ELSEVIER

Cardiovascular Research 53 (2002) 382–391

Cardiovascular Research

www.elsevier.com/locate/cardiores

Reduced level of serine[16] phosphorylated phospholamban in the failing rat myocardium: a major contributor to reduced SERCA2 activity

Jørn B. Sande[a,*], Ivar Sjaastad[a], Ingvild B. Hoen[a], Janny Bøkenes[a], Theis Tønnessen[a,b], Even Holt[a,c], Per K. Lunde[a], Geir Christensen[a]

[a] *Institute for Experimental Medical Research, Ullevaal University Hospital, Kirkeveien 166, N-0407 Oslo, Norway*
[b] *Department of Cardiothoracic Surgery, Ullevaal University Hospital, N-0407 Oslo, Norway*
[c] *Cardiological Department, Baerum Hospital, N-1306 Baerum Postterminal, Norway*

Received 21 June 2001; accepted 14 September 2001

Abstract

Objective: Heart failure is associated with alterations in contractile parameters and accompanied by abnormalities in intracellular calcium homeostasis. Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) and phospholamban (PLB) are important in intracellular calcium cycling. The aim of the present study was to examine mechanisms causing reductions in SERCA2 activity in the failing heart. **Methods:** Myocardial infarction (MI) was induced in male Wistar rats, and animals with congestive heart failure were examined 6 weeks after the primary operation. **Results:** Serine[16] monomeric and pentameric phosphorylated PLB were significantly downregulated (50 and 55%, respectively), whereas threonine[17] phosphorylated PLB was unchanged in failing compared to sham hearts. Protein phosphatases 1 and 2A were significantly upregulated (26 and 42%, respectively) and phosphatase 2C significantly downregulated (29%), whereas the level of protein kinase A regulatory subunit II remained unchanged during heart failure. Increasing PLB phosphorylation by forskolin in isolated cardiomyocytes after inhibition of the Na^{+}–Ca^{2+} exchanger activity had significantly greater effect on SERCA2 activity in failing than in sham cells (49 and 20% faster transient decline, respectively). Decreasing PLB phosphorylation by the protein kinase A inhibitor H89 had significantly less effect on SERCA2 activity in failing compared to sham cardiomyocytes (20 and 75% slower transient decline, respectively). **Conclusion:** The observed changes in SERCA2 activity after increasing and decreasing serine[16] PLB phosphorylation in cardiomyocytes from sham and failing hearts, suggest that the observed reduction in serine[16] PLB phosphorylation is one major factor determining the reduced SERCA2 activity in heart failure after MI.

Keywords: Calcium (cellular); Contractile function; Heart failure; Infarction; Myocytes; SR (function)

1. Introduction

Heart failure following myocardial infarction (MI) is associated with decreased myocardial contractility and relaxation abnormalities [1]. In individual cardiomyocytes isolated from the non-infarcted region of failing left ventricles, reduced shortening and prolonged relaxation have been observed [2,3]. The impaired myocyte function has been associated with a reduction in the ATP-dependent reuptake of cytosolic calcium by the sarcoplasmic reticulum (SR) [3,4]. However, the specific molecular mechanisms causing the reduction in SR calcium reuptake are not well defined.

The SR plays a critical role in the regulation of cytosolic calcium during myocardial contraction and relaxation. Calcium stored in the SR is released into the cytosol to activate the contractile apparatus, and the calcium is subsequently reaccumulated in the SR. The SR calcium reuptake rate is primarily determined by the SR-Ca^{2+} ATPase (SERCA2) pump capacity. A reduction in the amount of SERCA2 in the failing heart has been suggested as a mechanism for reduced SR calcium reuptake [5,6]. Although the SERCA2 mRNA level has been reported to be reduced both in the failing human [7–9] and rat

*Corresponding author. Tel.: +47-2301-6795; fax: +47-2301-6799.
E-mail address: j.b.sande@ioks.uio.no (J.B. Sande).

Time for primary review 20 days.

0008-6363/02/$ – see front matter
PII: S0008-6363(01)00489-8

myocardium [10], several reports indicate that the protein levels of both phospholamban (PLB) and SERCA2 are unchanged in heart failure [9,11–14].

Decreased SR calcium reuptake may also be caused by a reduction in SERCA2 pumping capacity without any change in the amount of SERCA2 or PLB. The unphosphorylated form of PLB inhibits SERCA2 activity, whereas the phosphorylated form of PLB dissociates from SERCA2 leading to increased pumping activity. PLB is phosphorylated at the serine[16] residue via the β-adrenergic pathway and at the threonine[17] residue primarily via calcium/calmodulin kinase II [15,16]. The degree of PLB phosphorylation is also regulated by protein phosphatases (PP); PP1 and PP2A especially have been reported to dephosphorylate PLB [17].

The aim of the present study was to examine the mechanisms causing the reduction in SERCA2 activity in heart failure after MI. In particular, alterations in the level of PLB phosphorylation were examined with special emphasis on the pathways involved in phosphorylation and dephosphorylation of PLB. The functional impact of alterations in PLB phosphorylation on SR calcium reuptake was examined in isolated cardiomyocytes after elimination of the Na^+–Ca^{2+} exchanger activity (NCX). Elimination of the NCX is important since its contribution is different in failing compared to sham cells [5,18,19].

2. Methods

2.1. Animal preparation

Male Wistar rats weighing ~300 g were purchased from Møllegaard Breeding and Research Center, Skensved, Denmark. MI was induced by ligation of the left coronary artery as previously described [20]. During the surgical procedure, the rats were intubated and ventilated on a Zoovent ventilator (Triumph Technical Services, Milton Keynes, UK) with a mixture of 68% N_2O, 29% O_2 and 2–5% isofluran (Abbot Laboratories, USA). The sham-operated animals (sham) were subjected to the same surgical procedure without ligation of the left coronary artery. A total of 57 animals were included in the study. The experimental protocol is in accordance with the Norwegian National Committee for Animal Welfare Act, which closely confirms to the NIH guidelines (NIH publication No. 85-23, revised 1996). The rats were anesthetized 6 weeks after the first operation, and left ventricular end-diastolic pressure (LVEDP) and left ventricular systolic pressure were measured with a 2F micromanometer tipped catheter (Model SPR-407, Millar Instruments, Houston, TX). Only rats with LVEDP≥15 mmHg were considered to have congestive heart failure (CHF). In a study from our laboratory it was shown that animals with LVEDP≥15 mmHg had echocardiographic characteristics and clinical signs typical for animals in heart failure [21]. Of the 33 animals with left coronary artery ligation, 26 fulfilled our criteria for CHF and were included in the study.

2.2. In vivo studies of phospholamban phosphorylation

A 1-μmol/l aliquot of the protein kinase A (PKA) inhibitor H89 dissolved in 1% DMSO/0.9% NaCl was infused for 5 min in vivo through the right vena jugularis in normal rats. At the end of infusion, the hearts were rapidly excised, divided into right ventricle, left ventricle and infarct area, and frozen on liquid nitrogen. Control rats did not receive H89.

2.3. Protein immunoblot analysis

Total protein and membrane protein fractions were isolated from the non-infarcted region of left ventricles from rats with CHF and sham rats [22]. Protein concentrations were determined by the bicinchoninic acid assay (Pierce 23235) using bovine serum albumin as standard. Immunoblot analysis was performed as previously described [23]. For inhibition of endogenous phosphatases, one protease inhibitor cocktail tablet (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany) dissolved in 50 ml homogenized buffer was added. Briefly, proteins were separated by 10 or 12% SDS–PAGE and transferred to polyvinyl difluorid membranes (Schleicher and Schuell, Dassel, Germany). Non-specific binding was blocked in non-fat dry milk at 4°C overnight before the blots were incubated with primary antibody for 1 h at room temperature, and then incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (rabbit anti-sheep IgG (Pierce, Rockford, UK), anti-mouse IgG or anti-rabbit IgG (both from Amersham Pharmacia Biotech, Buckinghamshire, UK)). The primary antibodies were anti-phospholamban A1, anti-phospholamban PS-16, anti-phospholamban PT-17 (all from Fluorescience, Leeds, UK), anti-mouse protein kinase A RII subunit and anti-protein phosphatase 1, 2A and 2C (all from Upstate, Lake Placid, NY). Immunoreactive proteins were visualized using ECL detection kit (Amersham). Luminescence was detected by LAS-1000 video detection system and quantified with the Image Gauge program (both from Fujifilm, Stockholm, Sweden).

2.4. Myocyte isolation

Myocytes were isolated from the viable part of the left ventricle as described in a previous publication from our laboratory [24]. Briefly, the method included collagenase perfusion via aorta in a modified Langendorff system. Only rod-shaped cells without blebs or other morphological alterations were included in the study. Myocytes from three or more animals were included in every experimental group.

2.5. Measurements of intracellular calcium and SERCA2 activity

Myocytes plated on coverslips were loaded with fluo-3 acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR) at room temperature for 40 min in a solution containing 10 μmol/l fluo-3 AM before washing for 15 min in standard Tyrodes solution containing (in mmol/l): HEPES, 5; NaCl, 140; $CaCl_2$, 1.8; KCl, 5.4; $MgCl_2$, 0.5; glucose, 5.5; NaH_2PO_4, 0.4; pH was adjusted to 7.4 with NaOH. The coverslips were then placed in an open perfusion chamber located on an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan). The microscope was attached to a PTI Delta Ram fluorescence system (Photon Technology International, Monmouth Junction, NJ). The cells were superfused with Tyrodes solution, and the test solutions were applied with a rapid solution switcher, exchanging the solution around the cell within 200 ms. The cells were field stimulated at 0.5 Hz. The myocytes were excited at 500 nm and emitted fluorescence was measured at 525 nm sampled with Felix software (PTI), and analyzed in MatLab (Mathworks, Nattick, MA). Background fluorescence was recorded for each cell and subtracted prior to any analysis. All experiments were performed at 23°C.

Removal of calcium from cytosol depends mainly on SERCA2 and the NCX [25–27]. Thus, the calcium transient decline after elimination of the NCX is almost entirely due to SERCA2 activity. To eliminate the NCX we superfused the cardiomyocytes with a solution containing (in mmol/l): KCl, 126.0; $MgCl_2$, 1.0; KOH, 13.0; HEPES, 24.0; EGTA, 2.0; probenecid, 0.5; and tetracaine, 0.3 [28]. Prior to exposure to that solution, the electrical pacing was stopped and the solution changed with the rapid solution switcher. The membrane was depolarized by high $[K^+]$ and maintained at ~0 mV, which induced a calcium transient with slower decline due to the elimination of the NCX as described by Yao et al. [28]. The rate of transient decline was estimated by calculating time from peak transient to 50% decay (T_0-T_{50}), from 50 to 90% decay ($T_{50}-T_{90}$) and from peak transient to 90% decay (T_0-T_{90}). The time constant τ, describing the transient decline, was derived from the formula $f(t) = a \exp^{-t/\tau}$, where '*a*' is a constant. Calculations of SERCA2 activity were based on the time constants calculated after elimination of the NCX. Since a high time constant value denotes low SERCA2 activity and vice versa, the time constants were inverted in order to illustrate the SERCA2 activity. The mean value for sham cells under control conditions was set to 100%, and the values obtained under decreased and increased PLB phosphorylation in sham and failing cardiomyocytes were normalized to the sham control value.

2.6. Statistics

Data are presented as means±S.E.M. Simple comparisons between groups were made using Student's *t*-test or two-way ANOVA when appropriate. A two-sided *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Body weight, heart weight and hemodynamic parameters

The heart weight 6 weeks after coronary artery ligation was significantly higher in rats with MI compared to sham (Table 1). The heart/body weight ratio was significantly increased in rats with MI, indicating that a significant myocardial hypertrophy had developed in the remaining viable part of the left ventricle. Left ventricular systolic pressure was significantly lower in rats with MI compared to sham. Together with a significantly increased lung weight, the findings are consistent with presence of CHF 6 weeks after ligation of the left coronary artery.

3.2. Serine[16] and threonine[17] phosphorylation of PLB in sham and failing hearts

Protein immunoblot analysis revealed a substantial reduction in the amount of serine[16] phosphorylated PLB in hearts from animals with CHF (Fig. 1A,B). Since the highly specific antibodies used allowed quantification of both the pentameric and monomeric forms, we were able to show that the reduction in serine[16] phosphorylation occurs both in the monomeric and the pentameric forms of PLB. Fig. 1A shows that in CHF, the amount of serine[16] phosphorylated pentameric form of PLB was reduced to 50±7% of the amount of serine[16] phosphorylated pentameric form of PLB found in sham hearts ($P<0.05$). The amount of the serine[16] phosphorylated monomeric form of PLB was reduced to 55±6% in failing hearts compared to sham ($P<0.05$) (Fig. 1B). The ratio of serine[16] phosphorylated pentameric to monomeric form in CHF was not different from sham. The amount of threonine[17] phosphorylated PLB was unchanged in CHF compared to sham (Fig. 1C,D). Both the pentameric and monomeric forms of threonine[17] phosphorylated PLB were unaltered in CHF compared to sham. Neither the amount of SERCA2 nor the

Table 1
Body weight, heart weight and hemodynamic parameters

	Sham	CHF	*P*
Heart weight (g)	1.51±0.04	2.66±0.07	<0.01
Body weight (g)	412.5±4.1	405.8±11.5	NS
Heart/body weight ratio (mg/g)	3.66±0.12	6.55±0.51	<0.01
Lung weight (g)	1.48±0.04	3.86±0.23	<0.01
LVSP (mmHg)	128.7±3.7	101.8±3.9	<0.01
LVEDP (mmHg)	5.3±0.3	24.8±1.7	<0.01

LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; NS, not significant; $n=31$ sham, $n=26$ CHF.

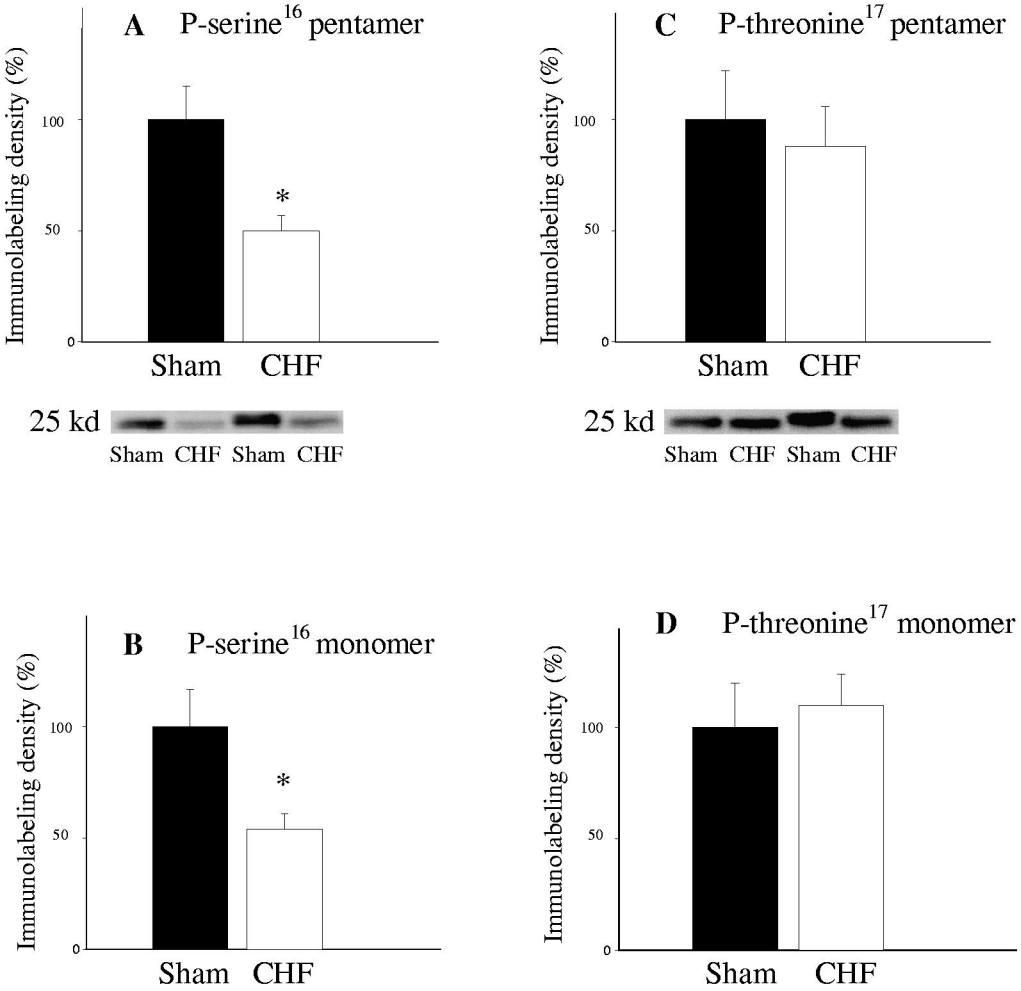

Fig. 1. Immunoblot analysis of serine[16] and threonine[17] phosphorylated phospholamban (PLB) (P-serine[16] and P-threonine[17], respectively). Immunolabeling density for sham was set to 100%. Panel A shows pentameric serine[16] phosphorylated PLB with representative immunoblot. Panel B shows monomeric serine[16] phosphorylated PLB. Panel C shows pentameric threonine[17] phosphorylated PLB with representative immunoblot. Panel D shows monomeric threonine[17] phosphorylated PLB. *$P<0.05$ vs. sham; $n=5$ sham, $n=6$ CHF.

total amount of PLB were altered in rats with CHF compared to sham (Table 2).

3.3. The amount of PKA-RII and phosphatases in sham and failing hearts

The amount of the regulatory subunit of PKA, PKA-RII, in hearts from rats with CHF and sham was examined by immunoblot analysis. No significant difference was found between animals with CHF and sham. The protein levels of phosphatase 1, 2A and 2C in hearts from rats with CHF and sham were examined by immunoblot analysis. Fig. 2 shows that the protein level of phosphatase 1 was 26±8% higher in hearts from CHF animals compared to sham ($P<0.05$). Furthermore, the protein level of phosphatase 2A was 42±13% higher in CHF than in sham ($P<0.05$). The protein level of phosphatase 2C was reduced by 29±7% in failing hearts compared to sham ($P<0.05$).

Table 2
Phospholamban (PLB) and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) protein levels in sham and failing hearts

	Sham	CHF	*P*
PLB-pentamer (au)	1.11±0.12	0.91±0.11	NS
PLB-monomer (au)	0.97±0.13	0.97±0.18	NS
PLB total (au)	1.04±0.09	0.94±0.01	NS
PLB-pentamer/PLB-monomer	1.67±0.24	1.83±0.57	NS
SERCA2 (au)	1.02±0.07	0.89±0.08	NS
SERCA2/PLB-pentamer	1.01±0.16	1.09±0.12	NS
SERCA2/PLB-monomer	1.18±0.20	1.10±0.18	NS

au, arbitrary units; NS, not significant; $n=5$ sham, $n=6$ CHF.

3.4. Effects of stimulation with forskolin and isoproterenol on SR calcium reuptake in cardiomyocytes isolated from sham and failing hearts

After elimination of the NCX in isolated cardiomyocytes, the initial transient decline (T_0-T_{50}) was unchanged (Table 3), but the terminal decline ($T_{50}-T_{90}$) was significantly prolonged. The prolongation was significantly greater in failing than in sham cardiomyocytes

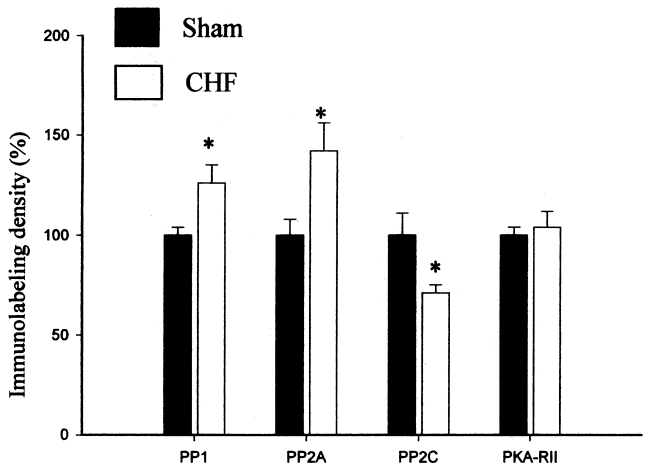

Fig. 2. Immunoblot analysis of protein phosphatases 1 (PP1), 2A (PP2A), 2C (PP2C) and protein kinase A regulatory subunit II (PKA-RII). Immunolabeling density for sham was set to 100%. *$P<0.05$ vs. sham; $n=5$ sham, $n=6$ CHF.

($P<0.05$), indicating a greater contribution of NCX in CHF compared to sham. Moreover, since SERCA2 and the NCX are the major contributors to calcium removal from the cytosol, the slower decline of the calcium transient (T_0-T_{90}) after elimination of the NCX in failing cardiomyocytes (Fig. 3) suggests an attenuated SR calcium reuptake by SERCA2 in CHF compared to sham.

To examine the effects of an increase in cAMP on SR calcium reuptake, cardiomyocytes were stimulated with 1 μmol/l forskolin and examined after elimination of the NCX. Forskolin increases adenylyl cyclase activity, which then generates more cAMP leading to an increased PKA activity and hence elevated PLB phosphorylation. In cardiomyocytes from sham hearts, the decline (T_0-T_{90}) of the calcium transient was 481±34 ms during stimulation with forskolin compared to 599±58 ms before stimulation ($n=5$). In cardiomyocytes from rats with CHF, the decline (T_0-T_{90}) of the calcium transient was 564±90 ms during stimulation with forskolin compared to 1104±146 ms before stimulation ($n=7$). Hence, stimulation with forskolin decreased the decline (T_0-T_{90}) of the calcium transient by 188±32 ms (20±4%) in sham cardiomyocytes, whereas a significantly greater reduction by 540±83 ms (49±8%) was observed in failing cardiomyocytes ($P<0.01$) (Fig. 4).

To examine the effects of β-adrenergic receptor stimulation on SR calcium reuptake, cardiomyocytes were stimulated with 200 nmol/l isoproterenol and examined after elimination of the NCX. In cardiomyocytes from sham hearts, the decline (T_0-T_{90}) of the calcium transient was 458±73 ms during stimulation with isoproterenol compared to 685±94 before stimulation ($n=6$). In failing cardiomyocytes, the decline (T_0-T_{90}) was 551±60 ms during stimulation with isoproterenol compared to 877±109 ms before stimulation ($n=11$). Hence, stimulation with isoproterenol decreased the transient decline by 228±43 ms (33±4%) and 326±120 ms (31±8%) in sham and failing cardiomyocytes, respectively. Although there was a significant shortening of the decline of the transients in sham and failing cells, the effect of isoproterenol on the calcium transient was not significantly greater in failing cardiomyocytes.

Table 3
Time from peak to 50% decline of the calcium transient (T_0-T_{50}), and from 50 to 90% decline of calcium transient ($T_{50}-T_{90}$) before (+NCX) and after elimination of the Na^+–Ca^{2+} activity (−NCX)

	+NCX		−NCX	
	T_0-T_{50}	$T_{50}-T_{90}$	T_0-T_{50}	$T_{50}-T_{90}$
Sham	175±9	263±15	197±22	458±40*
CHF	196±8	266±16	217±15	749±70*†

All values in ms. *$P<0.05$ vs. +NCX; †$P<0.05$ vs. sham; $n=19$ sham, $n=17$ CHF.

3.5. In vivo effects of PKA inhibition on PLB phosphorylation in normal hearts

The effects of PKA inhibition on serine[16] and threonine[17] phosphorylated forms of PLB were examined in vivo by infusing H89 ($n=6$). The protein level of serine[16] phosphorylated pentameric PLB was reduced to 25±12% of the control level ($P<0.05$). Serine[16] phosphorylated monomeric PLB was reduced to 44±14% of control after H89 infusion ($P<0.05$). Threonine[17] phosphorylated PLB was not significantly altered after H89 infusion.

3.6. Effects of PKA inhibition on calcium homeostasis and SR calcium reuptake in cardiomyocytes isolated from sham and failing hearts

Superfusion with H89 reduced the peak transient magnitude to 39±9% of control in cardiomyocytes from sham hearts. In contrast, the peak transient magnitude fell to only 80±11% of control in cells isolated from failing hearts (Fig. 5A,B). When the NCX was eliminated prior to PKA inhibition, the time constant for the transient decline was greater in failing cardiomyocytes (1386±157 ms, $n=12$) compared to cardiomyocytes from sham hearts (931±80 ms, $n=17$) ($P<0.01$). After elimination of the NCX during PKA inhibition, the time constant was 1661±94 ms in failing cardiomyocytes ($n=9$) and 1631±132 ms in sham ($n=16$) (Fig. 5C). These data show that the decline of the calcium transients was significantly less affected by inhibition of PKA in cardiomyocytes from failing hearts (20±5% increase in τ) compared to cardiomyocytes isolated from sham hearts (75±14% increase in τ) ($P<0.05$).

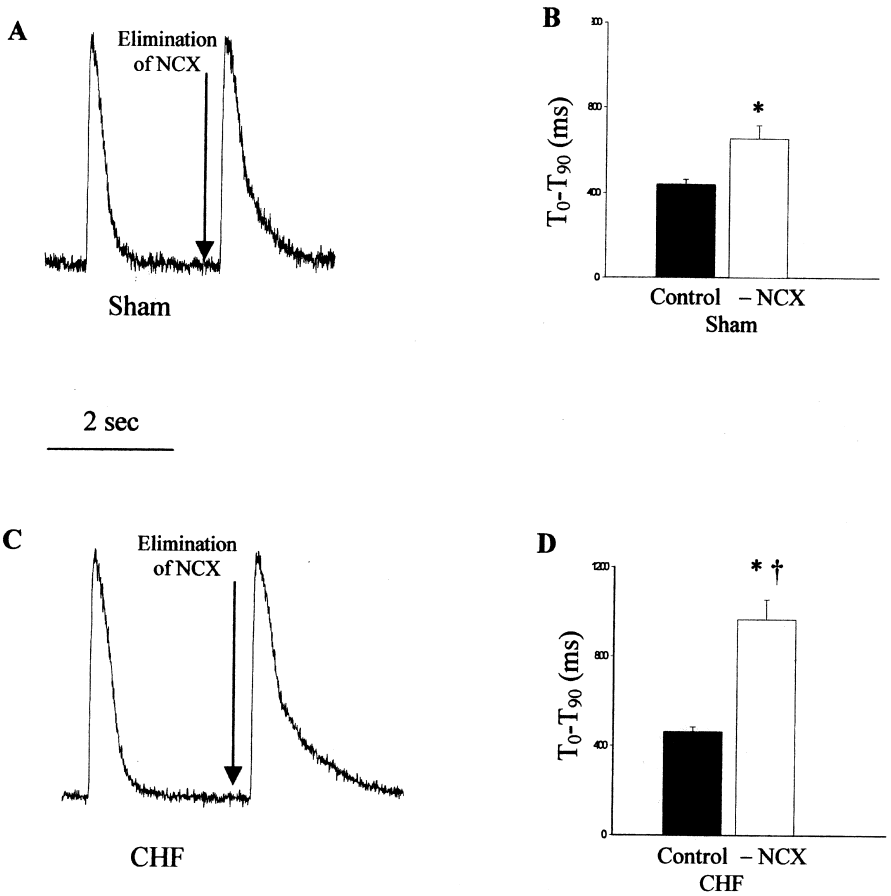

Fig. 3. Effect of inhibiting Na^+–Ca^{2+} exchanger activity (NCX) on calcium transients. Representative tracings from a sham (panel A) and a failing (panel C) cardiomyocyte. The arrows in panels A and C denote application of the 0-Na^+, 0-Ca^{2+} and 140 mmol/l K^+ solution simultaneously as the electrical pacing was shut off. The transients elicited reached the same magnitude as the preceding transients, but with significantly slower decay ($P<0.05$). Panels B and D show the time from peak to 90% decay of the calcium transient (T_0-T_{90}) after NCX elimination. *$P<0.05$ vs. control; †$P<0.05$ vs. sham; $n=19$ sham, $n=17$ CHF.

4. Discussion

Previous studies on PLB phosphorylation in heart failure after MI are not consistent. In a recent study, Netticadan et al. [29] did not show any reduction in serine[16] phosphorylated PLB 8 weeks after occlusion of the left coronary artery in rat. However, they observed a reduction in threonine[17] phosphorylated PLB and suggested that alterations in SR calcium reuptake may occur partly because of a defect in the calcium/calmodulin kinase-mediated phosphorylation. In a rat model of compensated hypertrophy with no evidence of heart failure, reduced levels of serine[16] and threonine[17] phosphorylated PLB have been reported [14]. In human end-stage heart failure, downregulation of serine[16] phosphorylated PLB [13] and decreased levels of both serine[16] and threonine[17] phosphorylation of PLB have been reported [30]. The finding in our study that serine[16] phosphorylated PLB was significantly reduced without any change in the level of threonine[17] phosphorylated PLB, indicates a reduction in PKA-dependent PLB phosphorylation in the failing myocardium.

A reduction in PKA-dependent PLB phosphorylation in heart failure may be explained by changes at various levels of β-adrenergic signaling [31,32]. An alteration in the amount of PKA-RII in failing hearts may be involved in the reduction of PLB phosphorylation. Zakhary et al. [33] have previously reported decreased expression of PKA-RII in failing human hearts. However, our data revealed similar amounts of PKA-RII in failing and sham hearts. This is consistent with the observation by Böhm et al. [34], who demonstrated similar cAMP-dependent protein kinase activity in failing and non-failing human hearts.

In addition to abnormalities in the PKA-dependent phosphorylation pathway, the reduction in serine[16] phosphorylated PLB could also result from an increase in the amount of PP. It is assumed that more than 90% of myocardial PP activity is accounted for by PP1 and PP2A [35]. Inhibition of PP1 and PP2A by okadaic acid in guinea pig papillary muscles has been shown to shorten the

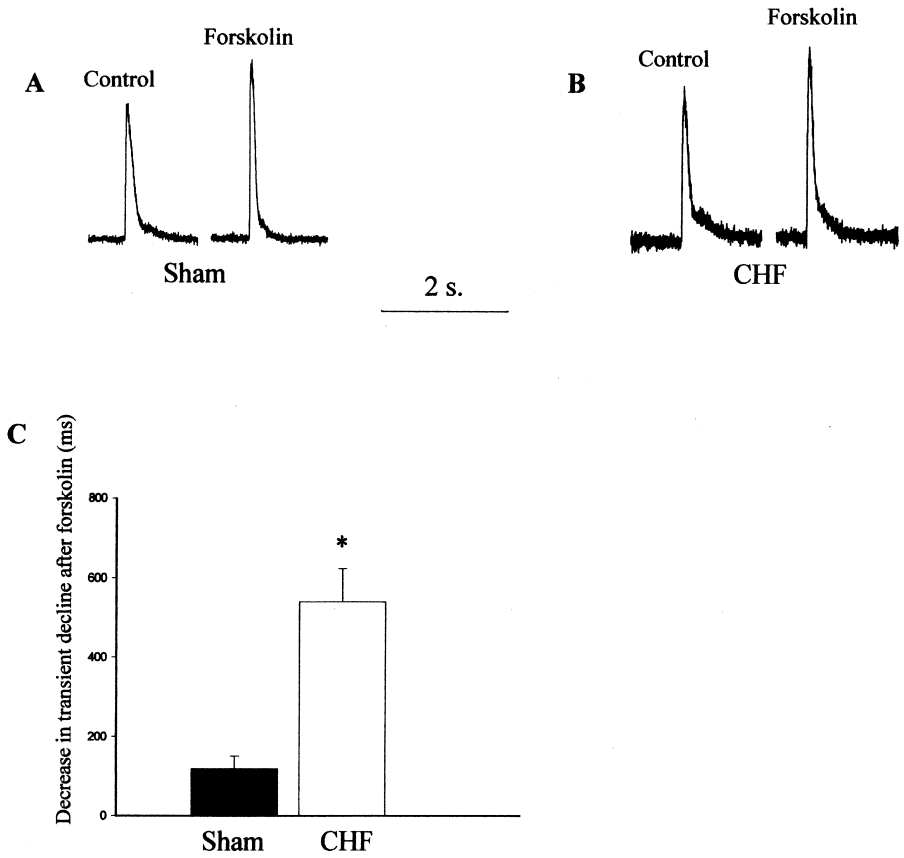

Fig. 4. Effect of stimulation with forskolin on isolated cardiomyocytes. Panel A shows transients recorded from a sham cardiomyocyte and panel B shows a failing cardiomyocyte before and after exposure to forskolin for 60 s. Panel C shows decrease in transient decline ($T_0 - T_{90}$) after exposure to forskolin in cardiomyocytes from sham and CHF. *$P<0.05$ vs. sham; $n=5$ sham, $n=7$ CHF.

relaxation and increase the force of contraction [36]. PP activity has been reported to be increased after long-term β-adrenergic stimulation [37] and in compensated hypertrophy in the rat [14]. In human end-stage heart failure Neumann et al. [38] have reported increased activity of PP1. The amounts of the various PP in the failing heart have to our knowledge not been known. Our study shows significant increase in the amounts of both PP1 and PP2A, and suggests that increased production of these PP dephosphorylates PLB at a more rapid rate in failing compared to sham hearts. However, the pathway(s) involved in the upregulation of PP1 and PP2A remains to be examined. It is intriguing that the level of threonine[17] phosphorylated PLB was not significantly reduced in hearts from rats with CHF. Although some controversy exists, a significant increase in calcium/calmodulin kinase II activity has been found in failing hearts [39,40]. Hence, it is possible that elevated kinase activity was able to balance the effect of increased levels of PP in failing hearts.

We also found that the level of PP2C was significantly lower in failing compared to sham hearts. The role of cardiac PP2C is at present unclear, but it may be capable of dephosphorylating PLB [17]. Whether downregulation of PP2C is important in other aspects of the heart failure process remains to be examined. Hanada et al. [41] have reported that PP2C selectively inhibits the stress activated protein kinases through suppression of several mitogen-activated protein kinase-kinases. Thus, it is possible that PP2C is involved in the process of cardiac hypertrophy.

Calcium removal from cytosol was significantly slower in cardiomyocytes from animals with CHF than from sham after elimination of the NCX. Our data suggest that increased Na^+–Ca^{2+} exchange in failing cardiomyocytes may have counteracted the reduction in SERCA2 pumping activity. The greater effect of forskolin on failing cardiomyocytes compared to sham indicates that the mechanisms for PLB phosphorylation downstream of adenylyl cyclase are intact and that the potential for PLB phosphorylation in failing cardiomyocytes is able to almost completely abolish the observed difference between sham and failing cells. In contrast to forskolin, stimulation with isoproterenol did not reveal a greater effect on the decline of the calcium transients in failing cardiomyocytes compared to sham. The difference between the effects of stimulation with isoproterenol and forskolin may be due to β-adrenergic receptor downregulation or uncoupling from adenylyl cyclase in the failing cardiomyocytes.

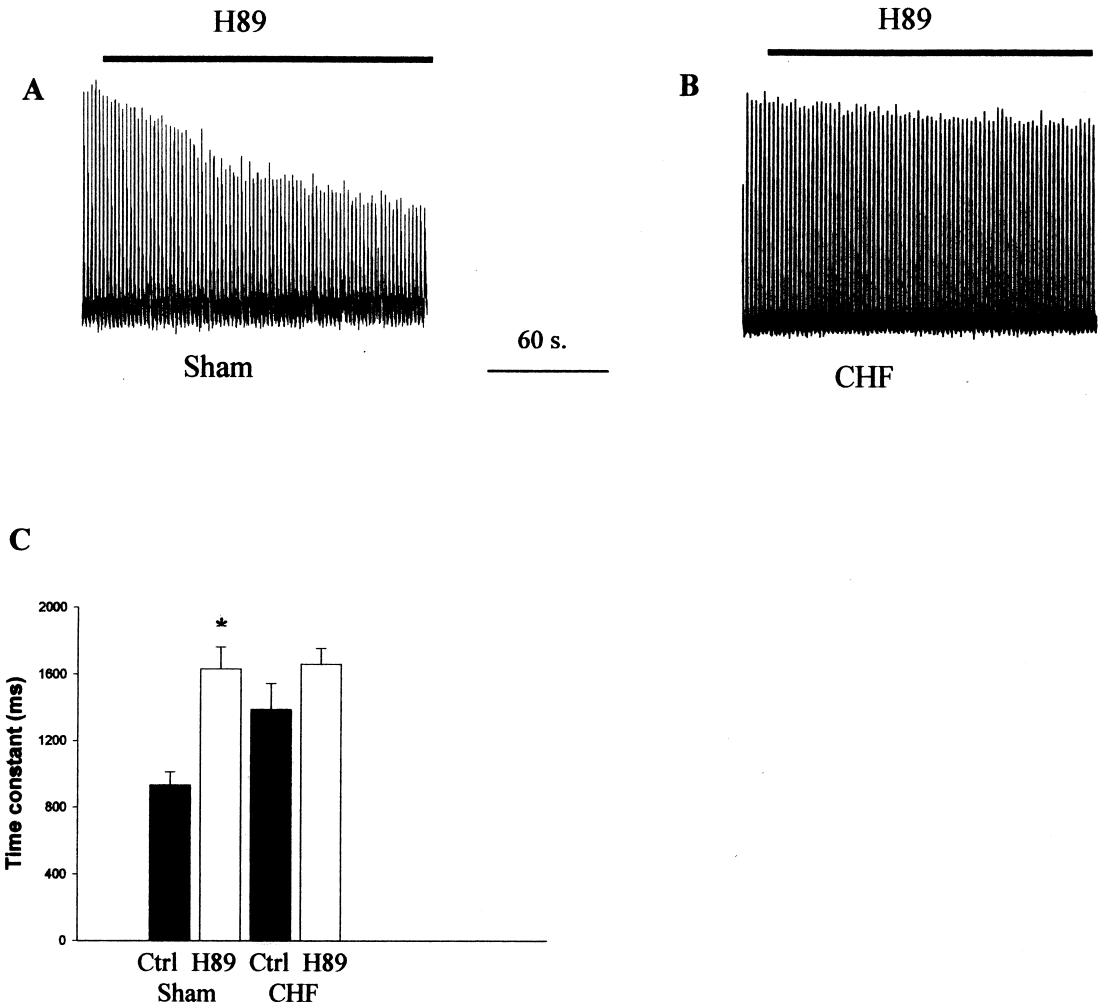

Fig. 5. Effect of protein kinase A (PKA) inhibition. Trains of calcium transients during perfusion with the PKA inhibitor H89. Panel A shows a sham and panel B shows a failing cardiomyocyte. Panel C shows changes in time constants (see Methods section) of transient decline after exposure with H89 for 150 s following inhibition of the Na^+–Ca^{2+} exchanger. *$P<0.05$ vs. control; $n=16$ sham, $n=9$ CHF.

Since H89 reduced serine[16] phosphorylation of PLB in vivo without any effect on threonine[17] phosphorylated PLB, we used H89 to examine the effect of a reduction in PLB phosphorylation on SR calcium reuptake in isolated cardiomyocytes. Our experiments showed that PKA inhibition induced significantly less reduction in SERCA2 activity in cardiomyocytes from rats with CHF than in those from sham. A possible explanation for this finding is that the low phosphorylation level in the failing cells was less affected by a further decrease in phosphorylation. Our finding supports the concept that the reduction in the level of serine[16] phosphorylation observed in our study is of major functional importance. Fig. 6 illustrates the functional implications of increasing and decreasing PLB phosphorylation on SERCA2 activity by using a method described in the Methods section to calculate SERCA2 activity.

In summary, this study demonstrates that: (i) in failing hearts, the level of serine[16] phosphorylated PLB was downregulated, whereas the level of threonine[17] phosphorylated PLB was unchanged; (ii) in failing hearts, the protein levels of SERCA2 and PLB were unchanged compared to sham; (iii) the protein levels of PP1 and PP2A were upregulated in failing hearts, whereas PP2C was downregulated; (iv) stimulation with forskolin increased SERCA2 activity significantly more in failing cardiomyocytes compared to sham; (v) inhibition of PKA had significantly less effect on SERCA2 activity in cardiomyocytes from rats with CHF than in cardiomyocytes from sham. Taken together, the reduction in serine[16] phosphorylated PLB in the failing rat heart may, at least in part, be explained by the observed increase in the level of PP1 and PP2A. Moreover, the observed changes in SERCA2 activity after increasing or decreasing phosphorylation in cardiomyocytes from sham and failing hearts suggest that the reduction in serine[16] phosphorylated PLB is one important factor determining the observed reduction in SR calcium reuptake in heart failure after MI.

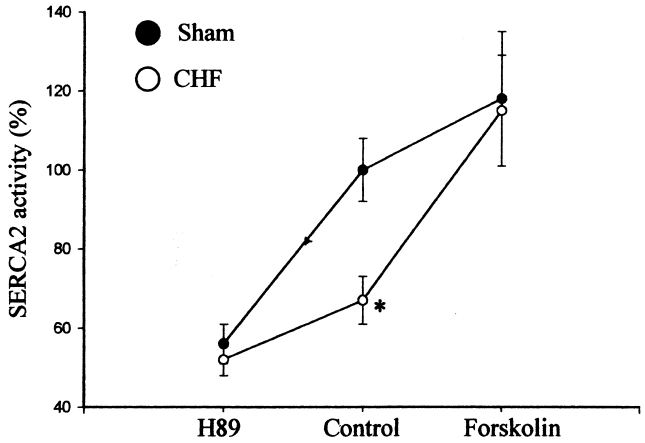

Fig. 6. Illustration of the relationship between SERCA2 activity and phospholamban (PLB) phosphorylation. SERCA2 activity in sham cardiomyocytes in the control situation was set to 100%. In sham cells, increasing PLB phosphorylation (forskolin) increased SERCA2 activity to 118%, while decreasing PLB phosphorylation (H89) decreased SERCA2 activity to 56%. In failing cardiomyocytes, SERCA2 activity was 67% of sham in the control situation, increased to 115% during stimulation with forskolin and decreased to 52% during PKA inhibition. $*P<0.05$ vs. sham; $n=22$ sham, $n=19$ CHF.

Acknowledgements

We are grateful to Kathrine Andersen and Geir Florholmen for isolation of cardiomyocytes, and we thank Bjørn Amundsen and Lisbeth H. Winer for technical assistance. We also thank Morten Eriksen and Line Solberg for animal care. This study was supported by grants from the Research Council of Norway and Anders Jahres fund for the Promotion of Science and Research Forum, Ullevaal Hospital.

References

[1] Braunwald E, Grossman W. Clinical aspects of heart failure. In: Braunwald E, editor, Heart diseases, Philadelphia, PA: Saunders, 1994, pp. 445–467.
[2] Li P, Park C, Micheletti R et al. Myocyte performance during evolution of myocardial infarction in rats: effects of propionyl-L-carnitine. Am J Physiol 1995;268:H1702–H1713.
[3] Holt E, Tønnessen T, Lunde PK et al. Mechanisms of cardiomyocyte dysfunction in heart failure following myocardial infarction in rats. J Mol Cell Cardiol 1998;30:1581–1593.
[4] Beuckelmann DJ, Nabauer M, Erdmann E. Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. Circulation 1992;85:1046–1055.
[5] Studer R, Reinecke H, Bilger J et al. Gene expression of the cardiac Na^+–Ca^{2+} exchanger in end-stage human heart failure. Circ Res 1994;75:443–453.
[6] Hasenfuss G, Reinecke H, Studer R et al. Relation between myocardial function and expression of sarcoplasmic reticulum Ca^{2+}-ATPase in failing and non-failing human myocardium. Circ Res 1994;75:434–442.
[7] Mercadier JJ, Lompre AM, Duc P et al. Altered sarcoplasmic reticulum Ca^{2+}-ATPase gene expression in the human ventricle during end-stage heart failure. J Clin Invest 1990;85:305–309.
[8] Takahashi T, Allen PD, Izumo S. Expression of A-, B-, and C-type natriuretic peptide genes in failing and developing human ventricles. Correlation with expression of the Ca^{2+}-ATPase gene. Circ Res 1992;71:9–17.
[9] Schwinger RH, Böhm M, Schmidt U et al. Unchanged protein levels of SERCA II and phospholamban but reduced Ca^{2+} uptake and Ca^{2+}-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with non-failing hearts. Circulation 1995;92:3220–3228.
[10] Zarain-Herzberg A, Afzal N, Elimban V, Dhalla NS. Decreased expression of cardiac sarcoplasmic reticulum Ca^{2+}-pump ATPase in congestive heart failure due to myocardial infarction. Mol Cell Biochem 1996;163–164:285–290.
[11] Movsesian MA, Karimi M, Green K, Jones LR. Ca^{2+}-transporting ATPase, phospholamban, and calsequestrin levels in non-failing and failing human myocardium. Circulation 1994;90:653–657.
[12] Schmidt U, Hajjar RJ, Kim CS, Lebeche D, Doye AA, Gwathmey JK. Human heart failure: cAMP stimulation of SR Ca^{2+}-ATPase activity and phosphorylation level of phospholamban. Am J Physiol 1999;277:H474–H480.
[13] Schwinger RH, Munch G, Bolck B, Karczewski P, Krause EG, Erdmann E. Reduced Ca^{2+}-sensitivity of SERCA 2a in failing human myocardium due to reduced serine[16] phospholamban phosphorylation. J Mol Cell Cardiol 1999;31:479–491.
[14] Huang B, Wang S, Qin D, Boutjdir M, El Sherif N. Diminished basal phosphorylation level of phospholamban in the postinfarction remodeled rat ventricle: role of β-adrenergic pathway, G_i protein, phosphodiesterase, and phosphatases. Circ Res 1999;85:848–855.
[15] Wegener AD, Simmerman HK, Lindemann JP, Jones LR. Phospholamban phosphorylation in intact ventricles. Phosphorylation of serine[16] and threonine[17] in response to β-adrenergic stimulation. J Biol Chem 1989;264:11468–11474, published erratum in J Biol Chem 1989;264(26):15738.
[16] Drago GA, Colyer J. Discrimination between two sites of phosphorylation on adjacent amino acids by phosphorylation site-specific antibodies to phospholamban. J Biol Chem 1994;269:25073–25077.
[17] MacDougall LK, Jones LR, Cohen P. Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. Eur J Biochem 1991;196:725–734.
[18] O'Rourke B, Kass DA, Tomaselli GF, Kaab S, Tunin R, Marban E. Mechanisms of altered excitation–contraction coupling in canine tachycardia-induced heart failure. I: experimental studies. Circ Res 1999;84:562–570.
[19] Wasserstrom JA, Holt E, Sjaastad I, Lunde PK, Ödegaard A, Sejersted OM. Altered E–C coupling in rat ventricular myocytes from failing hearts 6 weeks after MI. Am J Physiol Heart Circ Physiol 2000;279:H798–H807.
[20] Tønnessen T, Christensen G, Øie E et al. Increased cardiac expression of endothelin-1 mRNA in ischemic heart failure in rats. Cardiovasc Res 1997;33:601–610.
[21] Sjaastad I, Sejersted OM, Ilebekk A, Bjørnerheim R. Echocardiographic criteria for detection of postinfarction congestive heart failure in rats. J Appl Physiol 2000;89:1445–1454.
[22] Ploug T, Wojtaszewski J, Kristiansen S, Hespel P, Galbo H, Richter EA. Glucose transport and transporters in muscle giant vesicles: differential effects of insulin and contractions. Am J Physiol 1993;264:E270–E278.
[23] Semb SO, Lunde PK, Holt E, Tønnessen T, Christensen G, Sejersted OM. Reduced myocardial Na^+, K^+-pump capacity in congestive heart failure following myocardial infarction in rats. J Mol Cell Cardiol 1998;30:1311–1328.
[24] Holt E, Christensen G. Transient Ca^{2+} overload alters Ca^{2+} handling in rat cardiomyocytes: effects on shortening and relaxation. Am J Physiol 1997;273:H573–H582.
[25] Bers DM, Bridge JH. Relaxation of rabbit ventricular muscle by

Na^+–Ca^{2+} exchange and sarcoplasmic reticulum calcium pump. Ryanodine and voltage sensitivity. Circ Res 1989;65:334–342.
[26] Bers DM, Lederer WJ, Berlin JR. Intracellular Ca^{2+} transients in rat cardiac myocytes: role of Na^+–Ca^{2+} exchange in excitation–contraction coupling. Am J Physiol 1990;258:C944–C954.
[27] Bassani JW, Bassani RA, Bers DM. Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. J Physiol (Lond) 1994;476:279–293.
[28] Yao A, Su Z, Nonaka A et al. Abnormal myocyte Ca^{2+} homeostasis in rabbits with pacing-induced heart failure. Am J Physiol 1998;275:H1441–H1448.
[29] Netticadan T, Temsah RM, Kawabata K, Dhalla NS. Sarcoplasmic reticulum Ca^{2+}/calmodulin-dependent protein kinase is altered in heart failure. Circ Res 2000;86:596–605.
[30] Schwinger RH, Bolck B, Munch G, Brixius K, Muller-Ehmsen J, Erdmann E. cAMP-dependent protein kinase A-stimulated sarcoplasmic reticulum function in heart failure. Ann NY Acad Sci 1998;853:240–250.
[31] Bristow MR, Ginsburg R, Minobe W et al. Decreased catecholamine sensitivity and β-adrenergic-receptor density in failing human hearts. New Engl J Med 1982;307:205–211.
[32] Neumann J, Schmitz W, Scholz H, von Meyerinck L, Doring V, Kalmar P. Increase in myocardial G_i-proteins in heart failure. Lancet 1988;2:936–937.
[33] Zakhary DR, Moravec CS, Stewart RW, Bond M. Protein kinase A (PKA)-dependent troponin-I phosphorylation and PKA regulatory subunits are decreased in human dilated cardiomyopathy. Circulation 1999;99:505–510.
[34] Böhm M, Reiger B, Schwinger RH, Erdmann E. cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. Cardiovasc Res 1994;28:1713–1719.
[35] Lüss H, Klein-Wiele O, Boknik P et al. Regional expression of protein phosphatase type 1 and 2A catalytic subunit isoforms in the human heart. J Mol Cell Cardiol 2000;32:2349–2359.
[36] Neumann J, Boknik P, Herzig S et al. Evidence for physiological functions of protein phosphatases in the heart: evaluation with okadaic acid. Am J Physiol 1993;265:H257–H266.
[37] Boknik P, Fockenbrock M, Herzig S et al. Protein phosphatase activity is increased in a rat model of long-term β-adrenergic stimulation. Naunyn Schmiedebergs Arch Pharmacol 2000;362:222–231.
[38] Neumann J, Eschenhagen T, Jones LR et al. Increased expression of cardiac phosphatases in patients with end-stage heart failure. J Mol Cell Cardiol 1997;29:265–272.
[39] Currie S, Smith GL. Calcium/calmodulin-dependent protein kinase II activity is increased in sarcoplasmic reticulum from coronary artery ligated rabbit hearts. FEBS Lett 1999;459:244–248.
[40] Kirchhefer U, Schmitz W, Scholz H, Neumann J. Activity of cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase in failing and non-failing human hearts. Cardiovasc Res 1999;42:254–261.
[41] Hanada M, Kobayashi T, Ohnishi M et al. Selective suppression of stress-activated protein kinase pathway by protein phosphatase 2C in mammalian cells. FEBS Lett 1998;437:172–176.