Heart Failure

Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II and Protein Kinase A Differentially Regulate Sarcoplasmic Reticulum Ca\textsuperscript{2+} Leak in Human Cardiac Pathology

Thomas H. Fischer, MD; Jonas Herting, MS; Theodor Tirilomis, MD; André Renner, MD; Stefan Neef, MD; Karl Toischer, MD; David Ellenberger, DiplMath; Anna Förster, MD; Jan D. Schmitto, MD; Jan Gummert, MD; Friedrich A. Schöndube, MD; Gerd Hasenfuss, MD; Lars S. Maier, MD; Samuel Sossalla, MD

**Background**—Sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak through ryanodine receptor type 2 (RyR2) dysfunction is of major pathophysiological relevance in human heart failure (HF); however, mechanisms underlying progressive RyR2 dysregulation from cardiac hypertrophy to HF are still controversial.

**Methods and Results**—We investigated healthy control myocardium (n=5) and myocardium from patients with compensated hypertrophy (n=25) and HF (n=32). In hypertrophy, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA) both phosphorylated RyR2 at levels that were not different from healthy myocardium. Accordingly, inhibitors of these kinases reduced the SR Ca\textsuperscript{2+} leak. In HF, however, the SR Ca\textsuperscript{2+} leak was nearly doubled compared with hypertrophy, which led to reduced systolic Ca\textsuperscript{2+} transients, a depletion of SR Ca\textsuperscript{2+} storage and elevated diastolic Ca\textsuperscript{2+} levels. This was accompanied by a significantly increased CaMKII-dependent phosphorylation of RyR2. In contrast, PKA-dependent RyR2 phosphorylation was not increased in HF and was independent of previous β-blocker treatment. In HF, CaMKII inhibition but not inhibition of PKA yielded a reduction of the SR Ca\textsuperscript{2+} leak. Moreover, PKA inhibition further reduced SR Ca\textsuperscript{2+} load and systolic Ca\textsuperscript{2+} transients.

**Conclusions**—In human hypertrophy, both CaMKII and PKA functionally regulate RyR2 and may induce SR Ca\textsuperscript{2+} leak. In the transition from hypertrophy to HF, the diastolic Ca\textsuperscript{2+} leak increases and disturbed Ca\textsuperscript{2+} cycling occurs. This is associated with an increase in CaMKII- but not PKA-dependent RyR2 phosphorylation. CaMKII inhibition may thus reflect a promising therapeutic target for the treatment of arrhythmias and contractile dysfunction. (Circulation. 2013;128:970-981.)

Key Words: calcium–calmodulin-dependent protein kinase type 2 ■ heart failure ■ hypertrophy ■ protein kinases ■ sarcoplasmic reticulum

An increased diastolic ryanodine receptor type 2 (RyR2) Ca\textsuperscript{2+} leak is regarded as an important pathomechanism for the development of cardiac pump failure and arrhythmias in the failing heart.\textsuperscript{1,2} The diastolic loss of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) via spontaneously opening RyR2 clusters (Ca\textsuperscript{2+} sparks) leads to a depletion of SR Ca\textsuperscript{2+} storage and consecutively compromises systolic Ca\textsuperscript{2+} release. Additionally, leaky RyR2 is regarded as potently inducing proarrhythmic triggers\textsuperscript{3,4} through the following mechanism: The elimination of spontaneously released Ca\textsuperscript{2+} via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger during diastole causes a transient transsarcolemmal inward current and hence delayed afterdepolarizations as a substrate for cardiac arrhythmias.

**Clinical Perspective on p 981**

In animal models, protein kinase A (PKA) and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) have both been causally linked to a destabilization of diastolic RyR2 closure. The role of PKA in this context is controversial.\textsuperscript{5-9} In contrast, it has been shown in numerous animal models that increased CaMKII activity leads to RyR2 dysregulation.\textsuperscript{10-12

Received February 5, 2013; accepted June 25, 2013.


Dr Fischer and Mr Herting as first authors, and Drs Maier and Sossalla as last authors, contributed equally to this work, respectively.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.113.001746/-/DC1.

Correspondence to Samuel Sossalla, MD, Abteilung Kardiologie und Pneumologie/Herzzentrum, Georg-August-Universität Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany, E-mail ssossalla@med.uni-goettingen.de.

© 2013 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.113.001746
We have demonstrated recently that CaMKII inhibition can decrease diastolic SR Ca\(^{2+}\) leak in end-stage human heart failure (HF), thereby increasing contractility.\(^{13}\) It is, however, still unclear at what time point the SR Ca\(^{2+}\) leak appears in the development of human HF and what the specific underlying molecular mechanisms are. The functional alterations that precede HF and the respective contribution of both kinases in this context need to be elucidated.

Here, we explore the functional and molecular alterations in Ca\(^{2+}\) handling that accompany the disease progression from healthy (nonfailing, NF) to hypertrophied and end-stage failing (HF) human myocardium. We show that in hypertrophy, both PKA and CaMKII functionally modulate RyR2, whereas the pronounced increase of the diastolic SR Ca\(^{2+}\) leak in HF is dependent on CaMKII but independent of PKA activity. This may have clinical implications for the understanding and treatment of cardiac pump failure and arrhythmias in human myocardial disease.

**Methods**

**Human Myocardial Tissue**

All procedures were conducted in compliance with the local ethics committee, and written informed consent was received from all participants before inclusion.

**Heart Failure**

Left ventricular myocardial tissue was taken from explanted hearts of 32 patients (Table) with end-stage HF (New York Heart Association HF classification IV, mean ejection fraction 20.1±1.0%). The explanted hearts were acquired directly in the operating room during surgical procedures and immediately placed in precooled cardioplegic solution (in mmol/L: NaCl 110, KCl 16, MgCl\(_2\) 16, NaHCO\(_3\) 16, CaCl\(_2\) 1.2, glucose 11). Myocardial samples for Western blot analysis were frozen (−80°C) immediately after excision. The remaining heart tissue was stored for cell isolation in cooled cardioprotective solution containing (in mmol/L) Na\(^{+}\) 156, K\(^{+}\) 3.6, Cl\(^−\) 135, HCO\(_3\)\(^−\) 25, Mg\(^{2+}\) 0.6, HPO\(_4\)\(^{2−}\) 1.3, SO\(_4\)\(^{2−}\) 0.6, Ca\(^{2+}\) 2.5, glucose 11.2, 2,3-butanedimonoxime (BDM) 10, aerated with 95% O\(_2\) and 5% CO\(_2\). Control experiments showed that BDM did not act as chemical phosphatase under these conditions (Figure I in the online-only Data Supplement).

**Cardiac Hypertrophy**

Myocardial samples were obtained from 25 patients (Table) with severe aortic stenosis (mean aortic valve area 0.7±0.04 cm\(^2\)) undergoing aortic valve replacement. During surgery, a Morrow resection from the hypertrophied left ventricular septum was performed. Only patients without significant valvular regurgitation and with preserved ejection fraction (ejection fraction >50%, mean ejection fraction 58.1±1.0%) were included to ensure mere afterload-induced hypertrophy. Samples for Western blot analysis were frozen, and the remaining myocardium was kept for cell isolation in cardioplegic solution (in mmol/L: NaCl 110, KCl 16, MgCl\(_2\) 16, NaHCO\(_3\) 16, CaCl\(_2\) 1.2, glucose 11).

**NF Myocardium**

Frozen myocardial samples originated from 5 healthy donor hearts that could not be transplanted for technical reasons.

**Western Blots**

Left ventricular myocardium was homogenized in buffer containing (in mmol/L) Tris-HCl 20, pH 7.4, NaCl 200, NaF 20, Na\(_3\)VO\(_4\) 1, DTT 1, triton X-100 1%, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Denatured tissue (RyR2: 30 minutes, 37°C; troponin-I [TnI]: 5 minutes, 95°C) was subjected to Western blotting (4% to 15% gradient and 10% SDS polyacrylamide gels) with anti-TnI (1:1000; Cell Signaling Technology, Danvers, MA), anti-phospho-TnI Ser23/24 (1:1000; Cell Signaling Technology), anti-RyR2 (1:10000; Sigma-Aldrich, St Louis, MO), anti-phospho-RyR2 2809 (1:5000; Badrilla, Table.

**Characteristics and Echocardiographic Parameters of Patients Included in the 3 Different Study Groups**

<table>
<thead>
<tr>
<th></th>
<th>Hypertrophy (n=25)</th>
<th>HF (n=27)</th>
<th>HF Without β-Blockers (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>60.0</td>
<td>81.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Age, y</td>
<td>72.6±1.7</td>
<td>51.2±2.5</td>
<td>53.4±3.0</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>58.1±1.0</td>
<td>20.1±1.0</td>
<td>18.0±1.3</td>
</tr>
<tr>
<td>Interventricular septum, mm</td>
<td>15.2±0.5</td>
<td>8.6±0.5</td>
<td>6.0±0.7</td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>44.4±2.4</td>
<td>68.7±2.5</td>
<td>72.3±5.9</td>
</tr>
<tr>
<td>Aortic valve area, cm(^2)</td>
<td>0.7±0.04</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean AV pressure gradient, mmHg</td>
<td>53.5±4.4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>37.5</td>
<td>69.6</td>
<td>60.0</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>70.8</td>
<td>91.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Diuretics</td>
<td>65.2</td>
<td>91.3</td>
<td>80.0</td>
</tr>
<tr>
<td>Digoxin</td>
<td>4.3</td>
<td>30.4</td>
<td>20.0</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>4.3</td>
<td>34.8</td>
<td>80.0</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>4.3</td>
<td>26.1</td>
<td>40.0</td>
</tr>
<tr>
<td>AT1 receptor antagonists</td>
<td>13.0</td>
<td>13.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Aldosterone antagonists</td>
<td>0.0</td>
<td>69.6</td>
<td>40.0</td>
</tr>
<tr>
<td>PDE inhibitors</td>
<td>0.0</td>
<td>39.1</td>
<td>80.0</td>
</tr>
<tr>
<td>Ca(^{2+}) channel blockers</td>
<td>34.8</td>
<td>0.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM or percentages. Regarding echocardiographic parameters and medication, only available data could be included for each patient. ACE indicates angiotensin-converting enzyme; AT1, angiotensin II receptor, type 1; AV, aortic valve; HF, heart failure; LV, left ventricular; N/A, not applicable; and PDE, phosphodiesterase.
Leeds, United Kingdom), anti-phospho-RyR2 Ser2815 (1:5000; Badrilla), and anti-GAPDH (1:20000; Biotrend Chemikalien GmbH, Köln, Germany) antibodies. Chemiluminescent detection was performed with Immobilon Western (Millipore, Billerica, MA). For TnI Western blotting, calceinAM was used for normalization instead of GAPDH, because GAPDH and TnI have similar molecular weights. Phosphorylation values were normalized to protein expression.

Myocyte Isolation

Left ventricular myocardium was rinsed, cut into small pieces, and incubated at 37°C in a spinner flask filled with Joklik-MEM solution (JMEM; AppliChem, Darmstadt, Germany) that contained 1.0 mg/mL collagenase (Worthington type 1, 185 U/mg, CellSystems, Troisdorf, Germany) and 15% trypsin (Life Technologies, Carlsbad, CA). After 45 minutes, the supernatant was discarded, and fresh JMEM solution that contained only collagenase was added. The solution was incubated for 10 to 20 minutes until myocytes were disaggregated by use of a Pasteur pipette. The supernatant that contained disaggregated cells was removed and centrifuged (600 rpm, 3 min). Fresh JMEM with collagenase was added to the remaining tissue. This procedure was repeated 4 to 5 times. After every step, the centrifuged cells were resuspended in KB medium containing (in mmol/L) taurine 10, glutamic acid 70, KCl 25, KH2PO4 10, dextrose 22, EGTA 0.5, bovine calve serum 10% (pH 7.4, KOH, room temperature). Only cell solutions that contained elongated, not granulated cardiomyocytes with cross-striations were selected for experiments, plated on laminin-coated recording chambers, and left to settle for 1 hour.

Intracellular Ca2+ Imaging

Confocal Microscopy (Measurement of SR Ca2+ Sparks)

Isolated cardiomyocytes were incubated at room temperature for 30 minutes with a Fluo-3 AM loading buffer (10 μmol/L; Molecular Probes, Life Technologies, Carlsbad, CA) that also contained either the PKA inhibitor H89 (5 μmol/L), the CaMKII inhibitor autocamide-2–related inhibitory peptide (AIP; 1 μmol/L; Alexis Corp, Lausen, Switzerland), or no inhibitor in the control group. AIP was used in its myristoylated form to ensure cell permeability. Experimental solution contained (in mmol/L) NaCl 136, KCl 4, NaH2PO4 0.33, NaHCO3 4, CaCl2 2, MgCl2 1.6, HEPES 10, glucose 10 (pH 7.4, NaOH, room temperature), as well as 10 mmol/L isoproterenol and the respective inhibitors. Cells were continuously superfused during experiments. To wash out the loading buffer and remove any extracellular dye, as well as to allow enough time for complete deesterification of Fluo-3 AM, cells were superfused with experimental solution for 5 minutes before experiments were begun. Ca2+ spark measurements were performed with a laser scanning confocal microscope (LSM 5 Pascal, Zeiss, Jena, Germany) using a 40× oil-immersion objective. Fluo-3 was excited by an argon ion laser (488 nm), and emitted fluorescence was collected at a 505-nm long-pass emission filter. Fluorescence images were recorded in the line-scan mode with 512 pixels per line (width of each scan line: 38.4 μm) and a pixel time of 0.64 μs. One image consists of 10000 unidirectional line scans, which equates to a measurement period of 7.68 seconds. Experiments were conducted at resting conditions after the SR was loaded with Ca2+ by repetitive field stimulation (1 Hz, 20 V). Ca2+ sparks were analyzed with the program SparkMaster for ImageJ. The mean frequency of the respective cell resulted from the number of sparks normalized to cell width and scan rate (100 μm s⁻¹). Spark size was calculated as the product of spark amplitude (F/F0), duration, and width. From this, we inferred the average leak per cell by multiplication of spark size and spark frequency.

Epifluorescence Microscopy (Systolic Ca2+ Transients and SR Ca2+ Content)

Cardiomyocytes were isolated and plated as described above and incubated with a Fura-2 AM loading buffer (10 μmol/L; Molecular Probes) for 15 minutes. In the inhibitor groups, the loading buffer also contained H89 (5 μmol/L) or AIP (1 μmol/L), respectively. After staining, the cardiomyocytes were superfused with experimental solution (as described in Confocal Microscopy) for 5 minutes before measurements were begun to enable complete deesterification of intracellular Fura-2 and allow cellular rebalancing of Ca2+-cycling properties. During measurements, cardiomyocytes were continuously superfused with experimental solution. Measurements were performed with a Nikon Eclipse TE2000-U microscope equipped with a fluorescence detection system (IonOptix, Milton, MA). Cells were excited at 340 and 380 nm, and the emitted fluorescence was collected at 510 nm. The intracellular Ca2+ level was measured as the ratio of fluorescence at 340 and 380 nm (F340 nm/F380 nm, in ratio units). Systolic Ca2+ transients were recorded in steady-state conditions under constant field stimulation (0.5 Hz, 20 V). To assess SR Ca2+ content, we measured the amplitude of caffeine-induced Ca2+ transients. Two seconds after the stimulation was stopped during steady-state conditions, caffeine (10 μmol/L) was applied directly onto the cell leading to an immediate and complete SR Ca2+ release. The recorded Ca2+ transients were analyzed with the software IONWizard (IonOptix).

Statistical Analysis

All descriptive statistics and figures are presented as frequencies or means±SEM. Student t test was used for analysis of Western blots and epifluorescence data because the variance that was explained by the intrapatient correlation was relatively small compared with the total variance between different cells. For confocal measurements, statistical testing was performed according to a repeated-measures ANOVA to account for the clustering of measurements within a single patient. Because of the exchangeability of measurements, a compound symmetry covariance structure was assumed. When data were highly skewed along with a frequent occurrence of zeros because of nonsparking of cells, a nonparametric rank-based repeated-measures ANOVA was used. For analysis of the proportion of sparking cells, a logistic regression was performed analogously that accounted for the clustering of measurements. Analyses were made with GraphPad Prism or SAS 9.3 (PROC MIXED, PROC GLIMMIX), and P<0.05 was considered statistically significant.

Results

Ca2+ Handling in Compensated Hypertrophy and End-Stage HF

Ca2+-Handling Proteins

We performed Western blot analysis from tissue samples of patients with compensated afterload-induced cardiac hypertrophy and end-stage HF and compared them to NF myocardium to assess alterations in the expression and phosphorylation of key Ca2+-handling proteins. The characteristics of and mediators used by patients included in the respective groups are specified in the Table. We detected an increased expression of RyR2 protein by 95±24% in hypertrophy compared with NF myocardium (P<0.05; n=12 versus 5; Figure 1A). Neither PKA-dependent phosphorylation of RyR2 at Ser2809 (84±7%, n=12 versus 5, P=0.36; Figure 1B) nor CaMKII-dependent phosphorylation at Ser2815 (113±16%, n=12 versus 5, P=0.66; Figure 1C) was significantly altered when normalized to RyR2 protein expression. In contrast, in HF we found an unaltered RyR2 expression (89±9%, n=8 versus 4, P=0.56; Figure 1D) but a pronounced hyperphosphorylation at Ser2815 by 311±72% (P<0.05, n=8 versus 4; Figure 1F) compared with NF. The PKA-dependent site Ser2809, again,
lacked differential regulation (123±12%, n=4 versus 8, P=0.24; Figure 1E). Because the majority of HF patients received β-blocker medication, an iatrogenic modification of PKA-dependent target phosphorylation could not be excluded. We therefore additionally identified HF patients who had not received β-blockers before transplantation because of comorbidities or decompensation and compared them with HF patients taking β-blocker medication. Interestingly, we could not detect significant differences as to PKA-dependent RyR2 phosphorylation at Ser2809 (110±11%, n=4 versus 7, P=0.68; Figure 1G). To evaluate PKA activity in other subcellular compartments, we analyzed TnI phosphorylation at Ser23/24. We found an unaltered TnI protein expression in HF compared with NF (95±8%, n=8 versus 4; Figure 1H) but a decreased amount of TnI phosphorylated at Ser23/24 taking β-blocker medication.

Figure 1. Protein expression and phosphorylation of ryanodine receptor type 2 (RyR2) in cardiac hypertrophy (Hy) and heart failure (HF), influences of β-blocker (β-B) medication on phosphorylation of RyR2, and further protein kinase A (PKA) targets in HF compared with healthy myocardium (NF). Each panel shows representative Western blots (right) and the respective quantification (left). A, RyR2 expression normalized to GAPDH. RyR2 was higher expressed in Hy than in NF myocardium (n=12 vs 5; P<0.05). B, RyR2 phosphorylation at Ser2809 normalized to RyR2 expression. No significant regulation in Hy vs NF could be detected (n=12 vs 5). C, RyR2 phosphorylation at Ser2815 normalized to RyR2 expression; there was no significant regulation in Hy vs NF (n=12 vs 5). D, RyR2 expression normalized to GAPDH. RyR2 expression was not significantly altered in HF compared with NF (n=8 vs 4). E, RyR2 phosphorylation at Ser2809 normalized to RyR2 expression. No significant regulation in HF vs NF could be detected (n=8 vs 4). F, RyR2 phosphorylation at Ser2815 normalized to RyR2 expression. RyR2 was markedly hyperphosphorylated in HF vs NF (n=8 vs 4; P<0.05). G, Phosphorylation of RyR2 at Ser2809 normalized to RyR2 expression according to β-blocker medication. No significant difference was detected between HF patients with (β-B) and without (−) β-blocker medication (n=7 vs 4). H, Expression of troponin I (TnI) normalized to calsequestrin (Calsequ). TnI expression did not significantly differ in HF vs NF (n=8 vs 4). I, TnI phosphorylation at Ser23/24 normalized to calsequestrin. The amount of TnI phosphorylated at Ser23/24 is decreased in HF vs NF (n=7 vs 4; P<0.05).
by 48±9%, n=8 versus 4, P<0.05; Figure 1I), which indicates decreased PKA activity or increased phosphatase activity in this microdomain.

**Systolic Ca\(^{2+}\) Release and SR Ca\(^{2+}\) Content in Hypertrophy and HF**

To functionally investigate Ca\(^{2+}\) handling, we freshly isolated cardiomyocytes from both groups of patients and performed epifluorescence measurements (Fura-2 AM). Cardiomyocytes were stimulated at 0.5 Hz, and systolic Ca\(^{2+}\) transients were recorded (Figure 2A). Additionally, caffeine was applied to quantify SR Ca\(^{2+}\) content (Figure 2D). The amplitudes of systolic Ca\(^{2+}\) transients were reduced by 31±6% in HF compared with hypertrophy (F\(_{340}/F_{380}\): 0.71±0.03 versus 0.53±0.05, P<0.01; n=53/4 versus 15/6; Figure 2C). We also found decreased caffeine-induced Ca\(^{2+}\) transients by 26±6% in HF (F\(_{340}/F_{380}\): 0.25±0.02 versus 0.34±0.02, P<0.05, n=26/5 versus 11/5; Figure 2E), which suggests a reduced SR Ca\(^{2+}\) content.

**Diastolic SR Ca\(^{2+}\) Leak in Compensated Hypertrophy and HF**

We then addressed the diastolic SR Ca\(^{2+}\) leak using confocal microscopy (Fluo-3 AM). We paced the cardiomyocytes at 1 Hz for 10 beats and then scanned for diastolic Ca\(^{2+}\) sparks. In HF, the fraction of sparking cells was increased significantly compared with hypertrophy (n=63 of 103 versus 37 of 107, P<0.01; Figure 3B), which translated into an increase in Ca\(^{2+}\) spark frequency by 100±26% (1.46±0.19 versus 0.73±0.14 100 μm\(^{-1}\)·s\(^{-1}\), P<0.05; number of cells/patients=103/11 versus 107/10; Figure 3B). Furthermore, we detected a slight increase in spark amplitude by 5±1% (P=0.05, n=352/11 versus 186/9; Figure 3C) and maximal Ca\(^{2+}\) release flux by 21±3% (P<0.05, n=351/11 versus 186/9; Figure 3D) in HF compared with hypertrophy. Width (3.30±0.08 versus 3.18±0.12 μm, n=352/11 versus 186/9, P=0.41) and duration (50.00±1.94 versus 47.99±3.08 ms, n=352/11 versus 186/9, P=0.56) of the detected sparks were not significantly different between the 2 groups. Interestingly, we found a nearly 2-fold increase of the total calculated SR Ca\(^{2+}\) leak (by 93±38%, P<0.05, n=103/11 versus 107/10; Figure 3E) in HF compared with compensated hypertrophy despite the significantly lower SR Ca\(^{2+}\) load (Figure 2E).

**Influences of PKA and CaMKII on Ca\(^{2+}\) Homeostasis in Compensated Cardiac Hypertrophy**

To investigate the functional role of PKA and CaMKII in RyR2 regulation, we used the kinase inhibitors H89 and AIP and evaluated the resulting effects on Ca\(^{2+}\)-cycling parameters. The efficiency of PKA inhibition by H89 was verified by Western blot analysis (Figure II in the online-only Data Supplement). We first inhibited the respective kinase in cardiomyocytes isolated from cardiac hypertrophy and performed epifluorescence measurements. Specific inhibition of both CaMKII and PKA caused a reduction of the systolic Ca\(^{2+}\) transient amplitude (0.5 Hz) by 42±6% (AIP, F\(_{340}/F_{380}\): 0.14±0.02 versus 0.24±0.03, P<0.01; number of cells/patients=8/3 versus 8/4; Figure 4B) and 36±7% (H89, F\(_{340}/F_{380}\): 0.15±0.02 versus 0.24±0.03, P<0.05; n=6/3 versus

![Figure 2](http://circ.ahajournals.org/content/full/138/13/2565/F2.large.jpg)
8/4; Figure 4B) compared with untreated control myocytes. There was a tendency toward higher diastolic Ca$^{2+}$ concentrations after PKA inhibition (F$_{340}$/F$_{380}$ 0.60±0.07 versus 0.50±0.04; n=6/3 versus 14/6, P<0.20) and CaMKII inhibition (0.56±0.05 versus 0.50±0.06; n=13/4 versus 14/6, P=0.31; Figure 4C). Additionally, there was a tendency
toward slower Ca²⁺-elimination kinetics (half-maximal relaxation time) with either of the 2 inhibitors (control versus H89 versus AIP; 0.46±0.04 versus 0.52±0.05 versus 0.51±0.07 seconds, \( P<0.37 \) for H89; Figure 4D). CaMKII inhibition significantly reduced SR Ca²⁺ load in compensated hypertrophy as measured by caffeine-induced Ca²⁺ transients (\( F_{340/F380} \) 0.22±0.03 versus 0.34±0.02; \( n=7/4 \) versus 11/5, \( P<0.01 \); Figure 4F). Inhibition of PKA by H89, however, did not significantly reduce the SR Ca²⁺ load (\( F_{340/F380} \) 0.32±0.04 versus 0.34±0.02; \( n=9/3 \) versus 11/5, \( P=0.70 \); Figure 4F).

We then investigated the effects of PKA and CaMKII inhibition on the diastolic SR Ca²⁺ leak in hypertrophy. Inhibition of CaMKII yielded a reduction of the Ca²⁺ spark frequency by 46±12% compared with untreated control (spark frequency; 0.41±0.09 versus 0.76±0.15 100 \( \mu \text{m} \text{·s}^{-1} \), \( P<0.05 \); number of cells/patients=100/9 versus 91/9; Figure 5B). CaMKII inhibition reduced the total calculated SR Ca²⁺ leak by 62±14% (124.7±44.2 versus 325.3±94.1, \( P=0.05 \); \( n=100/9 \) versus 91/9; Figure 5D). AIP treatment did not influence Ca²⁺ spark amplitude (1.63±0.02 versus 1.64±0.02; number of sparks/patients=95/9 versus 162/8; \( P=0.82 \); Figure 5C), width (2.87±0.14 versus 3.24±0.13 \( \mu \text{m} \), \( n=95/9 \) versus 162/8; \( P=0.07 \)), or duration (46.70±3.54 versus 49.41±3.33 ms; \( n=95/9 \) versus 162/8; \( P=0.19 \)). Similar results were obtained when PKA was inhibited with H89. We detected a reduced spark frequency of 0.38±0.08 100 \( \mu \text{m} \text{·s}^{-1} \) in H89-treated myocytes compared with 0.68±0.14 100 \( \mu \text{m} \text{·s}^{-1} \) in control (\( n=129/9 \) versus 101/9, \( P<0.05 \); Figure 6B) and a significant reduction of the calculated SR Ca²⁺ leak by 61±12% (\( P<0.05 \); \( n=129/9 \) versus 101/9; Figure 6D), with no significant influence on Ca²⁺ spark amplitude (1.63±0.02 versus 1.64±0.02, \( P=0.77 \); Figure 6C), width (3.21±0.13 versus 2.87±0.12 \( \mu \text{m} \), \( P=0.07 \)), or duration (49.1±3.3 versus 40.9±2.9 ms, \( P=0.08 \)).

**Influences of PKA and CaMKII on Ca²⁺ Homeostasis in HF**

We have recently shown that inhibition of CaMKII exerts a beneficial effect on Ca²⁺-cycling properties in human end-stage HF. On CaMKII inhibition, the diastolic SR Ca²⁺ leak was found to be reduced, which led to a higher SR Ca²⁺ load and improved contractility. We confirmed these data in the course of the present study and again could show that the inhibition of CaMKII by AIP in human HF yields a reduction of the diastolic Ca²⁺ spark frequency by 50±15% compared with untreated control (Figure IIIA and IIIB in the online-only Data Supplement; \( P<0.05 \)). To evaluate the role of PKA in this context, we used H89 and measured the respective effects on Ca²⁺ handling. In contrast to CaMKII inhibition, PKA inhibition did not effectively restore the severely compromised Ca²⁺ cycling in myocytes from patients with HF. We even found a decrease of systolic Ca²⁺ transients from 0.16±0.02 to 0.11±0.01 \( F_{340/F380} \) in the presence of H89 at 0.5 Hz (\( P<0.05 \); number of cells/patients=30/4 versus 43/4; Figure 7B) and a tendency toward slower Ca²⁺ elimination (half-maximal relaxation time 0.63±0.04 versus 0.54±0.04 seconds, \( P=0.11 \); \( n=30/4 \) versus 43/4; Figure 7D). The caffeine-induced SR Ca²⁺ transient was also reduced to 0.19±0.02 \( F_{340/F380} \) after PKA inhibition compared with 0.24±0.02 \( F_{340/F380} \) in control myocytes (\( P<0.05 \); \( n=25/5 \) versus 27/5; Figure 7F). The diastolic Ca²⁺-cycling properties of the failing myocardium (by 62±14%; \( n=100/9 \) versus 91/9, \( P<0.05 \)) and improved contractility. We confirmed these data in the course of the present study and again could show that the inhibition of CaMKII by AIP in human HF yields a reduction of the diastolic Ca²⁺ spark frequency by 50±15% compared with untreated control (Figure IIIA and IIIB in the online-only Data Supplement; \( P<0.05 \)). To evaluate the role of PKA in this context, we used H89 and measured the respective effects on Ca²⁺ handling. In contrast to CaMKII inhibition, PKA inhibition did not effectively restore the severely compromised Ca²⁺ cycling in myocytes from patients with HF. We even found a decrease of systolic Ca²⁺ transients from 0.16±0.02 to 0.11±0.01 \( F_{340/F380} \) in the presence of H89 at 0.5 Hz (\( P<0.05 \); number of cells/patients=30/4 versus 43/4; Figure 7B) and a tendency toward slower Ca²⁺ elimination (half-maximal relaxation time 0.63±0.04 versus 0.54±0.04 seconds, \( P=0.11 \); \( n=30/4 \) versus 43/4; Figure 7D). The caffeine-induced SR Ca²⁺ transient was also reduced to 0.19±0.02 \( F_{340/F380} \) after PKA inhibition compared with 0.24±0.02 \( F_{340/F380} \) in control myocytes (\( P<0.05 \); \( n=25/5 \) versus 27/5; Figure 7F). The diastolic Ca²⁺-cycling properties of the failing myocardium (by 62±14%; \( n=100/9 \) versus 91/9, \( P<0.05 \)).
Fura ratio was decreased significantly under H89 treatment (F340/F380: 0.62±0.03 versus 0.71±0.03, P<0.05; n=34/4 versus 53/4; Figure 7C).

Furthermore, inhibition of PKA by H89 did not significantly modulate the diastolic SR Ca2+ leak in HF (H89 versus control: 456.3±124.5 versus 454±102.7, P=0.95; number of cells/patients=74/7 versus 85/7; Figure 8D). Ca2+ spark frequency (1.28±0.21 versus 1.29±0.2 100 μm-1*s-1, P=0.85; n=74/7 versus 85/7; Figure 8B), Ca2+ spark amplitude (1.66±0.02 versus 1.64±0.01, P=0.48; Figure 8C), duration (47.66±2.17 versus 46.80±2.23 ms, P=0.78) and width (3.23±0.11 versus 3.17±0.10 μm, P=0.70) were unaltered (each n=218/7 versus 258/7).

Discussion

To the best of our knowledge, this is the first study that functionally compares the 2 clinically relevant human cardiac pathologies of myocardial hypertrophy and HF with regard to the inherent alterations of Ca2+ handling and underlying regulatory activities of protein kinases, with the following major findings: (1) SR Ca2+ leak increases from human cardiac hypertrophy to HF and is accompanied by profound disturbances of excitation-contraction coupling. The transition from hypertrophy to HF is associated with a significant increase in the CaMKII-dependent phosphorylation of RyR2 at Ser2815, and CaMKII inhibition conclusively reduces the SR Ca2+ leak. (2) The transition from cardiac hypertrophy to HF is not associated with increased phosphorylation of the PKA-dependent site Ser2809, and PKA inhibition does not reduce the SR Ca2+ leak in HF. (3) In cardiac hypertrophy, CaMKII and PKA phosphorylate the RyR2 to an extent that does not differ significantly from healthy control myocardium and an inhibition of either of the 2 kinases reduces the basal SR Ca2+ leak.

Ca2+ Homeostasis in Compensated Hypertrophy and HF

We showed that the diastolic SR Ca2+ leak is nearly doubled in HF compared with hypertrophy despite a reduced SR Ca2+ load, which suggests a severely compromised diastolic RyR2 closure in HF. This leads to decreased systolic Ca2+ transients and an increase in diastolic Ca2+ levels. Interestingly, the progression of SR Ca2+ leak occurs although RyR2 protein expression is higher in hypertrophy than in HF, which suggests an even bigger SR Ca2+ leak per RyR2 in HF than the cumulative values indicate. It was postulated previously
that there is a positive correlation between left ventricular ejection fraction and SR Ca\textsuperscript{2+} load and that the SR Ca\textsuperscript{2+} load is decreased in human HF.\textsuperscript{15} The present data support this hypothesis and additionally suggest that an increased diastolic SR Ca\textsuperscript{2+} leak significantly contributes to the depletion of SR Ca\textsuperscript{2+} storage in human HF. Furthermore, the present results are in line with studies in animal models that showed an elevated diastolic SR Ca\textsuperscript{2+} leak in HF\textsuperscript{10,16–19} and thus contradict a previous study performed in human tissue that proposed that the frequency of Ca\textsuperscript{2+} sparks is significantly decreased in human HF compared with NF, rendering a causal involvement of the SR Ca\textsuperscript{2+} leak in the depletion of SR Ca\textsuperscript{2+} storage unlikely.\textsuperscript{20} Moreover, the increased maximal Ca\textsuperscript{2+} release flux of Ca\textsuperscript{2+} sparks in HF despite a significantly lower driving force (lower SR Ca\textsuperscript{2+} load, higher cytosolic Ca\textsuperscript{2+} concentration) demonstrates the dimension of RyR2 dysfunction in HF.

The present data further suggest that CaMKII-dependent hyperphosphorylation of RyR2 at Ser2815 may be the key factor evoking RyR2 dysfunction in human HF. We found a >4-fold increase of RyR2 phosphorylation in HF compared with compensated hypertrophy. Hyperphosphorylation of RyR2 via CaMKII phosphorylation has been widely shown to increase diastolic SR Ca\textsuperscript{2+} leak.\textsuperscript{4,10,11,13,21,22} The present data thus suggest that an increased activity of CaMKII sets in during disease progression and is responsible for the deterioration of left ventricular function. Moreover, the present findings support a recent study performed in a mouse model of cardiac hypertrophy and HF (transverse aortic constriction) that showed that CaMKII knockout mice equally developed cardiac hypertrophy after transverse aortic constriction surgery but were protected against the transition to HF.\textsuperscript{23} Importantly, the present data clearly show that in human failing myocardium, PKA-dependent RyR2 modification is not mechanistically relevant, RyR2-Ser2809 phosphorylation is not increased, and PKA inhibition does not reduce the SR Ca\textsuperscript{2+} leak. PKA-dependent TnI-Ser23/24 phosphorylation was even decreased significantly in HF. When we compared HF patients with and without β-blocker medication with regard to RyR2 phosphorylation, we could exclude that an intrinsically increased PKA activity would be masked iatrogenically.

As a consequence, the increased SR Ca\textsuperscript{2+} leak causes systolic contractile dysfunction but may also impair late diastolic relaxation because of an increased diastolic Ca\textsuperscript{2+} concentration, as was found in the present study. Moreover, an elevated SR Ca\textsuperscript{2+} leak can induce delayed afterdepolarizations, rendering the failing heart more prone to severe arrhythmias, particularly in the presence of reduced SERCA2a expression and an increased expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.\textsuperscript{24–26}

**Figure 8.** Effects of protein kinase A inhibition on the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak in heart failure. A. Representative confocal line scans of cardiomyocytes, with and without H89 treatment. Inhibition of protein kinase A in heart failure had no effect on Ca\textsuperscript{2+} spark frequency (B; number of cells/patients=74/7 vs 85/7, P=0.88) or spark amplitude (C; number of sparks/patients=218/7 vs 258/7, P=0.48), which led to an unchanged SR Ca\textsuperscript{2+} leak (D; CaSpF=xamplitude×duration×width, normalized to control group; n=74/7 vs 85/7, P=0.95).

**Relevance of CaMKII and PKA for RyR2 in Compensated Hypertrophy and HF**

To investigate the mechanisms underlying RyR2 regulation in both groups of patients, we modulated the respective kinase activity by suitable compounds, ie H89 for inhibition of PKA and AIP for inhibition of CaMKII, both of which are widely used for this purpose.\textsuperscript{13,17,23} In cardiac hypertrophy with preserved ejection fraction, SR Ca\textsuperscript{2+} leak could be reduced by both H89 and AIP. This implies that PKA and CaMKII are involved in RyR2 regulation in cardiac hypertrophy. The fact that both inhibitors reduced systolic Ca\textsuperscript{2+} transients suggests a physiologically important role of both kinases for the maintenance of sufficient systolic Ca\textsuperscript{2+} release in cardiac hypertrophy with preserved Ca\textsuperscript{2+}-cycling properties. Accordingly, it was shown in a mouse model that increased afterload (transverse aortic constriction) leads to CaMKII activation, which increases fractional systolic Ca\textsuperscript{2+} release.\textsuperscript{23} Another study showed that phosphorylation of RyR2 by CaMKII at Ser2814 may play an important role for a positive force-frequency relation, because this was blunted in healthy mice that harbored a loss-of-function mutation (S2814A) of RyR2 at this site.\textsuperscript{28} In addition, reduced phospholamban phosphorylation with increased SERCA inhibition may be involved. Thus, therapeutic CaMKII inhibition and PKA inhibition in cardiac hypertrophy with preserved contractility would most likely compromise inotropy. This is not the case in HF; previous reports showed that both transgenic and acute adenoviral overexpression of CaMKII were associated with reduced SR Ca\textsuperscript{2+} load caused by an increased SR Ca\textsuperscript{2+} leak.\textsuperscript{10,11} This was associated with impaired contractility and with activation of a transient inward current via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which caused delayed afterdepolarizations and triggered arrhythmias. Furthermore, disturbed Ca\textsuperscript{2+} cycling and contractile dysfunction could be prevented by inhibition of CaMKII.\textsuperscript{10,11,17} Therefore, inhibition of CaMKII could be a promising new approach for antiarrhythmic and positive inotropic therapy in HF.
As there is ongoing debate about the role of PKA-dependent RyR2 phosphorylation, we directly assessed the effect of PKA activity in human end-stage HF. Importantly, in failing human cardiomyocytes, acute PKA inhibition did not influence RyR2 Ca\textsuperscript{2+} leak. We even detected lower systolic Ca\textsuperscript{2+} transients, a decreased SR Ca\textsuperscript{2+} load, and a deceleration of diastolic Ca\textsuperscript{2+} elimination after PKA inhibition, which can be explained by reduced PKA-dependent phospholamban phosphorylation and thus an increased inhibitory effect of phospholamban on SERCA2a activity. No beneficial effect of PKA inhibition on Ca\textsuperscript{2+} homeostasis in human end-stage HF was detectable. The present data thus indicate that in human HF, PKA-dependent RyR2 modification is not mechanistically relevant. The present study contradicts the previously described PKA-dependent hyperphosphorylation of RyR2 in human HF\textsuperscript{3} and confirms a recent study showing that RyR2 is hyperphosphorylated at Ser2815 but not Ser2809 in human nonischemic HF.\textsuperscript{29} Our results thus indicate that PKA is not causally involved in RyR2 dysregulation in human HF and contradicts several studies performed in animal models.\textsuperscript{5,6,30} On the other hand, the present findings are in line with other previously published data: It was shown in an arrhythmogenic rabbit model of nonischemic HF that CaMKII inhibition but not PKA inhibition can significantly reduce SR Ca\textsuperscript{2+} leak.\textsuperscript{17} Additionally, more recent studies showed that RyR2-Ser2808 mutated mice lacking the PKA-dependent phosphorylation site did not differ from wild-type littermates with regard to global cardiac function and Ca\textsuperscript{2+}-cycling parameters after myocardial infarction\textsuperscript{9} and showed an intact β-adrenergic response and an unmodified progression toward HF after transverse aortic constriction.\textsuperscript{7} A significant impact of RyR-S2808A mutation on the development of an increased diastolic SR Ca\textsuperscript{2+} leak could not be detected.

The discrepancies discussed above may be related to methodological differences on the one hand and species differences on the other. We believe that the present study overcomes these weaknesses for the following reasons: (1) We compare healthy control with compensated hypertrophy and HF and show a transition from one disease state to the other, not only from healthy to diseased myocardium. The data clearly show that the present analysis is sensitive enough to detect changes and makes pseudodifferences that originate from dissimilar treatments of control and diseased myocardium. The data clearly show that the present analysis is sensitive enough to detect changes and makes pseudodifferences that originate from dissimilar treatments of control and diseased myocardium unlikely. (2) We exclusively use human myocardium to overcome problems related to transferability of animal to human pathophysiology and to realistically depict the human HF population. (3) We use functional and biochemical measurements in the same tissue and show consistent changes in the present study. Therefore, we believe that the present findings add important new mechanistic information regarding RyR2 phosphorylation and Ca\textsuperscript{2+} handling across disease severities from healthy control over compensated hypertrophy to HF in human tissue.

**Study Limitations**

The present study has several limitations. (1) Studies with explanted human tissue could be limited because of longer periods of ischemic arrest than with animal tissue, which might affect the posttranslational modification of proteins. We therefore tried to minimize ischemic damage in the present study by ensuring continuous cooling and the use of BDM (Methods). (2) The functional data of the present study are derived from short-term experiments investigating acute effects of modifications of kinase activities and may thus be limited as to conclusions regarding long-term effects of protein kinase inhibition. (3) As we did not have access to vital healthy human myocardium, we were not able to functionally investigate potential differences of Ca\textsuperscript{2+}-cycling properties between hypertrophy and NF. Our Western blot data, however, did not show any RyR2 hyperphosphorylation in hypertrophy compared with NF and thus suggest that a disruption of diastolic RyR2 closure may not yet be present at this stage of cardiac disease. (4) Subgroup analysis of ischemic and nonischemic HF did not yield any relevant differences as to RyR2 phosphorylation and Ca\textsuperscript{2+} cycling parameters but was limited because of the small number of patients with ischemic HF. (5) There is evidence in the literature that diastolic Ca\textsuperscript{2+} loss from the SR also occurs as non–spark-related continuous percolation of Ca\textsuperscript{2+} into the cytoplasm (quarky Ca\textsuperscript{2+} release).\textsuperscript{31} Although we did not directly measure quarky Ca\textsuperscript{2+} release in the present study, this would most likely not change its significance, because currently available data suggest that quarky Ca\textsuperscript{2+} release coexists with Ca\textsuperscript{2+} spark–mediated SR-Ca\textsuperscript{2+} leak and that both forms of diastolic Ca\textsuperscript{2+} loss are most likely not altered independently in certain cell types and diseases.\textsuperscript{32,33} (6) In addition to Ser2809 and Ser2815, there have been some studies performed in animal models (mouse, rat, rabbit) claiming that Ser2830 is likely the phosphorylation site that in human HF, PKA-dependent RyR2 modification is not mechanistically relevant. The present study contradicts the previously described PKA-dependent hyperphosphorylation of RyR2 in human HF and confirms a recent study showing that RyR2 is hyperphosphorylated at Ser2815 but not Ser2809 in human nonischemic HF.\textsuperscript{29} Our results thus indicate that PKA does not functionally regulate RyR2 gating in human hypertrophy but appears to have lost the ability to modulate RyR2 gating in human HF. Because SR Ca\textsuperscript{2+} leak impairs contractility and induces arrhythmias, inhibition of CaMKII in contrast to PKA may serve as a promising new target for future therapies to improve disturbed SR Ca\textsuperscript{2+} handling in HF.

**Acknowledgments**

We gratefully acknowledge the technical assistance of F. Steuer, T. Schulte, T. Sowa, and S. Watanabe.
Sources of Funding

Dr Sossalla is supported by the German Heart Foundation/German Foundation of Heart Research through a research grant. Drs Sossalla, Maier, Toischer, and Hasenfuss are funded by the Deutsche Forschungsgemeinschaft through the SFB 1002. Dr Maier is funded by the Deutsche Forschungsgemeinschaft through the SFB 1002. Dr Sossalla is supported by the German Heart Foundation/Deutsche Forschungsgemeinschaft through the SFB 1002. Additionally, Dr Maier is funded by the Deutsche Forschungsgemeinschaft through the SFB 1002. Dr Sossalla is supported by the German Heart Foundation/Deutsche Forschungsgemeinschaft through a research grant. None.

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Contractile dysfunction and sudden cardiac death caused by arrhythmias are the clinically most important phenotypes in subjects with structural heart disease. A better insight into the underlying molecular mechanisms and signaling pathways may provide new toeholds for the development of future clinical therapies. Recent studies have identified the emergence of spontaneous diastolic sarcoplasmic (SR) Ca\(^{2+}\) release events from the SR ryanodine receptor (RyR2) as a crucial factor for cardiac contractile dysfunction and arrhythmogenic triggers. In the present study, we clarify that this deleterious SR Ca\(^{2+}\) leak increases from cardiac hypertrophy to heart failure in human myocardium. This is accompanied by profound disturbances of Ca\(^{2+}\) homeostasis. We provide the first evidence that protein kinase A, which is generally activated via the \(\beta\)-adrenergic pathway, functionally regulates RyR2 gating in human cardiac hypertrophy but appears to have lost the ability to modulate RyR2 gating in human heart failure. In contrast, we identified the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) as being primarily responsible for an elevated SR Ca\(^{2+}\) leak occurring in the human failing heart. Thus, the present findings identify CaMKII instead of protein kinase A as a potent inducer of the SR Ca\(^{2+}\) leak, which suggests that inhibition of CaMKII may be a promising treatment option for contractile dysfunction and arrhythmias. As inhibition or ablation of CaMKII has been shown to attenuate or prevent the development of heart failure in animal models via additional pathways, future drug or gene therapy approaches should address key clinical aspects of CaMKII inhibition in patients with heart failure or arrhythmias.
Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II and Protein Kinase A Differentially Regulate Sarcoplasmic Reticulum Ca\textsuperscript{2+} Leak in Human Cardiac Pathology

Thomas H. Fischer, Jonas Herting, Theodor Tirilomis, André Renner, Stefan Neef, Karl Toischer, David Ellenberger, Anna Förster, Jan D. Schmitto, Jan Gummert, Friedrich A. Schöndube, Gerd Hasenfuss, Lars S. Maier and Samuel Sossalla

*Circulation*. 2013;128:970-981; originally published online July 19, 2013; doi: 10.1161/CIRCULATIONAHA.113.001746

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/128/9/970

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/07/19/CIRCULATIONAHA.113.001746.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/
Supp. Figure 1

A

control

AIP

B

CaSpF (100 pmol·ms⁻¹)

control

AIP

*
Supp. Figure 2

A

![RyR2 and GAPDH blots for CL and BDM conditions.

B

![RyR2-S2809 and GAPDH blots for CL and BDM conditions.

C

![RyR2-S2815 and GAPDH blots for CL and BDM conditions.]
Figure Supp. 1
Effects of CaMKII-Inhibition on the SR Ca\(^{2+}\)-leak in heart failure. (A) Representative confocal line scans of cardiomyocytes, with and without AIP treatment. (B) Inhibition of CaMKII in heart failure significantly reduces Ca\(^{2+}\)-spark-frequency (n=34 vs. 54, P< 0.05).

Figure Supp. 2
Analysis of RyR2-expression and -phosphorylation depending on transportation media. No difference could be detected as to RyR2-expression (A), PKA-dependent phosphorylation at Ser2809 (B) and CaMKII-dependent phosphorylation at Ser2815 between human heart failure tissue that had been transported in cooled BDM-free (Custodiol, CL, Dr. Köhler Pharma) vs. BDM-containing solution (BDM, n samples/hearts = 6/3 vs. 6/3 each).

Figure Supp. 3
Evaluation of the effect of H89 (5 µmol/L) on PKA-dependent phosphorylation of RyR2 (Ser2809) and TnI (Ser23/24); each panel shows representative Western blots (right) and the respective quantification (left). (A) and (C) The expression of RyR2 (n samples/hearts = 9/5 vs. 7/5, P=0.78) and TnI (n= 9/5 vs. 9/5; P=0.53) is not significantly changed by H89. (B) and (D) The PKA-dependent phosphorylation of RyR2- and TnI was significantly reduced by 46 ± 11% (n=8/5 vs. 8/5) and 38 ± 8% (n=8/4 vs. 8/4), respectively, upon treatment with H89 (P<0.05 each).