.36, $n = 15$) compared with control rats (3.25 $\pm$ 0.29, $n = 10$, $P < 0.01$) (Table 1).

Measurement of action potential-induced Ca^{2+} transients

Depolarization-induced long-lasting calcium current ($I_{Ca·L}$) and spatially resolved intracellular Ca^{2+} transients (action potential-induced Ca^{2+} transients, ACT) were measured in cardiomyocytes dialyzed with Fluo-3/AM. They show representative traces of $I_{Ca·L}$ and confocal line-scan images along with the spatial average of Ca^{2+} transients recorded during a depolarizing step from -40 to $+10$ mV in cardiac myocytes from control and failing hearts. The amplitude of $I_{Ca·L}$ was lower in HF myocytes (0.59 $\pm$ 0.05, $n = 20$ cells from ten HF hearts) compared with control

Table 1 Assessment of cardiac function in control and heart failure (HF) rats

	Control (sham)	HF
Number	10	15
BW (g)	229.1 $\pm$ 9.2	231.7 $\pm$ 9.6
HW/BW (mg/g)	3.25 $\pm$ 0.29	6.87 $\pm$ 0.36**
HR (beats/min)	405 $\pm$ 14	425 $\pm$ 21*
LVEDP (mmHg)	4.8 $\pm$ 1.5	8.3 $\pm$ 2.2**
dp/dt_{max} (mmHg/s)	4,554.79 $\pm$ 203. 26	2,001.54 $\pm$ 210.02**
dp/dt_{min} (mmHg/s)	$-2,516.23 \pm 231.87$	$-1,740.48 \pm 229.15$**

BW Body weight, *HW* heart weight, *HR* heart rate, *LVEDP* left ventricular end-diastolic pressure, dp/dt_{max} maximum change in systolic pressure over time, dp/dt_{min} maximum change in the rate of relaxation over time

Data are presented as mean $\pm$ SEM. 1 mmHg = 0. 133 kPa

** $P < 0.01$ HF versus control, * $P < 0.05$ HF versus control

Fig. 1 Recordings of $I_{Ca\cdot L}$ in control (**a**) and HF group (**b**). The average current was 1.28 ± 0.07 in control and 0.59 ± 0.05 in HF myocytes (**c**). ($P < 0.01$, $n = 16$ cells from eight control hearts, $n = 20$ cells from ten HF hearts). The current was obtained in response to a 400 ms depolarizing step to $+10$ mV after a prepulse to -40 mV for 300 ms
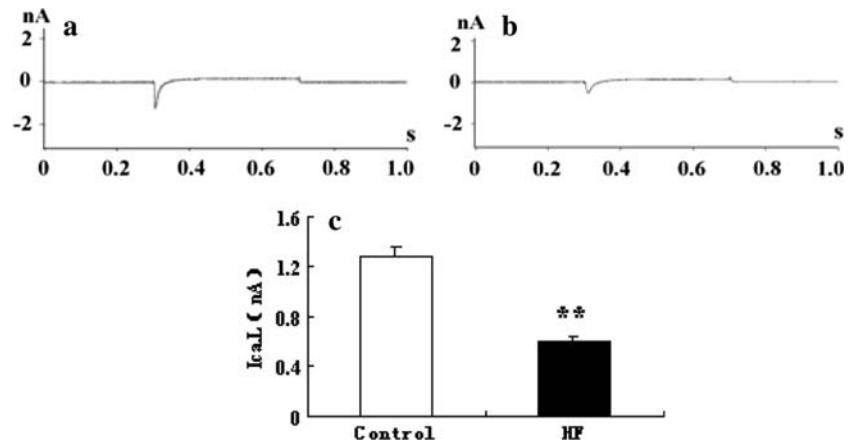

Fig. 2 Representative confocal line-scan images of $I_{ca\cdot L}$-induced Ca^{2+} transients in control (**a**) and HF group (**b**). 3D images of $I_{Ca\cdot L}$-induced Ca^{2+} transients in control (**c**) and HF group (**d**)
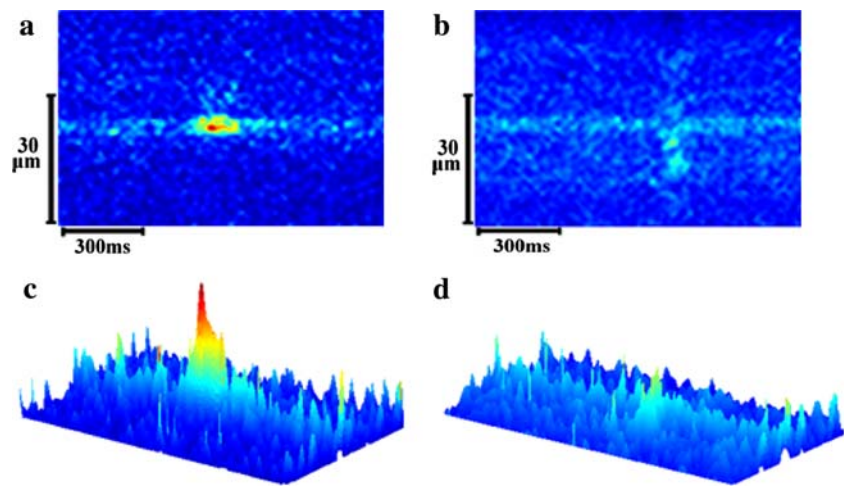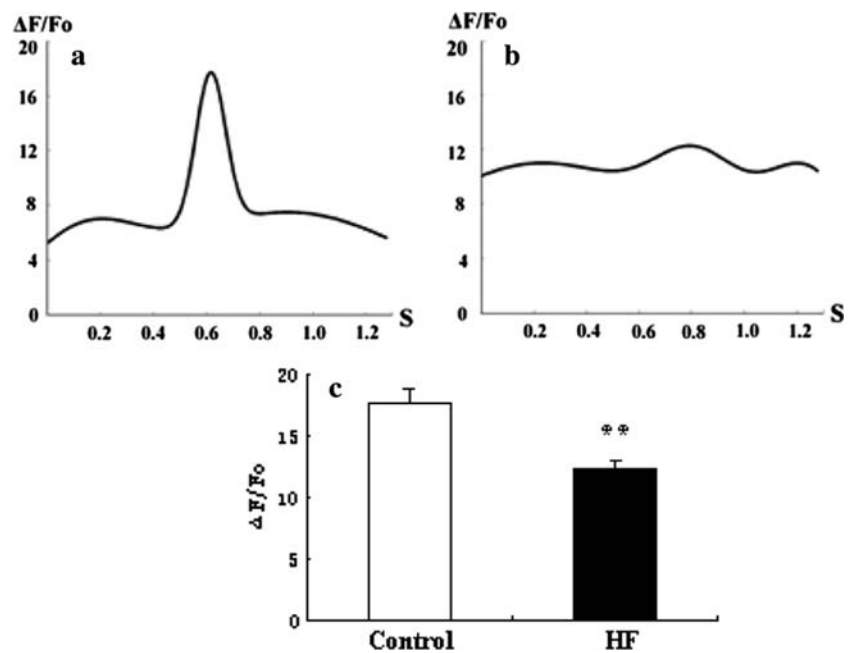

Fig. 3 The spatial averages of $I_{Ca\cdot L}$-induced Ca^{2+} transients in control (**a**) and HF group (**b**). The average amplitudes ($\Delta F/F_0$) of $I_{Ca\cdot L}$-induced Ca^{2+} transients were 17.7 ± 1.2 in control and 12.3 ± 0.8 in HF myocytes (**c**). ($P < 0.01$, $n = 10$ cells from five control hearts, $n = 12$ cells from six HF hearts)

$(1.28 \pm 0.07, n = 16$ cells from eight control hearts, $P < 0.01$) (Fig. 1a–c). Moreover the amplitude of Ca^{2+} transients was also reduced in the HF group (Fig. 2a–d). There was a significant decrease in spatial averages $(\Delta F/F_0)$ of $I_{Ca \cdot L}$-induced Ca^{2+} transients in HF $(12.3 \pm 0.8, n = 12$ cells from six HF hearts) compared with control $(17.7 \pm 1.2, n = 10$ cells from five control hearts, $P < 0.01$) (Fig. 3a–c).

Measurement of SR Ca^{2+} content

Isolated cardiomyocytes were loaded with Fluo-3/AM and rapidly perfused with caffeine (20 mM) using the dosing system. SR Ca^{2+} content can be measured in terms of caffeine-induced Ca^{2+} transients (CCT) during the diastolic phase [16]. The images show that the amplitude of Ca^{2+} transients was reduced in the HF group (Fig. 4a–d). Caffeine-induced Ca^{2+} transients $(\Delta F/F_0)$ were significantly decreased in HF $(12.4 \pm 1.1, n = 12$ cells from six HF hearts) compared with control

$(32.4 \pm 1.9, n = 10$ cells from five control hearts, $P < 0.01$) (Fig. 5a–c).

mRNA expression of Ca^{2+} handling genes

Real-time quantitative PCR was performed to determine the relative mRNA expression pattern of RyR2, FKBP12.6, SERCA2a, and LCTT. We found that the mRNA expression of RyR2 did not differ in the HF group compared with the control group. The down-regulation of FKBP12.6, SERCA2a, and LCTT was established by RT-PCR to be significantly different $(n = 8, P < 0.01)$ from the control (Fig. 6a–d).

Expression of SR Ca^{2+} handling proteins

Immunoblot analyses were performed to determine the contents of the major SR Ca^{2+} handling proteins RyR2, FKBP12.6, and SERCA2a in the control and HF groups. The association of FKBP12.6 with RyR2 was determined

Fig. 4 Representative confocal line-scan images of caffeine-induced Ca^{2+} transients in control (a) and HF group (b). 3D images of caffine-induced Ca^{2+} transients in control (c) and HF group (d)
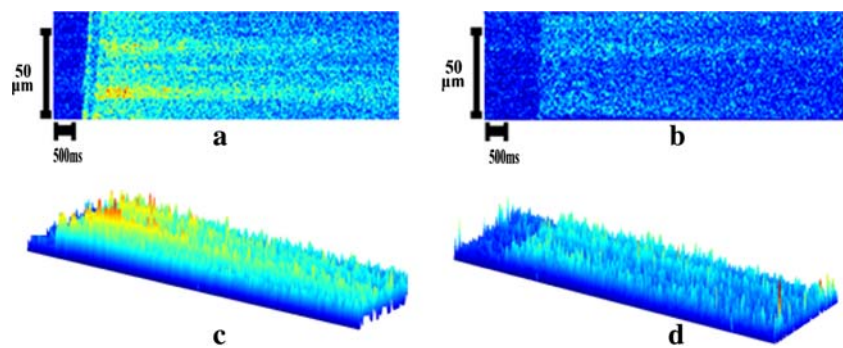

Fig. 5 The spatial averages of caffeine-induced Ca^{2+} transients in control (a) and HF group (b). The average amplitudes $(\Delta F/F_0)$ of caffeine-induced Ca^{2+} transients were 32.4 ± 1.9 in control and 12.4 ± 1.1 in HF myocytes (c). $(P < 0.01, n = 10$ cells from five control hearts, $n = 12$ cells from six HF hearts)
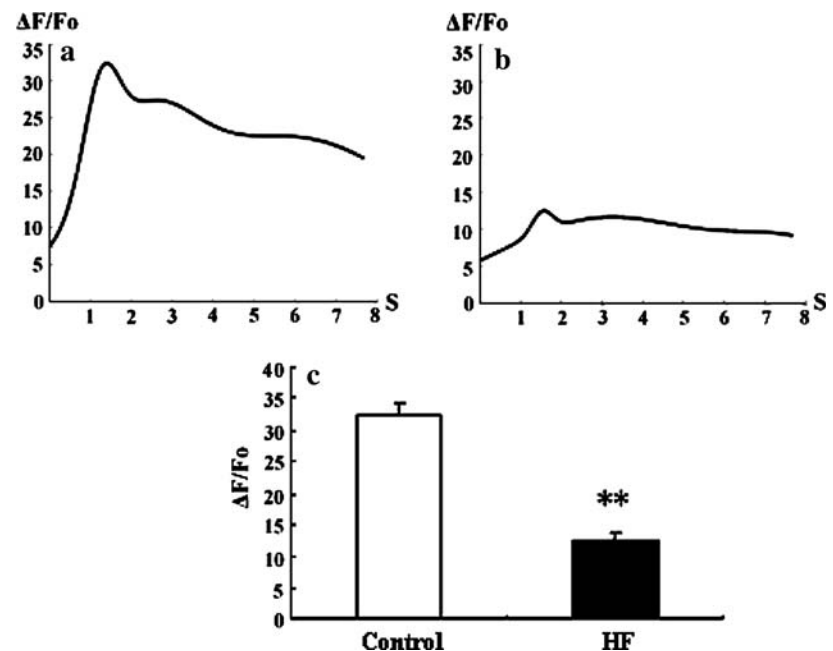
Fig. 6 Relative expression of Ca^{2+} handling genes RyR2 (**a**), FKBP12.6 (**b**), SERCA2a (**c**), and LTCC (**d**) in control and HF myocytes. ($P < 0.01$, $n = 8$)
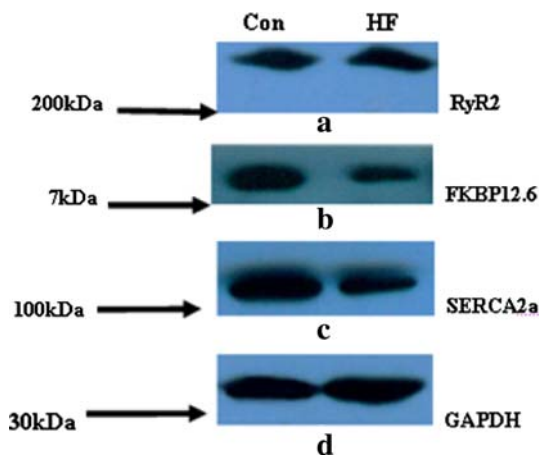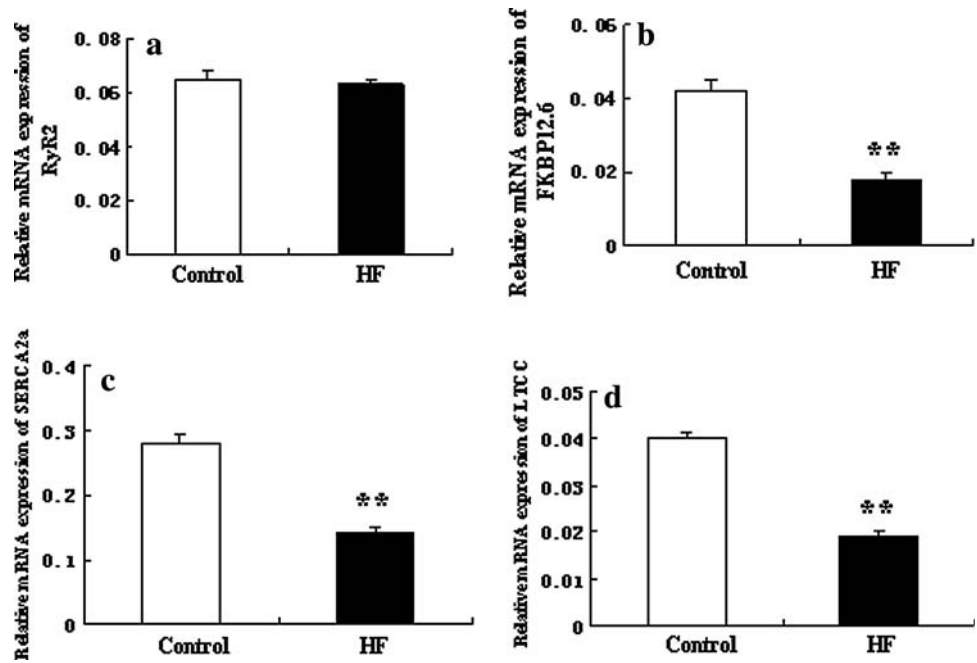

Fig. 7 Coimmunoprecipitation of RyR2 and FKBP12.6 in cardiac muscle. RyR2 was immunoprecipitated from solubilized myocytes. Immunoblots were probed with mouse anti-RyR and rabbit anti-FKBP antibodies for the presence of RyR2 (*a*) and FKBP12.6 (*b*), respectively. Expression of SERCA2a (*c*) and GAPDH (*d*) in control and HF myocytes. *Arrows on the left* indicate the position of molecular weight marker run in parallel to indicate protein migration on the gel

firstly by immunoprecipitation using an anti-RyR antibody, followed by immunoblotting with an anti-FKBP12 antibody. Compared with channel complexes from control rats, the expression of RyR2 was not different in HF rats ($n = 8$, $P > 0.05$) (Figs. 7a, 8a), whereas FKBP12.6 content was significantly lower in HF rats ($n = 8$, $P < 0.01$) (Figs. 7b, 8b). Furthermore, the expression of SERCA2a was also lower in HF rats compared with control ($n = 8$, $P < 0.01$) (Figs. 7c, 8c).

Discussion

In the present study, we investigated the cytosolic Ca^{2+} transients and SR Ca^{2+} content and assessed the expression of LTCC and SR Ca^{2+} handling proteins by using an SD-rat model of chronic HF. Our study shows for the first time that defective E-C coupling (due to down-regulation of LTCC), enhanced SR Ca^{2+} leak via RyR2s (due to dissociation of FKBP12.6 from RyR2), and reduced SR Ca^{2+} uptake via SERCA2a (due to down-regulation of SER-CA2a) are simultaneously involved in the progression of HF.

Calcium homeostasis is the maintenance of stable intracellular calcium levels during systole and diastole and is governed by numerous proteins that regulate the flow of calcium ions among the extracellular space, cytoplasm, and intracellular stores. Many studies have shown that the amplitude of the systolic Ca^{2+} transient depends on the SR Ca^{2+} content [17–19]. SR Ca^{2+} content depends directly on the relative power of Ca^{2+} uptake into the SR (by the SERCA2a) and release (by the RyR). However it will also depend indirectly on the Ca^{2+} efflux from the cell (largely by NCX) and the influx (largely by the L-type Ca^{2+} current). Therefore, potential contributors to the decreased SR Ca^{2+} content in HF include reduced SERCA2a function, enhanced NCX Ca^{2+} extrusion activity, and/or increased SR Ca^{2+} leak.

In our study, we found that the cytosolic Ca^{2+} transients were markedly reduced in amplitude in HF myocytes ($\Delta F/F_0 = 12.3 \pm 0.8$, $n = 12$ cells from six HF hearts) compared with control myocytes ($\Delta F/F_0 = 17.7 \pm 1.2$, $n = 10$

Fig. 8 Relative amount of Ca^{2+} handling proteins RyR2 (**a**), FKBP12.6 (**b**), and SERCA2a (**c**) in control and HF myocytes. ($P < 0.01$, $n = 8$)
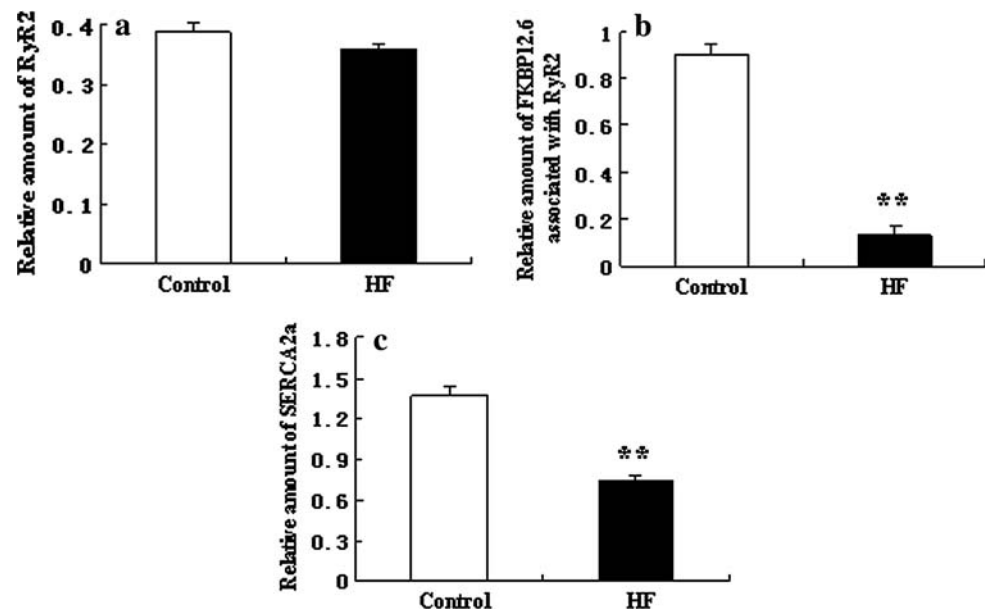
cells from five control hearts, $P < 0.01$), changes paralleled by a significant reduction in the SR Ca^{2+} content (HF: $\Delta F/F_0 = 12.4 \pm 1.1$, $n = 12$ cells from six HF hearts; control: $\Delta F/F_0 = 32.4 \pm 1.9$, $n = 10$ cells from five control hearts, $P < 0.01$). These alterations in Ca^{2+} handling are considered to be hallmarks of HF.

Although recent advances in the field of molecular biology have shed light on the close relationship between Ca^{2+} cycling abnormalities and the progression of HF, the exact molecular causes of HF are still uncertain. The aim of our study was to determine (1) whether a down-regulation of LTCC occurs that results in the decreased amplitude of $I_{Ca \cdot L}$ and the reduced Ca^{2+} release, (2) whether the dissociation of FKBP12.6 from RyR2 is required for Ca^{2+} leak, and (3) in addition to leaky RyR2, whether a down-regulation of the SERCA2a occurs that conspires with leaky RyR2 to deplete the SR Ca^{2+}.

Depolarization of the plasma membrane by an incoming action potential activates voltage-gated L-type Ca^{2+} channels in plasma membrane invaginations called T-tubules. In the heart, LTCC activation results in a plasma membrane Ca^{2+} influx current, which triggers RyR2 activation and SR Ca^{2+} release, referred to as CICR. The efficiency of the trigger (the size of the inward Ca^{2+} current) needed to cause Ca^{2+} release from the SR has been termed E-C coupling gain. In many cases of HF, the E-C coupling gain seems to be reduced by a functional defect in LTCC, an increase in the space between LTCC and RyR, a decrease in SR Ca^{2+}, and/or an abnormality in the channel-gating property of RyR. Not only the amount of Ca^{2+} released for a given Ca^{2+} release trigger but also the rate of Ca^{2+} release may be important for the contractility of the myofilaments. The present study shows that the expression

of LTCC seems to be reduced in HF rats ($n = 20$ cells from ten HF hearts, $n = 16$ cells from eight control hearts, $P < 0.01$), which leads to the decreased amplitude of $I_{Ca \cdot L}$ and the reduced Ca^{2+} release.

In addition to defects in E-C coupling, heart failure is characterized by changes in cardiac myofibrillar protein function. There is ample evidence to support the notion that the biochemical properties of the cardiac cell and indeed the cardiac sarcomere itself are responsible for mechanical properties of the heart [20, 21].

In mammalian cardiac muscle, the SR is the major source of the calcium that activates contraction. Calcium is released from the SR through a channel known as the RyR. There are three RyR isoforms in mammals. RyR1 is the major isoform in skeletal muscle, whereas RyR3 is the major isoform in the brain and skeletal muscle [22]. RyR2 is the major isoform found in cardiac muscle and the brain [23]; it is a macromolecular complex of 2.5 MDa molecular weight composed of four identical RyR2 monomers. Each RyR2 protomer contains a large N-terminal regulatory region that protrudes into the cytoplasm and a C-terminal that forms a transmembrane pore channel. The N-terminal domain serves as a scaffold for proteins that modulate RyR2 function. The cytoplasmic domain of each subunit has a binding site for several molecules such as FKBP12.6, PKA, protein phosphatase 1 and 2A, calmodulin, and sorcin. These RyR2 binding proteins function to regulate RyR2 activity [24]. On the luminal side of SR, the RyR2 interacts with calsequestrin, triadin, and junctin. Defective regulation of channel gating by these key proteins may render the channel leaky.

Many studies in recent years have suggested that the properties of the RyR can be altered under both normal and

abnormal conditions. Modulation of the RyR is critical to survival and consequently influenced by multiple regulatory mechanisms, including PKA-dependent phosphorylation of RyR [25] and Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) phosphorylation of RyR [26]; the mechanism of latter has not been clarified. The RyR does not exist in isolation but is coupled to other proteins, including FKBP12.6, junctin, triadin, and calsequestrin [27], which may affect its open probability (P_0).

FKBP12.6 is one of the important accessory proteins of RyR2. In the normal condition, FKBP12.6 binds tightly to RyR2 and stabilizes the channel. Marx et al. demonstrated that chronic and excess activation of beta-adrenergic receptor induces PKA-mediated hyperphosphorylation of RyR2 at serine 2808, which in turn dissociates FKBP12.6 from RyR2, thereby causing Ca^{2+} leak. So, it was suggested that beta-adrenergic stimulation plays an important role in modulating the effect of FKBP12.6 [28]. Both beta-blockers and angiotensin II receptor blockade (ARB) were found to suppress the hyperadrenergic state, thereby reversing PKA-mediated hyperphosphorylation of RyR2, restoring the FKBP12.6-mediated stabilization and inhibiting the Ca^{2+} leak [29]. Recently, Yano et al. [30] have shown that JTV519, a 1,4-benzothiazepine derivative, increased cardiac contractility in an experimental model of heart failure. JTV519 improved binding of FKBP12.6 to PKA phosphorylated RyR2, thereby inhibiting abnormal Ca^{2+} leak.

Several researchers, however, have challenged the role of FKBP12.6 as a channel stabilizer in various experimental conditions. They believe that phosphorylation at serine 2808 does not dissociate FKBP12.6 from RyR2, and the constitutive phosphorylation of serine 2808 by mutations (S2808D) failed to disrupt the FKBP12.6-RyR2 interaction [31]. In our study, we demonstrated that the expression of RyR2 did not differ between control and HF rats ($n = 8$, $P > 0.05$), while the expression of FKBP12.6 associated with RyR2 was significantly reduced in HF rats ($n = 8$, $P < 0.01$). In other words, RyR2 macromolecular complexes from HF rats were significantly more depleted of FKBP12.6. These results provide evidence for phosphorylation-induced detachment of FKBP12.6 from RyRs in HF.

In our experiment, the protein expression of FKBP12.6 was dramatically reduced compared with the mRNA expression. We do not know the exact reason. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product (protein). Several steps in the gene expression process may be modulated, including the transcription, RNA splicing, translation, and post-translational modification of a protein. We speculated that alteration occurred at the level of post-translational modification of FKBP12.6.

Another mechanism for restoring cytosolic calcium to diastolic levels is through reuptake of calcium by the SR via the SERCA2a, which (to some extent) competes with the NCX for cytoplasmic calcium. Some research has demonstrated that the SR Ca^{2+} uptake rate was not significantly altered and the expression of SERCA2a was unchanged in HF myocytes. We found, however, that the expression of SERCA2a was decreased in HF rats compared with controls ($n = 8$, $P < 0.01$) and was also a critical factor determining the reduced SR Ca^{2+} content in HF. Calcium reuptake via SERCA2a is significantly influenced by phosphorylation. PKA-dependent phosphorylation of phospholamban (PLB) is the likely mechanism of accelerated relaxation associated with catecholamines [32]. CaMKII can also accelerate SR calcium reuptake by phosphorylation of PLB and, possibly, by a mechanism that does not require PLB [33]. On the other hand, some studies have suggested that although NCX activity was accelerated in HF myocytes, these changes could not account for the altered Ca^{2+} transients. In other words, NCX is not a major contributor to the reduced SR Ca^{2+} content in HF [34]. Further investigation is still needed to clarify the role of NCX and other SR Ca^{2+} handling proteins.

In conclusion, diastolic SR Ca^{2+} leak (due to dissociation of FKBP12.6 from RyR2) along with reduced SR Ca^{2+} uptake (due to down-regulation of SERCA2a) and defective E-C coupling (due to down-regulation of LTCC) could contribute to the decreased contractility observed in failing hearts associated with reduced amplitude and slowed decay of the intracellular Ca^{2+} transients.

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