Ca$^{2+}$/calmodulin-dependent protein kinase II increases the susceptibility to the arrhythmogenic action potential alternans in spontaneously hypertensive rats

Hirofumi Mitsuyama, Hisashi Yokoshiki, Masaya Watanabe, Kazuya Mizukami, Junichi Shimokawa, and Hiroyuki Tsutsui

Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Submitted 18 May 2012; accepted in final form 17 May 2014

Mitsuyama H, Yokoshiki H, Watanabe M, Mizukami K, Shimokawa J, Tsutsui H. Ca$^{2+}$/calmodulin-dependent protein kinase II increases the susceptibility to the arrhythmogenic action potential alternans in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 307: H199–H206, 2014. First published May 23, 2014; doi:10.1152/ajpheart.00387.2012—Action potential duration alternans (APD-ALT), defined as long-short-long repetitive pattern of APD, potentially leads to lethal ventricular arrhythmia. However, the mechanisms of APD-ALT in the arrhythmogenesis of cardiac hypertrophy remain undetermined. Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is known to modulate the function of cardiac sarcoplasmic reticulum and play an important role in Ca$^{2+}$ cycling. We thus aimed to determine the role of CaMKII in the increased susceptibility to APD-ALT and arrhythmogenesis in the hypertrophied heart. APD was measured by high-resolution optical mapping in left ventricular (LV) anterior wall from normotensive Wistar-Kyoto (WKY; n = 10) and spontaneously hypertensive rats (SHR; n = 10) during rapid ventricular pacing. APD-ALT was evoked at significantly lower pacing rate in SHR compared with WKY (382 ± 43 vs. 465 ± 45 beats/min, P < 0.01). These changes in APD-ALT in SHR were completely reversed by KN-93 (1 μmol/l; n = 5), an inhibitor of CaMKII, but not its inactive analog, KN-92 (1 μmol/l; n = 5). The magnitude of APD-ALT was also significantly greater in SHR than WKY and was completely normalized by KN-93. Ventricular fibrillation (VF) was induced by rapid pacing more frequently in SHR than in WKY (60 vs. 10%; P < 0.05), which was also abolished by KN-93 (0%, P < 0.05). Western blot analyses indicated that the CaMKII autophosphorylation at Thr287 was significantly increased in SHR compared with WKY. The increased susceptibility to APD-ALT and VF during rapid pacing in hypertrophied heart was prevented by KN-93. CaMKII could be an important mechanism of arrhythmogenesis in cardiac hypertrophy.

Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is one of the important regulators of intracellular Ca$^{2+}$ cycling by phosphorylation of various proteins such as L-type Ca$^{2+}$ channel (LTCC), ryanodine receptor (RyR), and phospholamban (PLB) (22). It has been shown to be enhanced in hypertrophied hearts (2, 9). Moreover, the simulation study demonstrated that CaMKII could initiate intracellular Ca$^{2+}$ alteration and induce APD-ALT (21). We thus hypothesized that the enhanced CaMKII activity in hypertrophied heart might increase the susceptibility to APD-ALT, leading to its arrhythmogenesis. The aim of this study was to examine the occurrence of APD-ALT in hypertrophied hearts and to investigate the relation between its occurrence and ventricular arrhythmias.

MATERIALS AND METHODS

All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guidelines for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Experimental preparation. Male 18- to 24-wk-old Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were anesthetized with the inhalation of diethyl ether, and heparin sodium (400 IU/kg) was injected. The hearts were rapidly excised and perfused with Langendorff apparatus with oxygenated (100% O$_2$) Tyrode’s solution (37°C) containing (in mmol/l) 143 NaCl, 5.4 KCl, 0.33 NaH$_2$PO$_4$, 5 HEPES, 5.5 glucose, 0.5 MgCl$_2$, and 1.8 CaCl$_2$ (pH 7.4 adjusted by using NaOH). Perfused heart was placed in a custom-built chamber in which the temperature was constantly equal to the perfusion temperature. The bipolar pacing electrode was attached at the left ventricular apex, and two Ag/AgCl electrodes were attached to record an ECG. The perfusion pressure was kept at 70 cmH$_2$O for WKY and 100 cmH$_2$O for SHR to adjust coronary flow per weight as described previously (16). After a stabilization period of 10 min, the solution was switched to 100 ml Tyrode’s solution containing the voltage-sensitive dye, di-4-ANEPPS (2.5 μmol/l), and perfused for 10 min. The heart was then perfused with Tyrode’s solution containing blebbistatin (10 μmol/l), an excitation-contraction uncoupler, to eliminate motion artifacts.
Optical mapping. An optical mapping system was used to measure APDs. Illumination of the voltage-sensitive dye’s fluorescence was provided using a 531 ± 20 nm light emitted by a 150-W halogen light source. The output signals from the anterior epicardial surfaces of left ventricle were filtered with a 590-nm interference bandpass filter and recorded by charge-coupled device (CCD) camera (MiCAM02, BrainVision, Tokyo, Japan). The imported data were analyzed with 0.2 mm spatial and 2.2 ms temporal resolutions by a personal computer. Three × three spatial filter was used to minimize the distortions and artifacts of the images before analysis.

Stimulation protocol. Hearts were paced from left ventricular apex at twice diastolic threshold current with a silver bipolar electrode. The optical mapping of APD and ECG recording was made during steady-state pacing at a cycle length of 300 ms, and the pacing cycle length was shortened (250 ms, 200 ms, and reduced in 10-ms steps each 30 s) until the ventricular arrhythmias were induced or 1:1 capture was lost. We defined rapid ventricular arrhythmia as sustained ventricular fibrillation (VF) when it lasted more than 10 s. APD was measured at 90% repolarization (APD90). APD-ALT was measured by calculating the difference in APD of two consecutive beats. The APD-ALT threshold interval was defined as the longest pacing interval required to produce more than 10 ms of APD-ALT.

Pharmacological effect of an inhibitor of CaMKII. To determine the effect of CAMKII on APD-ALT, KN-93 (1 μmol/l), an inhibitor of CaMKII, was added to Tyrode’s solution and perfused for 10 min. Optical mapping was obtained with the same pacing protocol as described above. KN-92 (1 μmol/l), an inactive analog of KN-93, was used to exclude the effects of KN-93 on other enzymes than CaMKII. Each experiment was completed within 100 min.

Western blot analyses. Western blot analyses for CaMKII were performed in WKY and SHR. The ventricular tissues were excised just after thoracotomy for analysis of CaMKII activity or during pacing at the pacing cycle length of 150 ms for 10 min for analysis of effect of KN-93 on CaMKII activity. Those were snap-frozen, homogenized, and dissolved in 1× cell lysis buffer (Cell Signaling, Danvers, MA), supplemented with 1× Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After centrifugation at 15,000 rpm for 20 min at 4°C, the supernatants were separated into aliquots and stored at −80°C until the time of assay. Protein concentrations were determined using a standardized colorimetric assay. Proteins were fractionated by SDS-PAGE, transferred to PVDF membrane, blocked with 5% BSA, and blotted with anti-CaMKII (A-17, 1:100; Santa Cruz Biotechnology), anti-phospho-CaMKII Thr286/287 (1:1,000; Cell Signaling), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000; Cell Signaling) overnight at 4°C. Membranes were washed with blocking buffer and incubated with secondary antibodies directed against the primary and conjugated with horseradish peroxidase. Bands were detected with the enhanced chemiluminescence assay and quantified using ImageJ. Band intensity for the protein under question was normalized to the intensity of GAPDH in each lane.

Fig. 1. The hypertrophied heart is susceptible to action potential duration alternans (APD-ALT). A: optical action potentials from epicardial surface of left ventricle in Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Long (●, △)-short (○, ◊)-long repetitive pattern of APD appeared as pacing cycle length was shortened. B: the measured APD90 in WKY and SHR. APD in SHR was significantly longer than in WKY. C: representative plots of APD90 and APD-ALT obtained from WKY and SHR. APD-ALT occurred at pacing interval of 150 ms in SHR (●, ○) and 130 ms in WKY (△, ◊). Long (●, △)-short (○, ◊) repetitive APD appeared at a shorter pacing rate than that of APD-ALT threshold. D: threshold pacing interval for APD-ALT in WKY (n = 10) and SHR (n = 10). *P < 0.001, WKY vs. SHR.
ROLE OF CaMKII IN ACTION POTENTIAL ALTERNANS IN HYPERTROPHY

Statistical analysis. All data are expressed as means ± SE. Significance (P < 0.05) was determined by using unpaired Student’s t-test or two-way repeated-measures ANOVA as appropriate. Categorical data were compared with the Fisher’s exact test or χ² test when appropriate.

RESULTS

Left ventricular weight per body weight was significantly greater in SHR than in WKY (4.04 ± 0.07 mg/g for n = 10 vs. 3.06 ± 0.12 mg/g for n = 10; P < 0.01).

Figure 1A shows representative examples of the APD recorded at five different pacing rates. Until the pacing interval was shortened to 200 ms, the APD was stable over time and no alternans was observed. APD-ALT was observed in SHR at a pacing interval of 150 ms and in WKY at a pacing interval of 100 ms. Before the occurrence of APD-ALT, APD₀₉₀ was significantly longer in SHR than in WKY (P < 0.001) (Fig. 1B). The threshold pacing interval for APD-ALT was significantly longer in SHR than in WKY (169 ± 6 ms for n = 10 vs. 140 ± 4 ms for n = 10; P < 0.001) (Fig. 1D), indicating that SHR is susceptible to APD-ALT.

The addition of KN-93 facilitated rate-dependent shortening of APD in SHR. KN-93 significantly shortened APD₀₉₀ in SHR (P < 0.05) (Fig. 2, A and B), whereas it did not affect APD₀₉₀ in WKY (Fig. 2B). The threshold interval for APD-ALT was shortened in SHR by KN-93 (134 ± 7 vs. 169 ± 6 ms, P < 0.01) (Fig. 2C). KN-92, an inactive analog of CaMKII inhibitor, did not affect the degree of APD shortening and the threshold interval for APD-ALT in SHR (Fig. 2C). KN-93 did not affect the threshold interval for APD-ALT in WKY (Fig. 2D).

Figure 3A shows the representative contour maps of APD-ALT in WKY, SHR, and SHR + KN-93 on left ventricular anterior surface. The magnitude of APD-ALT was significantly greater in SHR compared with WKY (P < 0.01), which was also ameliorated by KN-93 (P < 0.01) (Fig. 3A and B). KN-93 did not affect the magnitude of APD-ALT in WKY (Fig. 3B).

Sustained VF induced by the shortening of pacing intervals was more frequently observed in SHR than in WKY (60% vs. 10%; P < 0.05), and VF did not occur in SHR + KN-93 (60% vs. 0%; P < 0.05) (Fig. 4A). The pacing interval at which sustained or nonsustained VF was induced was significantly shorter in WKY than in SHR (96 ± 4 ms vs. 129 ± 7 ms; P < 0.01). The discordant APD-ALT, defined as the phenomenon of spatially opposite phase of alternans, tended to occur more frequently in SHR (7/10) than in WKY (2/10) and SHR + KN-93 (2/5) (P = 0.078) (Fig. 4B). When the discordant APD-ALT occurred, VF was induced more frequently in SHR (5/7) than WKY (0/2) and SHR + KN-93 (0/2) (P = 0.073) (Fig. 4C).

Fig. 2. KN-93, an inhibitor of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), decreases the threshold interval for APD-ALT only in SHR. A: representative plots of APD₀₉₀ and APD-ALT in SHR in absence of (●) and presence of 1 μM KN-93 (○). B: changes of APD₀₉₀ at pacing cycle length of 300, 200, 180, and 170 ms from 4 groups of SHR (n = 10), SHR + KN-93(n = 5), WKY (n = 10), and WKY + KN-93 (n = 5). C: threshold interval for APD-ALT in SHR (n = 10), SHR + KN-93 (n = 5), and SHR + KN-92 (n = 5). KN-93 shortened the threshold interval for APD-ALT in SHR but not by KN-92. D: threshold for APD-ALT in WKY (n = 10) and WKY + KN-93 (n = 5). KN-93 did not affect the threshold for APD-ALT in WKY. *P < 0.01, SHR vs. SHR + KN-93.
To examine whether CaMKII activity was increased in SHR compared with WKY, we assessed CaMKII protein expression and phosphorylation of CaMKII in the left ventricular myocardium. Western blot analysis revealed that protein expression of CaMKII was not different between WKY and SHR (Fig. 5A). On the other hand, phosphorylation of CaMKII at Thr287 was increased in SHR compared with WKY (P < 0.01) (Fig. 5B).

In addition, we examined the inhibitory effect of KN-93 on the phosphorylation of CaMKII to estimate the CaMKII activity. The Langendorff-perfused hearts from SHR were continuously paced at the cycle length of 150 ms in the absence or presence of KN-93 (1 μmol/l) for 10 min. Western blot analysis revealed that KN-93 inhibited the phosphorylation of CaMKII at Thr287 (P < 0.01) (Fig. 6).

DISCUSSION

In the present study, APD-ALT was induced at lower pacing rate and its magnitude was greater in the hearts from SHR, which was associated with higher incidence of VF. This susceptibility to APD-ALT and VF in SHR was completely inhibited by CaMKII inhibitor, KN-93. These results suggest that the increased susceptibility to APD-ALT may lead to the arrhythmogenesis in hypertrophied heart via CaMKII activation.

Rate adaptation of APD. The main electrophysiological feature of LVH is the prolongation of APD (10, 30). In agreement with previous studies, we demonstrated the prolongation of APD under rapid pacing in LVH by optical mapping (Fig. 1B). Moreover, the APD was gradually shortened in both WKY and SHR as pacing interval was increased. In contrast, rate-dependent prolongation of APD was shown in isolated rat ventricular cells (6). The discrepancy of the response of APD to high pacing rate may arise from the differences in experimental conditions such as Ca2+ concentration and/or the method of APD measurement (optical mapping or patch-clamp technique).

APD-ALT in hypertrophied heart. We have demonstrated that the hypertrophied heart has low threshold of APD-ALT (Fig. 1). Guo et al. (8) reported that APD-ALT could be
induced at various pacing cycle lengths from 150 ms to 1,000 ms in single myocytes from rabbits with LVH. In agreement with their study, we also showed the susceptibility to APD-ALT in perfused hypertrophied hearts by optical mapping system, but it was not observed until the pacing cycle length was shortened to the threshold interval (169 ± 6 ms in SHR, 140 ± 4 ms in WKY). More recently, Kozhevnikov et al. (17) showed APD-ALT was frequently observed in the perfused LVH model of guinea pig. Based on our data and those from previous studies, it is evident that the hypertrophied heart has increased susceptibility to APD-ALT.

Role of CaMKII in APD-ALT. Although the mechanisms of APD-ALT have not been understood completely, intracellular Ca\(^{2+}\) alternans may be responsible for initiation of APD-ALT (4, 34, 37, 40). Several mechanisms of Ca\(^{2+}\) alternans have been postulated in normal hearts and simulation models, including 1) alternation of sarcoplasmic reticulum (SR) Ca\(^{2+}\) content due to the steep dependence of Ca\(^{2+}\) release on SR Ca\(^{2+}\) content (4, 2) Ca\(^{2+}\)-mediated refractoriness of RyR (27), and 3) Ca\(^{2+}\) uptake by the SERCA/PLB complex (13).

CaMKII, a multifunctional protein kinase, which can phosphorylate several Ca\(^{2+}\) transport proteins in heart (1, 5) such as RyR (39), LTCC, and PLB (18), has been demonstrated to be enhanced in hypertrophied heart (2, 9). The present results that CaMKII inhibition elevated the threshold of APD-ALT in hypertrophied heart indicate that CaMKII may play an important role in the induction of APD-ALT by modifying the response to intracellular Ca\(^{2+}\) of LTCC, RyR, and PLB. In general, the amplitude of the Ca\(^{2+}\) transient and the level of diastolic Ca\(^{2+}\) increase, as stimulation frequency is increased (19). Wan et al. (38) analyzed the relationship between diastolic Ca\(^{2+}\) and the onset of Ca\(^{2+}\) alternans and showed the onset of Ca\(^{2+}\) alternans was consistently preceded by a significant elevation of diastolic Ca\(^{2+}\).

To elucidate mechanisms of APD alternans in hypertrophied heart, we examined the phosphorylation of CaMKII and demonstrated that it was significantly increased in SHR compared with WKY (Fig. 5B). Considering the fact that past reports demonstrated that CaMKII increases diastolic Ca\(^{2+}\) and SR Ca\(^{2+}\) leak (15, 32), it is reasonable to think the rate-dependent exaggerated elevation of cytosolic Ca\(^{2+}\) produced by the increased CaMKII activity in SHR may lead to incomplete restoration of cytosolic Ca\(^{2+}\) during each beat. This possible mechanism could cause cytosolic Ca\(^{2+}\) alternans, thereby inducing APD (repolarization) alternans. The inhibition of enhanced CaMKII in hypertrophied heart may attenuate the increase in diastolic Ca\(^{2+}\) evoked by rapid pacing, which could suppress the appearance of Ca\(^{2+}\) alternans and APD-ALT.

In this report, KN-93 and KN-92 were used at a concentration of 1 μmol/l. Blockade of the CaMKII pathway was reported to be achieved by direct inhibition of the kinase activity with 1 μmol/l of KN-93 (36), and this concentration of KN-93 has been chosen in an attempt to specifically inhibit CaMKII in many experiments (14, 39). In consistency with these reports, we demonstrated that 1 μmol/l of KN-93 inhibited the phosphorylation of CaMKII in SHR (Fig. 6). CaMKII inhibition had little effect on APD in a transgenic mouse with increased endogenous CaMKII activity when the pacing rate was low (0.5 Hz) (41). Interestingly, KN-93, an inhibitor of CaMKII, contributed to the shortening of APD only in SHR, not in WKY (Fig. 2B) in our study. In hypertrophied heart, rate-dependent increase in inward current of Na\(^{+}/\)Ca\(^{2+}\) exchange (NCX) may play an important role in the shortening of APD. As a pacing rate increases, NCX current predominantly increase in hypertrophied heart compared with...
normal heart, thereby restricting the rate-dependent shortening of APD. CaMKII inhibition could relieve Ca\(^{2+}\) overload by suppression of L-type calcium current (I\(_{\text{CaL}}\)) and Ca\(^{2+}\) leak from SR, which may lead to decreased NCX current and accelerate rate-dependent shortening of APD. Another mechanism of shortening of APD may include the lack of positive feedback to I\(_{\text{CaL}}\) by CaMKII inhibition. I\(_{\text{CaL}}\) is regulated by positive feedback mechanism, which is related to CaMKII activation (1), and negative feedback mechanism, which is related to Ca\(^{2+}\)-dependent inactivation. The lack of positive feedback to I\(_{\text{CaL}}\) by CaMKII inhibition, thereby decreasing I\(_{\text{CaL}}\), might lead to the shortening of APD at high pacing rate.

**APD-ALT for arrhythmogenesis of LVH.** The mechanism linking APD-ALT with ventricular arrhythmias has been recently described, involving the development of spatially discordant APD-ALT. During discordant APD-ALT, marked spatial dispersion of repolarization emerges and pathophysiological heterogeneities of repolarization produces unidirectional conduction block and reentry (40). However, we were not able to demonstrate whether discordant APD-ALT directly induced VF. This is because the optical mapping could not be obtained at the timing of VF induction.

In the present study, APD-ALT occurred nonuniformly throughout the heart, and discordant APD-ALT was observed frequently in SHR (Fig. 4B: 70% in SHR, 20% in WKY, and 40% in SHR + KN-93). Interestingly, when discordant APD-ALT was observed, VF occurred in 71.4% (5 of 7) of SHR, whereas it did not occur in WKY (0 of 2) and SHR + KN-93 (0 of 2) (Fig. 4C). Since the magnitude of APD-ALT was larger in SHR than WKY and SHR + KN-93 (Fig. 3B), it could create steeper gradients of repolarization in SHR when the discordant APD-ALT occurs, which would induce unidirectional block, reentry, and VF as previously reported (25).

Previous studies reported that CaMKII was proarrhythmic in cardiac hypertrophy, but these studies proposed EADs as a triggering mechanism for ventricular arrhythmias (14, 41). In present study, we consider another mechanism that CaMKII contributes to the susceptibility of APD-ALT, thereby producing arrhythmogenic substrate for random reentry in hypertrophied heart.

**Expression and phosphorylation of CaMKII in hypertrophied heart.** There is growing evidence for a pathophysiological role of CaMKII in cardiac hypertrophy. CaMKII expression and activity have been reported to be altered in a variety of animal models of cardiac hypertrophy (43). Studies examining hypertrophied myocardium from SHR showed increase in mRNA expression of some isoforms of CaMKII (9) and increase in CaMKII activity in presence of 1 mM Ca\(^{2+}\) and calmodulin (2). We examined the protein levels of CaMKII and phosphorylation of CaMKII at Thr287 in myocardium of SHR and WKY (Fig. 5). Although there were no differences in the total protein levels of CaMKII between SHR and WKY, phosphorylation of CaMKII was significantly increased in SHR compared with WKY in this study. Our data were in agreement with the past report in which CaMKII activity was increased in SHR (2). These results suggest that the increased phosphorylation of CaMKII in LVH, not the total expression of CaMKII, could play an important role of early emergence of APD-ALT and its arrhythmogenesis. However, we should be aware that amount of autophosphorylated CaMKII did not always reflect the specific CaMKII activity especially under the pathological condition such as Ca\(^{2+}\) overload (42).
**Limitation of the study.** We could not evaluate the transmural and interventricular heterogeneity of action potential duration because we examined the epicardial surface of anterior left ventricle. Wan et al. (38) have demonstrated that the transmural heterogeneities in expression and function of calcium cycling proteins underlies heterogeneities in APD-ALT, in which endocardial myocytes exhibited greater susceptibility to cellular alternans than epicardial myocytes. Transmural heterogeneity of APD-ALT may be linked to cardiac arrhythmogenesis. However, it is not clear in the present study whether transmural difference is more augmented in SHR.

**Conclusion.** We demonstrated that CaMKII increases the susceptibility to the arrhythmogenic action potential alternans in hypertrophied heart. CaMKII may be an important therapeutic target against arrhythmogenesis seen in cardiac hypertrophy.

**ACKNOWLEDGMENTS**

We thank M. Fujii, A. Aita, and N. Ikeda for technical assistance in the experiments. We are grateful to Dr. S. Miyoshi, head of Miyoshi Clinic, Tokyo, Japan, for helpful advice at the initiation of optical mapping in our laboratory.

**GRANTS**

This work was supported, in part, by a grant from Grant-in-Aid for Scientific Research (23591075) from the Japan Society for the Promotion of Science to H. Yokoshiki.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: H.M., H.Y., M.W., J.S., and H.T. conception and design of research; H.M., H.Y., M.W., K.M., and J.S. performed experiments; DISCLOSURES

**REFERENCES**

32. Sag CM, Wadsack DP, Khabbazzadeh S, Abesser M, Grefe C, Neef S, Maier SK, Maier LS. Calcium/calmodulin-dependent protein kinase II contrib-
utes to cardiac arrhythmogenesis in heart failure. *Circ Heart Fail* 2:


