Increased Phosphorylation of Ca\(^{2+}\) Handling Proteins as a Proarrhythmic Mechanism in Myocarditis

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**Background:** Because fatal arrhythmia is an important cause of death in patients with myocarditis, we investigated the proarrhythmic mechanisms of experimental autoimmune myocarditis.

**Methods and Results:** Myocarditis was induced by injection of 2 mg porcine cardiac myosin into the footpads of adult Lewis rats on days 1 and 8 (Myo, n=15) and the results compared with Control rats (Control, n=15). In an additional 15 rats, 6 mg/kg prednisolone was injected into the gluteus muscle before the injection of porcine cardiac myosin on days 1 and 8 (MyoS, n=15). Hearts with myocarditis had longer action potential duration (APD), slower conduction velocity (CV; P<0.01 vs. Control), higher CV heterogeneity, greater fibrosis, higher levels of immunoblotting of high-mobility group protein B1, interleukin 6 and tumor necrosis factor-α proteins. Steroid treatment partially reversed the translations for myocarditis, CV heterogeneity, reduced APD at 90% recovery to baseline, increased CV (P<0.01), and reversed fibrosis (P<0.05). Programmed stimulation triggered sustained ventricular tachycardia in Myo rats (n=4/5), but not in controls (n=0/5) or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) inhibitor (KN93) treated Myo rats (n=0/5, P=0.01). CaMKII autoprophosphorylation at Thr287 (201%), and RyR2 phosphorylation at Ser2808 (protein kinase A/CaMKII site, 126%) and Ser2814 (CaMKII site, 21%) were increased in rats with myocarditis and reversed by steroid.

**Conclusions:** The myocarditis group had an increased incidence of arrhythmia caused by increased phosphorylation of Ca\(^{2+}\) handling proteins. These changes were partially reversed by an antiinflammatory treatment and CaMKII inhibition. (Circ J 2014; 78: 2292–2301)

**Key Words:** Arrhythmia; Ca\(^{2+}\)/calmodulin-dependent protein kinase II; Inflammation; Myocarditis

Myocarditis, and subsequent dilated cardiomyopathy (DCM), is a major cause of heart failure and arrhythmia in young patients.\(^1\)\(^2\) This condition is characterized by infiltration of inflammatory cells into the myocardium with cellular injury, consequent loss of myocytes and development of fibrosis and necrosis.\(^3\)\(^4\) In a significant number of patients, the long-term sequelae of cardiomyocyte loss are ventricular remodeling, permanent ventricular wall dysfunction, DCM, and consequently chronic heart failure. In particular, life-threatening ventricular arrhythmia or sudden death, occurring mainly during acute flare-up of myocarditis, is a serious complication of myocarditis. Therefore, understanding the mechanism of ventricular arrhythmia in myocarditis is important to developing treatment and improving prognosis.\(^5\)

Experimental autoimmune myocarditis in the rat is a unique and useful model for understanding myocarditis and subsequent DCM. Myocarditis rats exhibit high inducibility of ventricular arrhythmia and prolonged action potential duration (APD).\(^6\) However, detailed elucidation of how inflammation of the heart contributes to an increased APD and causes ventricular arrhythmia has not been reported. In myocarditis, inflammation by immune cells increases oxidative stress by excessive production of free radicals from inflammatory cells. Notably, oxidative stress in cardiomyocytes has been shown to activate Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII),\(^7\) sequentially inducing afterdepolarization by prolonging APD.\(^8\) Therefore, we hypothesized that myocarditis-induced arrhythmias could be attributable to CaMKII activation triggered by inflammation and oxidative stress. To prove this hypothesis, we evaluated arrhythmic events and mechanisms using an in-vivo myocarditis model and Langendorff-perfused isolated hearts. We evaluated whether the level of inflammation and CaMKII ac-
Activation were increased in myocarditis, and reversed by pretreatment with an antiinflammatory agent and CaMKII inhibition.

**Methods**

**Induction of Myocarditis**

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011). The study protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine and Cardiovascular Research Institute (approval reference no. 2011-0136), and conformed to the guidelines of the American Heart Association.

The myocarditis model was produced by the method described by Inomata et al. Briefly, to induce inflammation, we used purified cardiac myosin (M0531, Sigma Aldrich, Schnelldorf, Germany) from porcine ventricular muscle as the antigen. Purified cardiac myosin at a concentration of 7 mg/ml was emulsified with an equal volume of complete Freund’s adjuvant (BD Biosciences, Heidelberg, Germany) supplemented with Mycobacterium tuberculosis H37 Ra (Difco, Detroit, MI, USA) at a concentration of 10 mg/ml. The 6-week-old male Lewis rats were immunized by subcutaneous injection of 2 mg of purified cardiac myosin in each of their footpads on days 1 and 8 (Myo group, n=15). Another group was injected with 6 mg/kg prednisolone into the gluteus muscle prior to the immunization (MyoS group, n=15). The control rats received injections of 0.5 ml of complete Freund’s adjuvant in the same manner (Control group, n=15). Ambulatory Holter monitoring was performed for 24 h using a telemetry system (Telemetry Research, Auckland, New Zealand).

**Echocardiographic Examination**

On the 21st day after the initial immunization, transthoracic echocardiography was performed while the rats were anesthetized by intraperitoneal administration of pentobarbital sodium (0.25 mg/kg, Dainihon Chemical Co, Osaka, Japan). The echocardiographic examination was done with a 15-MHz transducer (Vivid Q, General Electric-Vingmed, Milwaukee, WI, USA) and the M-mode echocardiogram was evaluated along the short-axis view of the left ventricle (LV) at the level of the papillary muscles. Left ventricular end-diastolic dimension and left ventricular endsystolic dimension were measured and the left ventricular ejection fraction (LVEF) was calculated from the M-mode echocardiograms.
Activation and repolarization time-points at each site were determined from the fluorescence (F) signals by calculating (dF/dt)$_{\text{max}}$ and (d$^2$F/dt$^2$)$_{\text{max}}$, which have been shown to coincide with approximately 97% repolarization to baseline and recovery from refractoriness. APD was measured from (dF/dt)$_{\text{max}}$ to 90% recovery to baseline, APD$_{90}$. Mean APD$_{90}$ was calculated for each heart by averaging APD$_{90}$ from a region of atrium consisting of 10$\times$10 pixels or 100 APD$_{90}$ from each heart for a minimum of 5 hearts. APD dispersion is defined as the difference between maximum and minimum APDs. The duration of calcium transients was determined from the maximum first derivative of the Cai upstroke to the time point of 90% recovery of Ca$^2+$ to its original baseline. Local conduction velocity (CV) vectors were calculated for each pixel from the differences in activation time-points of that pixel (determined from (dF/dt)$_{\text{max}}$) and its 7$\times$7 nearest neighbors, as previously described. Local CVs were averaged and calculated as mean$\pm$standard error of the mean (SEM). Local CV can be overestimated when 2 wave fronts collide, transmural propagation breaks through the surface or when activation appears synchronous over a region of the atrium because of its proximity to the pacing electrode. To avoid overestimations of CV, values $>$1.25 m/s were deleted from the analysis.

**Optical Mapping**

On the 21st day, rats from 5 groups (250–300 g; Control, n=6; Myo, n=6; MyoS, n=6; Myo + KN92, n=5; Myo + KN93, n=5) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). The chests were opened via median sternotomy and the hearts were rapidly excised and immersed in cold Tyrode’s solution (composition in mmol/L: 125 NaCl, 4.5 KCl, 0.25 MgCl$_2$, 24 NaHCO$_3$, 1.8 NaH$_2$PO$_4$, 1.8 CaCl$_2$, and 5.5 glucose). The ascending aorta was immediately cannulated and perfused with 37°C Tyrode’s solution equilibrated with 95% O$_2$ and 5% CO$_2$ to maintain a pH of 7.4. Coronary perfusion pressure was regulated between 80 and 95 mmHg. For optical recording, the contractility of the heart was inhibited by 10–17 µmol/L of blebbistatin. For dual membrane voltage ($V_m$) and intracellular Ca$^{2+}$ (Ca) recordings, the hearts were stained with Rhod-2 AM and RH237 (Molecular Probes, Eugene, OR, USA) and excited with laser light at 532 nm. Fluorescence was collected using 2 cameras (MiCAM Ultima, BrainVision, Tokyo, Japan) at 1 ms/frame and 100$\times$100 pixels with spatial resolution of 0.35$\times$0.35 mm$^2$/pixel. The mapped area included parts of the right and left ventricular free walls. Optical mapping were performed in 6, 6 and 6 rats in the Control, Myo and MyoS groups, respectively. To evaluate the effect of CaMKII activation on arrhythmia, optical mapping was performed in additional rats with myocarditis after active CaMKII blockade (KN 93, 1 µmol/L infusion) for 20 min (Myo+KN93 group, n=5) or inactive CaMKII blockade (KN 92, 1 µmol/L infusion) for 20 min (Myo+KN92 group, n=5).

**Programmed Stimulation**

To test for vulnerability to ventricular tachycardia (VT), each
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Abcam Reagents, Cambridge, MA, USA, anti-IL-6 (1:1,000, Abcam Reagents), anti-TNF-α (1:1,000, Abcam Reagents), anti-iNOS (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cox-2 (1:1,000, Santa Cruz Biotechnology) and anti-GAPDH (1:1,000, Santa Cruz Biotechnology).

After development, the densities of each band in the digitized images were measured using the ImageJ program.

Blood was obtained from the abdominal aorta of each rat in the 3 groups on day 21. Enzyme-linked immunosorbent assay was performed to determine the levels of HMGB1, IL-6 and TNF-α in serum. According to the manufacturer’s instructions, protein levels in serum were quantified with HMGB1 (IBL International, Hamburg, Germany), IL-6 (R&D System, Minneapolis, MN, USA) and TNF-α (R&D System) kits.

Immunoblot Analysis of Ca2+ Handling Proteins

The protein levels of total CaMKIIδ (1:1,000, Abcam Reagents), anti-IL-6 (1:1,000, Abcam Reagents), anti-TNF-α (1:1,000, Abcam Reagents), anti-iNOS (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cox-2 (1:1,000, Santa Cruz Biotechnology) and anti-GAPDH (1:1,000, Santa Cruz Biotechnology). After development, the densities of each band in the digitized images were measured using the ImageJ program.

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Histology and Assay of Inflammatory Cytokines and Oxidative Stress

On the 21st day after initial immunization, hearts from 3 groups were immediately separated and weighed. The ratio of heart weight to body weight was calculated. In randomly selected rats, the heart was transversely sliced and fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin or Masson’s trichrome for histological evaluation. Quantification of inflammation and fibrotic area was expressed as the percentage of stained area in comparison with the total area of fields examined, using ImageJ, image analysis software (National Institutes of Health, Bethesda, MD, USA).

The immunoblotting of high-mobility group protein B1 (HMGB1), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2) was performed to evaluate the level of inflammation and oxidative stress of tissue. Targeted antigens were probed with the following primary antibodies: anti-HMGB1 (1:1,000, Abcam Reagents, Cambridge, MA, USA), anti-IL-6 (1:1,000, Abcam Reagents), anti-TNF-α (1:1,000, Abcam Reagents), anti-iNOS (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cox-2 (1:1,000, Santa Cruz Biotechnology) and anti-GAPDH (1:1,000, Santa Cruz Biotechnology). After development, the densities of each band in the digitized images were measured using the ImageJ program.

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Heat was paced at the LV using a programmed stimulation protocol consisting of 20 S1 pulses at 250ms cycle length (CL) followed by a premature S2 pulse with progressively shorter S1–S2 interval steps: 250 to 100ms in 20ms steps; 100 to 70ms in 10ms steps and 60 to 35 in 5ms steps, until loss of capture or the initiation of VT. Optical mapping and VT induction studies were performed in 5 rats in each group.

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The protein levels of total CaMKIIδ (1:1,000, Santa Cruz Biotechnology), Thr287 and Thr306/Thr307 phosphorylated CaMKIIδ (1:1,000, Abcam Reagents), GAPDH (1:100,000, Abcam Reagents), total RyR2 (1:1,000, Abcam Reagents), Ser2808 and Ser2814 phosphorylated RyR2 (1:500 and 1:1,000, Badrilla, Leeds, UK), total phospholamban (PLB) (1:1,000, Santa Cruz Biotechnology), Thr17 phosphorylated PLB (1:1,000, Santa Cruz Biotechnology) and L-type calcium channel subunits α (1:1,000, Santa Cruz Biotechnology) were quantified by western blotting. Targeted antigens were visualized with...
PARK H et al. in Myo rats ($2\pm1\%$ vs. $28\pm5\%$, $P<0.001$). The infiltration of inflammatory cells was partially prevented in the MyoS group ($15\pm5\%$, $P=0.006$). Myo rats showed increased levels of fibrosis compared with controls ($6\pm2\%$ vs. $20\pm2\%$, $P<0.001$). However, the MyoS group had less fibrosis than the Myo group ($11\pm2\%$, $P=0.013$).

Figure 1C shows the Kaplan-Meier survival curves of the 3 groups. The Myo group had lower survival rates than the controls ($P=0.03$). Figure 1D shows the ventricular premature beats and VT recorded by ambulatory Holter monitoring in the Myo group. Although arrhythmias were not observed in control rats, they were observed in 5 (56%) of the 9 surviving rats and 0 (0%) of 14 surviving rats in the Myo and MyoS groups, respectively ($P=0.03$).

Compared with controls, the myocarditis model showed decreased LVEF ($83\pm3\%$ vs. $58\pm7\%$, $P<0.001$) and increased LVDD ($6.6\pm0.1$ vs. $7.6\pm0.3$, $P=0.005$) and LVDS ($5.3\pm0.6$ vs. $3.4\pm0.4$, $P=0.003$) (Figure S1).

**Increased Inflammation and Oxidative Stress in Myocarditis Model**

Compared with the controls, HMGB1, IL-6, TNF-α, iNOS, Cox-2 expressions in the Myo group were increased by 3.1- ($P<0.001$), 2.8- ($P=0.003$), 2.2- ($P=0.005$), 1.8- ($P<0.001$) and 4.7-fold ($P<0.001$), respectively. After steroid treatment, compared with the Myo group, HMGB1, IL-6, and TNF-α expres-
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Increased Ventricular Arrhythmias in Myocarditis Model

Figure 4 shows an example of spontaneous triggered activity and VT observed in the myocarditis model. Beats 1–3 each had the same normal conduction pattern, originating from the left upper left side of the recording window, while beat 4 was a spontaneously triggered beat originating from apex of the LV (site 2). Beat 5 also had the same normal conduction pattern. The subsequent 3 beats (6–9) also originated from the same site (site 2).

Spontaneous triggered activities were observed in 1 (17%), 5 (83%) and 2 (33%) rats in the Control (n=6), Myo (n=6) and MyoS (n=6) groups, respectively. Spontaneous triggered activities were more frequently observed in the Myo group than in the Control group (P=0.02). The Myo+KN 93 group had a significantly lower incidence of spontaneous VT (P=0.02) than the Myo group.

By programmed stimulation, VT were induced in 0, 4 (80%) and 0 (0%) rats in the Control (n=5), Myo (n=5), and MyoS (n=5) groups, respectively. Hearts with myocarditis had higher inducible VT or ventricular fibrillation (VF) than controls (P=0.01). Steroid treatment prevented inducible VT or VF after myocarditis was induced (P=0.01). Inducible ventricular arrhythmias were not observed in the Myo+KN 93 group, but in 2 of 3 hearts from the Myo+KN 92 group.

Conduction Heterogeneities in Myocarditis Model

The CV vector maps showed that conduction was significantly slower in the Myo group than in the other groups and more heterogeneous. There was increased conduction heterogeneity in the Myo group compared with other groups. *P<0.001. CL, cycle length; Myo, myocarditis group; MyoS, myocarditis group treated with steroid.

Increased APD and APD Dispersion in Myocarditis Model

Figure 3A shows the Vm and Cai tracings recorded at the base of the LV during pacing CLs of 300 ms in the Langendorff-perfused hearts. The Myo group had longer APD with increased Cai transient duration than the Control and MyoS groups. Figure 3B shows the activation and APD maps. Compared with the Control and MyoS groups, the Myo group showed crowding of activation isochronal lines, suggesting increased conduction time of the ventricle. Compared with controls (13±4 ms), APD dispersion was increased in the Myo group (43±16 ms, P=0.001), but not in the MyoS group (17±5 ms, P>1.0). To evaluate the relationship between myocarditis-induced arrhythmia and CaMKII activation, we recorded the action potential after pretreatment with CaMKII inhibitor. Myocarditis-induced APD prolongation was prevented by pretreatment with KN 93 (1 μmol/L) for 20 min, but not by KN 92 (1 μmol/L) for 20 min. The comparison of mean APD90 among the groups is presented in Figure 3C and the comparison of mean Cai transient duration is shown in Figure S2. The Myo group had a longer mean APD90 with increased Cai transient duration than either the Control or MyoS group.

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Conduction Heterogeneities in Myocarditis Model

The CV vector maps showed that conduction was significantly slower in the Myo group than in the other groups and conduction was more heterogeneous in the Myo group than in the other groups (Figures 5A,B). To quantify this heterogeneous of conduction, the conduction heterogeneity index across 5 tested pacing CLs was calculated. There was a significantly

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Increased p-CaMKII, RyR2 and p-PLB in Myocarditis

We assessed CaMKII, RyR2 and PLB expressions and relative phosphorylation levels by western blotting. Figure 7 shows the Ca\(^{2+}\) handling protein assay in rat ventricular tissues. CaMKII and Thr287-CaMKII protein expressions were significantly increased in myocarditis tissue lysates (P<0.001), with unaltered Thr306-CaMKII (Figure 7A). With slightly increased total RyR2 (P=0.01), both Ser2808-RyR2 and Ser2814-RyR2 protein expression was significantly increased in myocarditis tissue lysates (P<0.001). CaMKII autophosphorylation at Thr287 (201\%), RyR2 phosphorylation at Ser2808 (protein kinase A/ CaMKII site, 126\%) and Ser2814 (CaMKII site, 21\%) were increased in myocarditis and reversed after steroid treatment. Moreover, the levels of the ratio of Thr17-PLB to total PLB, which decreased in Myo hearts (80\%), were restored by steroid treatment (Figure 7C). However, the protein levels of L-type calcium channel \(\alpha\)-subunits were unaltered in myocarditis (Figure S4).

Discussion

Main Findings

Firstly, rats with myocarditis showed decreased survival and increased incidence of fatal VT. Secondly, spontaneous triggered activity and VT inducibility were increased in myocarditis, and partially reversed by treatment with steroid and CaMKII inhibitor, KN93. Thirdly, the myocarditis model showed increased activity of Ca\(^{2+}\) handling proteins, including p-CaMKII and p-RyR2. Finally, myocarditis-related arrhythmias and activation of Ca\(^{2+}\) handling proteins were partially attenuated by steroid pretreatment. Our results suggest that the mechanism of arrhythmia in myocarditis might be related with the increased phosphorylation of Ca\(^{2+}\) handling proteins caused with the inflammation and oxidative stress.
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Increased Phosphorylation of Ca$^{2+}$ Handling Proteins in Myocarditis

Abundant evidence now supports an important role of CaMKII in promoting heart failure and arrhythmias by its actions on SR Ca$^{2+}$ uptake and release.\textsuperscript{15} The mechanism of CaMKII hyperactivity in heart failure is likely attributable to either auto-phosphorylation of threonine 287 and/or oxidation of methionines 281 and 282.\textsuperscript{16} In the present study, we found that the phosphorylation of CaMKII at Thr287 was increased in myocarditis, suggesting the mechanism of CaMKII hyperactivity is attributable to autophosphorylation of threonine 287.

RyR2 is a SR Ca$^{2+}$ release channel that is activated by a trigger of Ca$^{2+}$ from $I_{Ca}$.\textsuperscript{17} RyR2 phosphorylation by both protein kinase A and CaMKII enhances $I_{Ca}$ and RyR2 Ca$^{2+}$ release. CaMKII helps coordinate this physiological process of Ca$^{2+}$-induced Ca$^{2+}$ release by phosphorylation of CaV1.2 and RyR2. However, in failing myocytes the cell membrane ultrastructure supporting Ca$^{2+}$-induced Ca$^{2+}$ release is distorted\textsuperscript{18} and CaMKII hyperphosphorylation of CaV1.2 and RyR2 becomes arrhythmogenic. RyR2 is phosphorylated by CaMKII (serine 2814) and PKA (serine 2814 and 2808). In this study, myocarditis was associated with increased phosphorylation of serine 2814 and serine 2808 of RyR2. The hyperphosphorylation of RyR2 promotes RyR2 Ca$^{2+}$ leak and arrhythmia-triggering delayed afterdepolarizations\textsuperscript{19} while depleting SR Ca$^{2+}$ to impair inotropy.\textsuperscript{20–24}

Previous studies have reported initial reduction of $I_{Ca}$-related molecules, such as the expression levels of Kv4.2, Kv1.5, frequenin and KChIP2, in a myocarditis model.\textsuperscript{25,26} However, the role of Ca$^{2+}$ handling protein has not been evaluated in myocarditis. To our knowledge, ours is the first study to suggest that the activation of Ca$^{2+}$ handling proteins might be a mechanism of myocarditis-induced arrhythmias.

Mechanism of Ventricular Arrhythmia in Myocarditis

In this study, the myocarditis model showed electrophysiolog-
Circulation Journal Vol.78, September 2014

Conflict of Interest

The authors have declared that no conflict of interest exists.

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Attenuation of Myocarditis-Induced Arrhythmia by Antiinflammatory Therapy

Inflammatory cytokines, such as HMGB-1, TNF-α or IL-6, and oxidative stress were overexpressed in rats with myocarditis. As these inflammatory cytokines are strong inducers of reactive oxygen species, this inflammatory process may promote cardiac injury and electrical remodeling.36,38 Niwano et al reported that the N-acetylcysteinle treatment suppressed ventricular remodeling in myocarditis rats.36 This study consistently showed that steroid pretreatment was related to improved survival and suppression of arrhythmias in the rats with myocarditis. Therefore, the prevention of inflammation might suppress arrhythmia by preventing either remodeling or myocarditis itself.

Study Limitations

We induced myocarditis by injection of cardiac myosin, so our results cannot explain the exact mechanisms of myocarditis-related arrhythmia, which is mostly caused by viral infection or other etiologies. However, autoimmunization to myosin might be a common pathway of myocarditis.37

Conclusions

A rat myocarditis model showed increased arrhythmia and increased activity of Ca2+ handling proteins, including p-CaMKII and p-RyR2, suggesting that the mechanism of arrhythmia in myocarditis might be related to increased phosphorylation of Ca2+ handling proteins caused by inflammation and oxidative stress.

Acknowledgments

This study was supported in part by research grants from the Korean Heart Rhythm Society (2011-3), the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (NRF-2010-0021993, NRF-2012R1A2A2A02045367), and a grant of the Korean Healthcare technology R&D project funded by Ministry of Health & Welfare (HI12C1552). We thank Michael Hahn for his English correction.

Disclosures

None.
Ca\textsuperscript{2+}/calmodulin kinase II-dependent phosphorylation of ryanodine receptors suppresses Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves in cardiac myocytes. 


**Supplementary Files**

**Supplementary File 1**

**Figure S1.** Myocarditis-induced cardiac dysfunction.

**Figure S2.** Comparison of mean Ca transient duration among groups.

**Figure S3.** Aggravated spatially discordant alternans in autoimmune myocarditis.

**Figure S4.** Change in the protein expression levels of the L-type calcium channel (LTCC).

Please find supplementary file(s): http://dx.doi.org/10.1253/circj.CJ-14-0277