# Defective $Ca^{2+}$ Handling Proteins Regulation During Heart Failure

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**Summary**

Abnormal release of $Ca^{2+}$ from sarcoplasmic reticulum (SR) via the cardiac ryanodine receptor (RyR2) may contribute to contractile dysfunction in heart failure (HF). We previously demonstrated that RyR2 macromolecular complexes from HF rat were significantly more depleted of FK506 binding protein (FKBP12.6). Here we assessed expression of key $Ca^{2+}$ handling proteins and measured SR $Ca^{2+}$ content in control and HF rat myocytes. Direct measurements of SR $Ca^{2+}$ content in permeabilized cardiac myocytes demonstrated that SR luminal $[Ca^{2+}]$ is markedly lowered in HF (HF: $\Delta F/F_0$ = 26.4±1.8, $n$=12; control: $\Delta F/F_0$ = 49.2±2.9, $n$=10; $P$<0.01). Furthermore, we demonstrated that the expression of RyR2 associated proteins (including calmodulin, sorcin, calsequestrin, protein phosphatase 1, protein phosphatase 2A), $Ca^{2+}$ATPase (SERCA2a), PLB phosphorylation at Ser16 (PLB-S16), PLB phosphorylation at Thr17 (PLB-T17), L-type $Ca^{2+}$ channel (Cav1.2) and $Na^{+}$-$Ca^{2+}$ exchanger (NCX) were significantly reduced in rat HF. Our results suggest that systolic SR reduced $Ca^{2+}$ release and diastolic SR $Ca^{2+}$ leak (due to defective protein-protein interaction between RyR2 and its associated proteins) along with reduced SR $Ca^{2+}$ uptake (due to down-regulation of SERCA2a, PLB-S16 and PLB-T17), abnormal $Ca^{2+}$ extrusion (due to down-regulation of NCX) and defective $Ca^{2+}$-induced $Ca^{2+}$ release (due to down-regulation of Cav1.2) could contribute to HF.

**Key words**

Heart failure • Sarcoplasmic reticulum • Ryanodine receptor

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## Introduction

Heart failure (HF) is characterized by a complex disorder that leads to a disturbance of the normal pumping of blood to the peripheral organs to meet the metabolic demands of the body. A growing body of evidence has accumulated concerning the altered intracellular $Ca^{2+}$ cycling that plays a key role in the development of HF (Meyer *et al*. 1995, Houser and Margulies 2003). 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 $Ca^{2+}$ cycle is a definite regulator of myocyte contraction and relaxation. During a normal cardiac cycle, membrane depolarization leads to $Ca^{2+}$ entry into the cell via L-type $Ca^{2+}$ channel. The entry of $Ca^{2+}$, in close proximity to the sarcoplasmic reticulum (SR), triggers a calcium-induced calcium release (CICR) through the SR ryanodine-sensitive receptors (RyRs); $Ca^{2+}$ diffuses to the myofilaments and activates calcium-sensitive proteins leading to myofibrillar contraction. The subsequent relaxation is initiated by dissociation of $Ca^{2+}$ from troponin C, followed by its reuptake into the SR through phospholamban-regulated (PLB-regulated) sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA2a) and subsequent trans-sarcolemmal $Ca^{2+}$ removal through the $Na^{+}/Ca^{2+}$ exchanger (NCX) operating in its forward mode (Bassani *et al*. 1994). The whole process of $Ca^{2+}$ movement is characterized by a transient increase in intracellular $[Ca^{2+}]$ from 100 nM to about 1μM.

Systolic HF is a disease state associated with weakening of myocardial contractility which ultimately



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results in catastrophic deterioration of ventricular pump function. Although it is clear that reduced SR $Ca^{2+}$ content could lead to reduced systolic $Ca^{2+}$ transients and weakened contractility, the specific mechanisms responsible for the reduced SR $Ca^{2+}$ content in HF remain to be defined. Intracellular free calcium concentration is kept within the physiological limits by maintaining a delicate balance between influx and efflux of $Ca^{2+}$. 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 (Shannon *et al*. 2003). 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 (Marx *et al*. 2000). 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 simultaneously is lacking. Therefore, further studies are needed to determine the mechanisms of altered $Ca^{2+}$ handling in HF.

Here we investigated alternations in SR $Ca^{2+}$ content and the expression of the major $Ca^{2+}$ regulatory proteins. Our aims were to assess: (1) L-type calcium current (LTCC); (2) SR $Ca^{2+}$ content; (3) expression of RyR2 and RyR2 interacting protein, including calmodulin, sorcin, calsequestrin, PP1 and PP2A; (4) expression of SERCA2a, PLB, PLB-S16 and PLB-T17; (5) expression of Cav1.2; and (6) expression of NCX.

## Materials and Methods

*Surgical procedure and animal models*

Animal care and all procedures were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University and were performed in accordance with the "Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, revised 1985). Adult male Sprague-Dawley (SD) rats (200-250 g) were randomized to receive either myocardial infarction (MI) (Tanonakak 2001) or a sham procedure. Rats were anesthetized by intraperitoneal injection of 10 % chloral hydrate (3.5 ml/kg), cannulated, and ventilated with a tidal volume of 1.5-2 ml/100 g (80 strokes/min). A left lateral thoracotomy was performed between the third and fourth ribs, and the left anterior descending (LAD) coronary artery was permanently ligated proximally with a 5-0 silk suture. Sham-operated animal sunder went the same procedure without ligation of the LAD. The electrocardiogram (ECG) of MI and sham group is clear as shown in Figure 1. The chest was closed, the lung was reinflated and the animal was moved to a prone position until spontaneous breathing occurred.

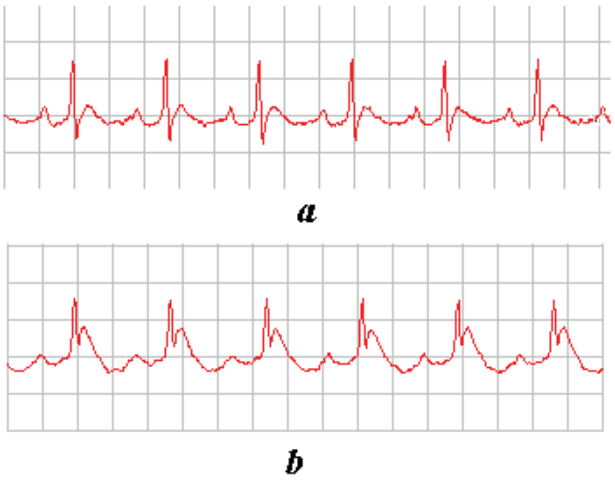


**Fig. 1.** ECG of sham (***a***) and myocardial infarction (***b***) group.

*Assessment of heart function*

Twenty-eight days after MI, 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 (d*p*/d*t*max) and relaxation (d*p*/d*t*min) were derived from the left ventricular pressure pulse (Van 2004). Each hemodynamic evaluation was completed within 20-30 minutes. At the termination of the functional study, the heart was removed and weight was obtained.

*Cell dissociation*

Single cardiomyocytes (left ventricle cell) of the rat heart were isolated by an enzymatic dissociation method (Ohmoto-Sekine 1999). The heart was removed from the open chest 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

**Table 1.** Primer sets for real-time quantitative RT-PCR.

| Gene names | Forward primers | Reverse primers |
|---|---|---|
| *RyR2* | 5'-TAACCTACCAGGCCGTGGAT-3' | 5'-GCTGCGATCTGGATAAGTTCAA-3' |
| *Calmodulin* | 5'-CTGCTGGTCCATTGGTGTCA-3' | 5'-GCTTTCTCGCAGGTGTATCGT-3' |
| *Calsequestrin* | 5'-AGTATGATGGCGAGTTTTCCG-3' | 5'-CTCGTAGGCTTTGTAATGCTCTG-3' |
| *Sorcin* | 5'-TCTGGCATAGCGGGAGGA-3' | 5'-TGTCGAAGCTGATGAAGTGTTG-3' |
| *SERCA2a* | 5'-CTGGCCGACGACAACTTCTC-3' | 5'-TGAGGTAGCGGATGAACTGCTT-3' |
| *PLB* | 5'-CCTTACTCGCTCGGCTATTAGG-3' | 5'-GATGAGACAGAAATTGATAAAGAGGTTCT-3' |
| *NCX* | 5'-TGGAACCTCAGTGCCAGACA-3' | 5'-GCTTCCGGTGACATTGCCTAT-3' |
| *Cav1.2* | 5'-CATCTTTGGATCCTTTTTCGTTCT-3' | 5'-TCCTCGAGCTTTGGCTTTCTC-3' |
| *Actin* | 5'-CCCATCTATGAGGGTTACGC-3' | 5'-TTTAATGTCACGCACGATTTC-3' |

albumin (BSA) for about 8-10 min. After digestion, ventricular myocardium tissue was cut into small pieces in the modified Kraft-Bruhe (KB) solution (Nakaya 1993) and gently shaken to dissociate cells. All experiments were conducted at room temperature (20-22 °C).

*Patch-clamp study*

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 compute. In the end, data analysis was performed.

*Laser scanning confocal microscope analysis*

Intra-SR $Ca^{2+}$ imaging was performed using a Leica TCS SP2 confocal microscope (Leica, Germany) in XY mode. To monitor the intra-SR $Ca^{2+}$ levels, myocytes were loaded with 5 μM Fluo-5N AM for 3-6 h at 37 °C. When measured, Fluo-5N was excited by 488 nm laser line, and fluorescence was acquired at wavelengths of 500-530 nm.

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 determination of $Ca^{2+}$ handling genes*

The mRNA levels of RyR2, calmodulin, sorcin, calsequestrin, SERCA2a, PLB, Cav1.2 and NCX were measured using real-time quantitative RT-PCR. Total RNA was extracted using the TRIzol reagent and purified to remove any contaminating genomic DNA by on-column DNase digestion. First-strand synthesis and real-time amplification were performed using the Platinum qRT-PCR ThermoScript One-Step System (Invitrogene) and with the TaqMan Assays-on-Demand primer/probes pairs specific for RyR2, calmodulin, sorcin, calsequestrin, SERCA2a, PLB, Cav1.2, NCX and Actin. Standard amplification curves were generated from Actin. Optimal PCR curves were observed within 40 cycles using the real-time quantitative PCR system (FTC2000, Canada). Three sets of RNA isolations were evaluated and each measurement was performed in triplicate. The threshold value (Ct) was set with the amplification-based threshold algorithms from Stratagene. Actin expression of each sample was used as endogenous control. All primer sets are listed in Table 1.

*Immunoprecipitation and western blotting analysis*

RyRs protein was immunoprecipitated from 500 μg (total protein) samples by overnight incubation with mouse anti-RyR (Jayaraman 1992) antibody, followed by incubation with protein G PLUS-Agarose at 4 °C for an additional 1h, and 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 dodecyl sulphate gel electrophoresis (SDS/PAGE) (6 %

**Table 2.** Assessment of cardiac function in control and heart failure (HF) rats.

| | Control (sham) | HF |
|---|---|---|
| *Number* | 10 | 18 |
| *BW (g)* | 235.6±11.3 | 241.7±9.1 |
| *HW/BW (mg/g)* | 2.97±0.28 | 4.62±0.25** |
| *HR (beats/min)* | 417±16 | 438±19* |
| *LVEDP (mmHg)* | 4.6±1.3 | 9.1±2.6** |
| $dp/dt_{max}$ *(mmHg/s)* | 4,237.82±215.39 | 2,174.42±229.02** |
| $dp/dt_{min}$ *(mmHg/s)* | −2,733.25±236.14 | −1,626.46 ±229.24** |

*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 ± S.E. 1 mmHg = 0.133 kPa; $^{**}P<0.01$ HF versus control, $^{*}P<0.05$ HF versus control.

separation gel for RyR2, 15 % separation gel for calmodulin, 12 % separation gel for sorcin, 10 % separation gel for calsequestrin, PP1 and PP2A). The SDS/PAGE-resolved proteins were transferred to nitrocellulose membranes at 30 mA for 16.5 h at 4 °C. The nitrocellulose membranes containing the transferred proteins were blocked for 1h with PBS containing 0.5 % Tween 20 and 5 % (w/v) skimmed milk. The blocked membranes were then incubated with respective antibodies (1:1,000) (Wehrens 2003) 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 peroxidase-conjugated secondary antibody (1:5,000) for 1 h. After washing three times for 5 min each in PBS containing 0.5 % Tween 20, these proteins were detected by enhanced chemiluminescence (ECL). Band densities were quantified by using LabWorks4.6 software (PerkinElmer). The level of NCX, Cav1.2, SERCA2a, PLB, PLB-S16 and PLB-T17 protein were also determined by immunoblot analysis. Cell lysate proteins were subjected to SDS 6 %, 8 % and 12 % PAGE, blotted onto nitrocellulose membranes, and probed with anti-NCX1, anti-DHPRα1, anti-SERCA2a, anti-PLB and anti-PLB-S16, anti-PLB-T17, respectively.

*Drugs and solutions*

Fluo-5N was purchased from Biotium; TRIzol Reagent was purchased from Invitrogen Life Technologies (USA), Protein G PLUS-Agarose (lot number #C1009) and Normal Mouse IgG (lot number #I2208) were purchased from Santa Cruz, RyR antibody (lot number 713354), calmodulin antibody (lot number 663553), sorcin antibody (lot number 716045), calsequestrin antibody (lot number 399596), NCX1 antibody (lot number 686846), SERCA2a antibody (lot number 629766), PLB antibody (lot number 669574), PLB-S16 antibody (lot number 647789) and DHPRα1 antibody (lot number 717054) were purchased from Abcam, PLB-T17 antibody (lot number #C2009) was purchased from Santa Cruz, PP1 antibody (lot number #DAM1518966), PP2A antibody (lot number #DAM1416548) were purchased from Millipore; and collagenase 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 Kraft-Bruhe (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*

Data are expressed as mean ± S.E. for all the experiments. Statistically significant differences between the HF and the control were determined by *t*-test. The statistical significance was set at $^{*}P<0.05$ and $^{**}P<0.01$.

## Results

*Characterization of cardiac function*

According to the experiment, infarct size of rat heart is more than 40 % and afteroperation mortality is 20 % in HF group. Hemodynamic responses were

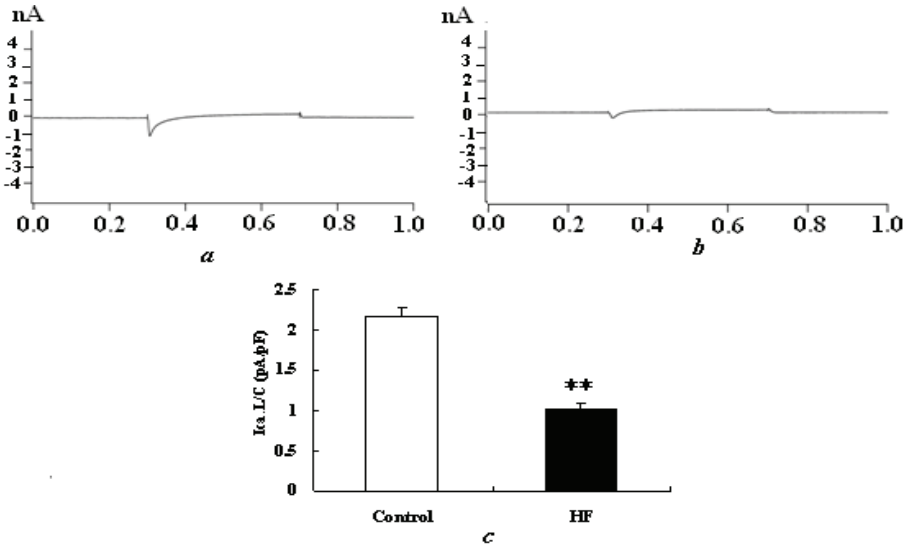


**Fig. 2.** Recordings of $I_{Ca\cdot L}$ in control (***a***) and HF group (***b***). The average current/capacitance (pA/pF) were 2.18±0.09 in control and 1.01±0.08 in HF myocytes (***c***) ($P$<0.01, n=18 cells from nine control hearts, n=20 cells from ten HF hearts). The current was obtained in response to 400 ms depolarizing step to +10 mV after a prepulse to –40 mV for 300 ms inactivated sodium current from a –40 mV holding potential.

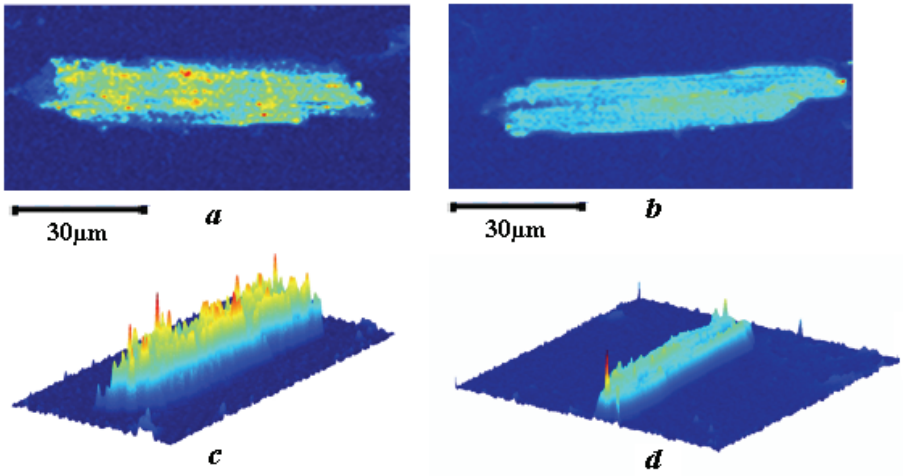


**Fig. 3.** Images of control (***a***) and HF (***b***) permeabilized myocytes with SR-entrapped fluo-5N, 3D images of control (***c***), HF (***d***).

assessed in control rats and HF rats (Table 2). There was a significant decrease in the maximal change of systolic pressure over time ($dp/dt_{max}$) in HF rats (2,174.42±229.02 mmHg/s, $n$=18) compared with control rats (4,237.82±215.39 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 (4.62±0.25, $n$=18) compared with control rats (2.97±0.28, $n$=10, $P$<0.01) (Table 2).

*Measurement of L-type calcium current*

It showed representative traces of $I_{Ca\cdot L}$ recorded during a depolarizing step from –40 to +10 mV in cardiac myocytes from control and failing hearts. The amplitude of $I_{Ca\cdot L}$ was reduced in HF myocytes (current/capacitance: 1.01±0.08, n=20 cells from ten HF hearts) compared with control (2.18±0.09, n=18 cells from nine control hearts, $P$<0.01) (Figure 2*a*-*c*).

*Measurement of SR $Ca^{2+}$ content*

Isolated cardiomyocytes were loaded with Fluo-5N and SR $Ca^{2+}$ content can be measured directly. The images showed that the SR $Ca^{2+}$ content was dramatically reduced in HF myocytes (Figure 3*a*-*d*). There was a significant decrease in spatial averages ($\Delta F/F_0$) in HF (26.4±1.8, $n$=12 cells from six HF hearts) compared with control (49.2±2.9, $n$=10 cells from five control hearts, $P$<0.01) (Figure 4*a*-*c*).

*mRNA expression of $Ca^{2+}$ handling genes*

Real time quantitative RT-PCR was performed to determine the relative mRNA expression pattern of RyR2, calmodulin, sorcin, calsequestrin, SERCA2a, PLB, Cav1.2 and NCX. We found that the mRNA expression of RyR2 and PLB failed to alter in HF compared with control group ($P$>0.05). The down-regulation of calmodulin, sorcin, calsequestrin SERCA2a, Cav1.2 and NCX was established by RT-PCR to be

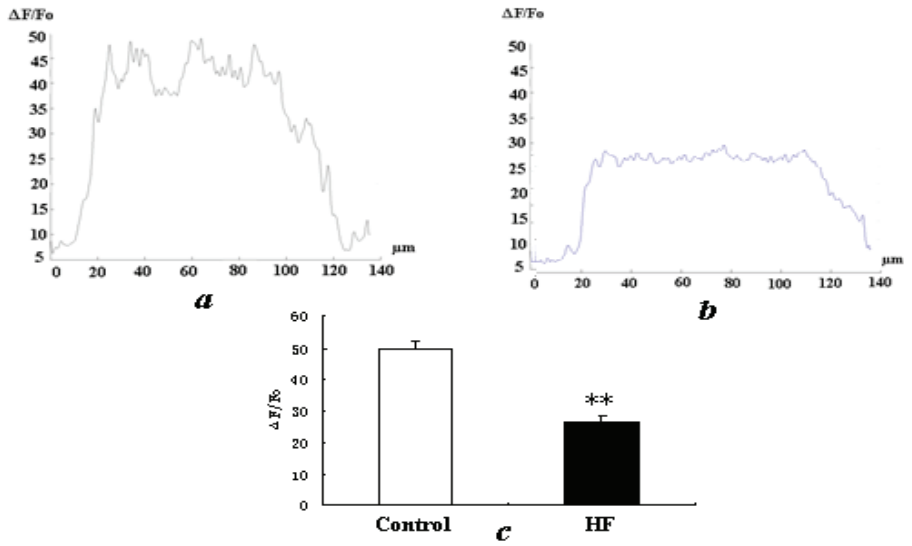


**Fig. 4.** Plots of fluorescence profiles in control (***a***) and HF myocytes (***b***). The average amplitudes ($\Delta F/F_0$) were 49.2±2.9 in control and 26.4±1.8 in HF myocytes (**c**). ($P$<0.01, n=10 cells from five control hearts, n=12 cells from six HF hearts)

significant ($n$=6, $P$<0.01) relative to the control, respectively (Figure 5*a*-*h*).

*Expression of SR proteins*

Immunoblot analyses were performed to determine the contents of the major SR $Ca^{2+}$ handling proteins RyR2, calmodulin, sorcin, calsequestrin, PP1, PP2A, NCX, SERCA2a, PLB, PLB-S16, PLB-T17 and Cav1.2 in control and HF group. The association of calmodulin, sorcin, calsequestrin, PP1, PP2A with RyR2 were determined firstly by immunoprecipitation using an anti-RyR antibody, and followed by immunoblotting with respective antibody. Compared with channel complexes from control rat, the expression of RyR2 and PLB failed to alter ($n$=6, $P$>0.05) (Figure 6*a*,6*i*,7*a*,7*i*), whereas calmodulin, sorcin, calsequestrin, PP1 and PP2A contents were significantly reduced from HF rat ($n$=6, $P$<0.01) (Figure 6*b*-*f*, 7*b*-*f*). Furthermore, the expression of NCX, SERCA2a, PLB-S16, PLB-T17 and Cav1.2 were decreased ($n$=6, $P$<0.01, $P$<0.05) (Figure 6*g*,6*h*,6*j*,6*k*,6*l*, 7*g*,7*h*,7*j*,7*k*,7*l*).

## Discussion

Numerous studies have suggested that abnormal $Ca^{2+}$ homeostasis may play a key role in 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 contractile dysfunction in HF is attributable largely to reduced myocyte $Ca^{2+}$ transients that ultimately arise from decreased SR $Ca^{2+}$ load (Pogwizd *et al*. 2001, Hobaiia and O'Rourke 2001, Lindner *et al*. 1998).

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) L-type calcium current (LTCC); (2) SR $Ca^{2+}$ content; (3) expression of RyR2 and RyR2 interacting proteins including calmodulin, sorcin, calsequestrin, PP1 and PP2A; (4) expression of SERCA2a, PLB, PLB-S16 and PLB-T17; (5) expression of Cav1.2; and (6) expression of NCX.

In our study, we found that a significant reduction in the SR $Ca^{2+}$ content (HF: $\Delta F/F_0$ = 26.4±1.8, $n$=12 cells from six HF hearts, control: $\Delta F/F_0$ =49.2±2.9, $n$=10 cells from five control hearts, $P$<0.01). The alteration in SR $Ca^{2+}$ content is considered to be hallmarks of HF.

Depolarization of the plasma membrane by an incoming action potential activates voltage-gated L-type $Ca^{2+}$ channels (DHPR or Cav1.2) in plasma membrane invaginations called T-tubules. In the heart, Cav1.2 activation results in a plasma membrane $Ca^{2+}$ influx current, which triggers RyR2 activation and SR $Ca^{2+}$ release, referred to as $Ca^{2+}$-induced $Ca^{2+}$ release (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 excitation-contraction coupling (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

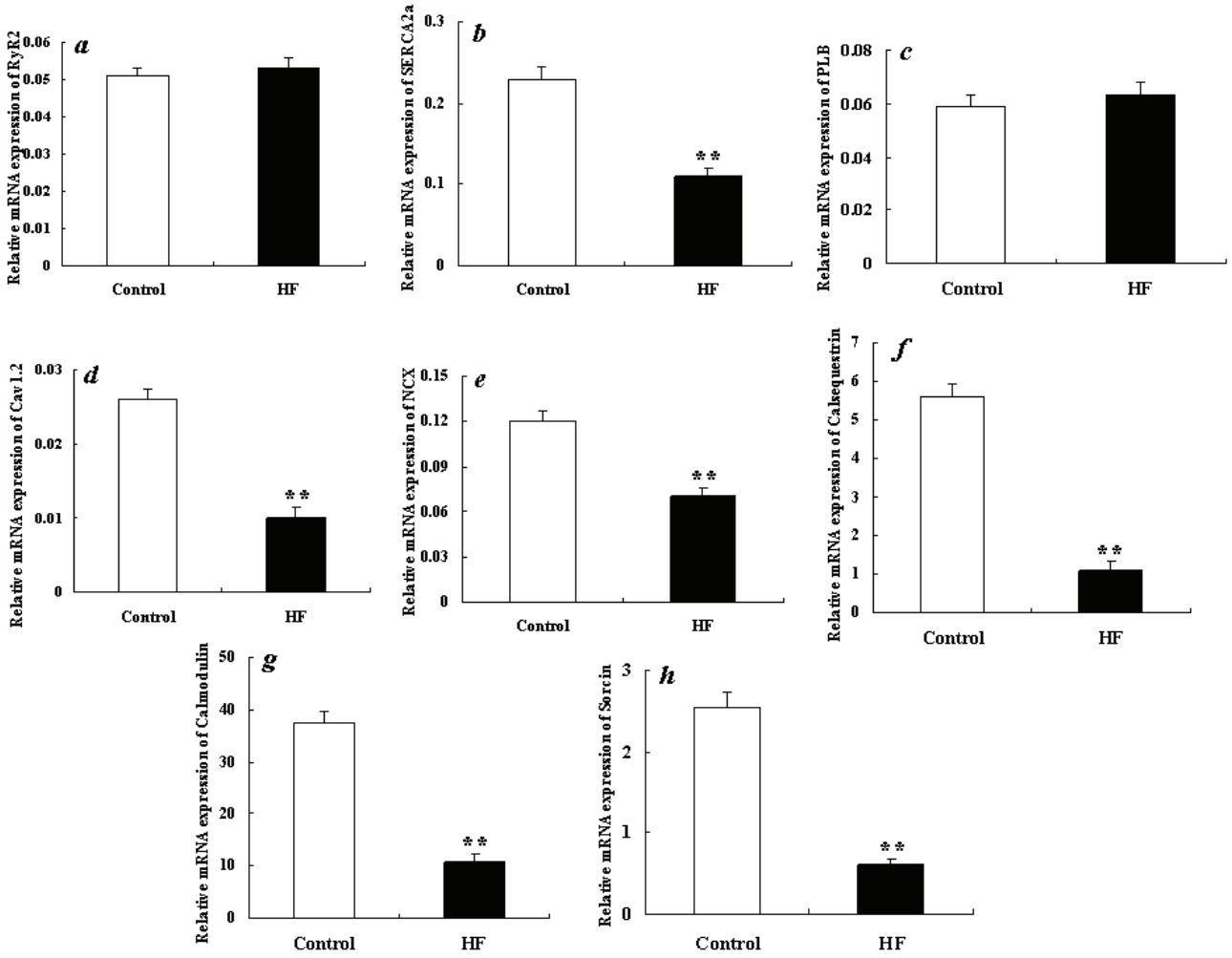


**Fig. 5.** Relative expression of $Ca^{2+}$ handling genes RyR2 (***a***), SERCA2a (***b***), PLB (***c***), Cav1.2 (***d***), NCX (***e***), Calsequestrin (***f***), Calmodulin (***g***) and Sorcin (***h***) in control and HF myocytes.

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 Cav1.2 seems to be reduced, which lead to the decreased amplitude of $I_{Ca\cdot L}$.

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. The approximately 4,000 amino acid large N-terminal domain of the RyR is exposed to the junctional cytosolic compartment and serves as a scaffold for protein-protein interactions and channel modulation (Bers 2004). The RyR bounds with many accessory proteins, such as calmodulin, FKBP12.6, PKA, PP1 and PP2A, producing a huge macromolecular complex. They act as an important modulator for maintaining normal $Ca^{2+}$ cycling. Defective regulation of channel gating by these key proteins may render the channel leaky. Modulation of the RyR is critical to survival, and consequently influenced by multiple regulatory mechanisms, include PKA-dependent phosphorylation of RyR (Xiao *et al*. 2005) and $Ca^{2+}$-Calmodulin dependent protein kinase (CaMKII) phosphorylation of RyR (Guo *et al*. 2006), the mechanism of latter is not known. The RyR does not exist in isolation but is coupled to other proteins, include FKBP12.6, junctin, triadin and calsequestrin (Wehrens *et al*. 2003, Terentyev *et al*. 2007), which may affect its open probability ($P_0$).

In our previous study, we demonstrated that the expression of RyR2 failed to alter, while the expression of FKBP12.6 associated with RyR2 was significantly reduced in HF rat. The present study explored the alterations in regulation and function of the RyR2 macromolecular complex that occur in HF. In the heart, the majority of phosphatase activity is attributed to type 1 protein

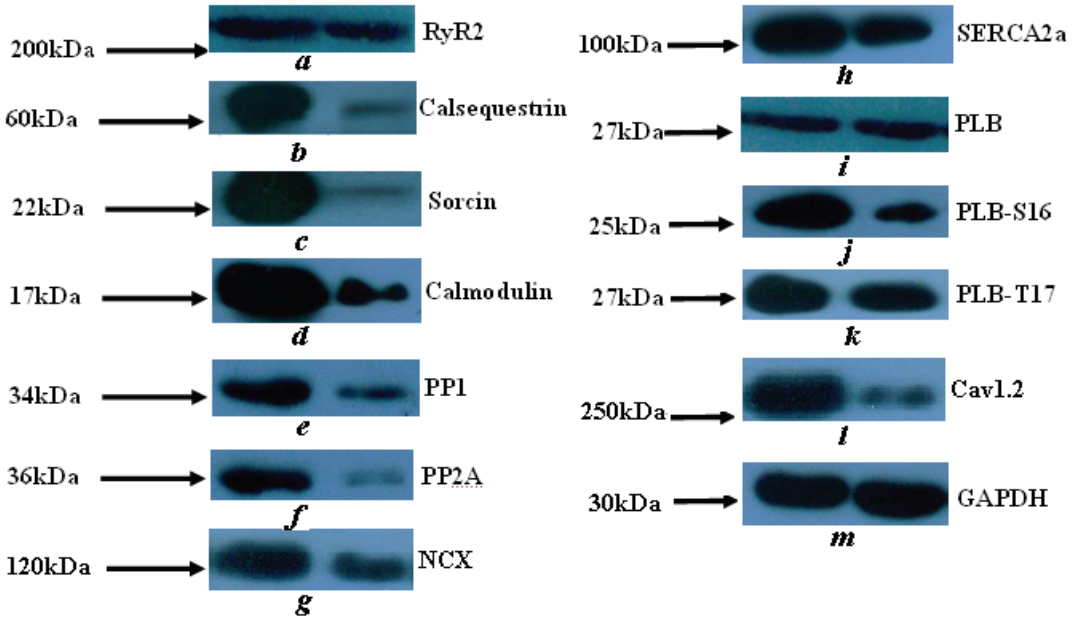


**Fig. 6.** Co-immunoprecipitation of RyR2, Calsequestrin, Sorcin, Calmodulin, PP1 and PP2A in cardiac muscle. RyR2 was immunoprecipitated from solubilised myocytes. Immunoblots were probed with mouse anti-RyR, rabbit anti-Calsequestrin, anti-Sorcin, anti-Calmodulin, anti-PP1 and anti-PP2A antibodies for the presence of RyR2 (***a***) Calsequestrin (***b***), Sorcin (***c***), Calmodulin (***d***), PP1 (***e***) and PP2A (***f***), respectively. Expression of NCX (***g***), SERCA2a (***h***), PLB (***i***), PLB-S16 (***j***), PLB-T17 (***k***), Cav1.2 (***l***) and GAPDH (***m***) 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.

phosphatase PP1, and type 2 phosphatases, PP2A and PP2B. Among these phosphatases, PP1 is of particular importance since it has been implicated as an important negative regulator of cardiac function. In fact, alterations in its levels and activity play a major role in the pathogenesis of heart failure. In the immunoprecipitated RyR2 complex in HF, we found reduced PP1 and PP2A. These changes would explain the enhanced RyR2 phosphorylation at sites of PKA action. We also found reduced calmodulin, sorcin and calsequestrin. Calmodulin inhibits RyR2 open probability at physiological [$Ca^{2+}$] (Marx *et al*. 2000), and the lower calmodulin associated with the RyR2 in HF here may contribute to increased SR $Ca^{2+}$ leak in HF. Sorcin is a cytosolic $Ca^{2+}$-binding protein that associates with RyR2 and may facilitate channel closure. Reduced expression of sorcin may also contribute to increased SR $Ca^{2+}$ leak in HF. Calsequestrin is a major intra-SR $Ca^{2+}$ binding protein. It forms a complex with the RyR2, triadin, and junction. In addition to its $Ca^{2+}$ buffering action, calsequestrin has been shown to regulate RyR2 function as a luminal $Ca^{2+}$ sensor of RyR2 (via protein-protein interactions involving triadin and junctin) (Györke *et al*. 2004). Reduced calsequestrin may decrease the open probability of RyR, which result in decreased SR $Ca^{2+}$ release in HF.

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 (Hasenfuss 1998). We found, however, the expression of SERCA2a was decreased in HF rat compare with control rat. It is also a critical factor determining the reduced SR $Ca^{2+}$ content in HF. Calcium reuptake via SERCA2a is significantly influenced by phosphorylation (PLB), a protein that, in its unphosphorylated form, inhibits SERCA2a, while in the phosphorylated state favors SERCA2a activity. PKA-dependent phosphorylation of PLB (PLB-S16) is the likely mechanism of accelerated relaxation associated with catecholamines (Li *et al*. 2000). CaMKII can also accelerate SR calcium reuptake by phosphorylation of PLB (PLB-T17) and, possibly, by a mechanism that does not require PLB (Desantiago *et al*. 2002). In the present study, the expression of PLB failed to alter, while the expression of PLB-S16 and PLB-T17 were reduced in HF, which leads to inhibition of SERCA2a. Furthermore, the protein expression of SERCA2a relative to PLB was diminished in HF. This ratio (the protein expression of SERCA2a relative to PLB) also indicates the extent of $Ca^{2+}$ pump inhibition, and hence the basal level of SERCA2a activity is at a

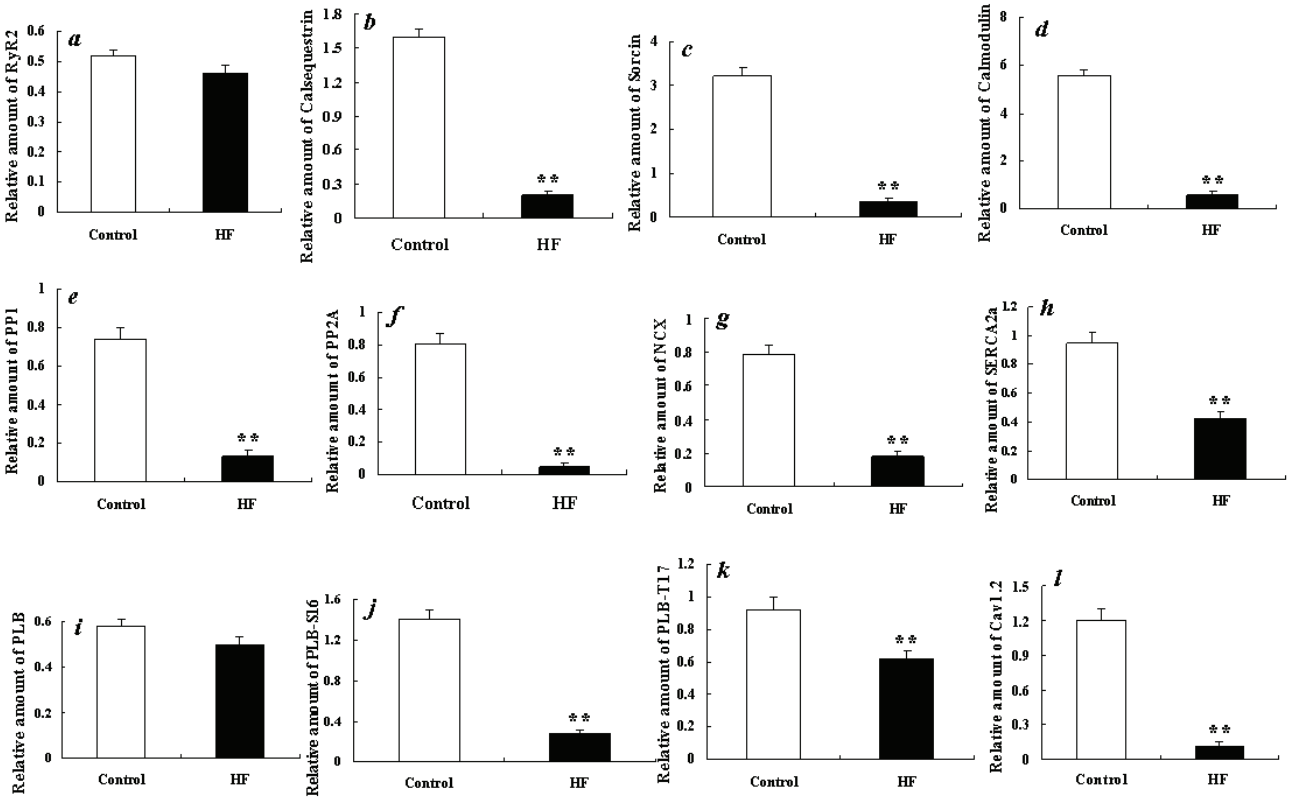


**Fig. 7.** Relative amount of $Ca^{2+}$ handling proteins RyR2 (***a***), Calsequestrin (***b***), Sorcin (***c***), Calmodulin (***d***), PP1 (***e***), PP2A (***f***), NCX (***g***), SERCA2a (***h***), PLB (***i***), PLB-S16 (***j***), PLB-T17 (***k***) and Cav1.2 (***l***) in control and HF myocytes.

lower level in failing hearts than in normal hearts.

On the other hand, the cardiac $Na^+/Ca^{2+}$ exchanger (NCX1) is almost certainly the major $Ca^{2+}$ extrusion mechanism in cardiac myocytes, although the driving force for $Ca^{2+}$ extrusion is quite small. The functional consequences of NCX overexpression in heart failure are controversial (Litwin and Bridge 1997). Previous studies in failing myocytes from the rabbit indicate that NCX overexpression is responsible for a reduction in sarcoplasmic reticulum $Ca^{2+}$ content and contractile dysfunction (Pogwizd *et al*. 2001). Using a canine model of cardiac hypertrophy, Sipido *et al.* (2000) showed that enhanced NCX function resulted in improved contractility. More recent studies in human failing myocytes suggest that NCX may be responsible for maintaining [Ca] during diastole (Piacentino *et al*. 2003). Assessment of this controversy requires that two points be considered: species-dependence of NCX function and the degree of hypertrophy and/or associate failure. We found the expression of NCX was decreased in our experiment. This may possibly be explained by differences in the stage and/or severity of the HF. Our hypothesis is that the downregulation of NCX fails to maintain function in late stages of heart failure. Further investigation is still needed to clarify the role of NCX and other SR $Ca^{2+}$ handling proteins.

In conclusion, systolic SR reduced $Ca^{2+}$ release and diastolic SR $Ca^{2+}$ leak (due to defective protein-protein interaction between RyR2 and its associated proteins) along with reduced SR $Ca^{2+}$ uptake (due to down-regulation of SERCA2a, PLB-S16 and PLB-T17), abnormal $Ca^{2+}$ extrusion (due to down-regulation of NCX) and defective CICR (due to down-regulation of Cav1.2) could contribute to heart failure.

## Conflict of Interest

There is no conflict of interest.

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## Abbreviations

$Ca^{2+}$, Calcium; CaMKII, Calmodulin kinase II; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; E-C coupling, Excitation-

contraction coupling; FKBP12.6, 12.6 kDa FK506 binding protein; HF, heart failure; $I_{Ca\cdot L}$, Long lasting calcium current; LTCC (Cav1.2), cardiac L-type $Ca^{2+}$ channel; NCX, $Na^{+}$-$Ca^{2+}$ exchanger; PKA, Protein kinase A; PLB, Phospholamban; PLB-S16, PLB phosphorylation at Ser16; PLB-T17, PLB phosphorylation at Thr17; PP, Protein phosphatase; RyR, Ryanodine receptor; RyR2, cardiac RyR; SERCA2a, SR-$Ca^{2+}$ATPase; SR, Sarcoplasmic reticulum.

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