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Original Contribution

Sustained CaMKII activity mediates transient oxidative stress-induced long-term facilitation of L-type Ca^{2+} current in cardiomyocytes

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ABSTRACT

Oxidative stress remodels Ca^{2+} signaling in cardiomyocytes, which promotes altered heart function in various heart diseases. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was shown to be activated by oxidation, but whether and how CaMKII links oxidative stress to pathophysiological long-term changes in Ca^{2+} signaling remain unknown. Here, we present evidence demonstrating the role of CaMKII in transient oxidative stress-induced long-term facilitation (LTF) of L-type Ca^{2+} current ($I_{Ca,L}$) in rat cardiomyocytes. A 5-min exposure of 1 mM H_2O_2 induced an increase in $I_{Ca,L}$, and this increase was sustained for ~1 h. The CaMKII inhibitor KN-93 fully reversed H_2O_2-induced LTF of $I_{Ca,L}$, indicating that sustained CaMKII activity underlies this oxidative stress-induced memory. Simultaneous inhibition of oxidation and autophosphorylation of CaMKII prevented the maintenance of LTF, suggesting that both mechanisms contribute to sustained CaMKII activity. We further found that sarcoplasmic reticulum Ca^{2+} release and mitochondrial ROS generation have critical roles in sustaining CaMKII activity via autophosphorylation- and oxidation-dependent mechanisms. Finally, we show that long-term remodeling of the cardiac action potential is induced by H_2O_2 via CaMKII. In conclusion, CaMKII and mitochondria confer oxidative stress-induced pathological cellular memory that leads to cardiac arrhythmia.

Oxidative stress-induced cell signaling is implicated in ischemia/reperfusion-induced cardiac arrhythmia and other heart diseases [1–3]. Importantly, oxidative burst occurring at the initial moment of reperfusion induces subsequent ion channel modulation and Ca^{2+} dysregulation that result in altered heart functions [4–6]. However, the exact mechanism by which transient oxidative stress is converted to sustained alteration of heart function remains largely unknown. Recently, our group and others suggested that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) mediates H_2O_2- and ischemia/reperfusion-induced Ca^{2+} dysregulation, arrhythmogenesis, and cardiomyocyte death [7–12].

CaMKII is a multifunctional serine/threonine kinase that has central roles in regulating ion channels and Ca^{2+} signaling in cardiomyocytes in both physiological and disease states [13–15]. Activation of CaMKII is initiated by binding of Ca^{2+}/calmodulin (Ca/CaM), which causes conformational changes to disrupt interactions between the autoinhibitory sequence in the regulatory domain and the N-terminal catalytic domain [14,16]. Interestingly, CaMKII can show autonomous kinase activity even after dissociation from Ca/CaM when threonine 287 in the autoinhibitory sequence is phosphorylated [14,16]. Because Ca^{2+} signals are usually brief and so is Ca/CaM binding, autophosphorylation-dependent autonomous activity is believed to be crucial for CaMKII to act on target proteins and bring about cellular responses, such as long-term potentiation evoked by high-frequency stimulation in hippocampal neurons [17] and frequency- and Ca^{2+}-dependent facilitation (CDF) of Ca^{2+} channels in cardiomyocytes [7,18].

Recently, Erickson et al. reported that reactive oxygen species (ROS) can oxidize methionine 281/282 in the regulatory domain, causing activation of CaMKII [9]. The oxidation-dependent CaMKII activation requires initial Ca/CaM binding but the subsequent autonomous activity seems to be independent of autophosphorylation because the mutation of threonine 287 to alanine did not inhibit autonomous CaMKII activity induced by H_2O_2 in purified CaMKII in vitro [9]. Consistent with this finding, our group showed that the H_2O_2-induced increase in L-type Ca^{2+} current ($I_{Ca,L}$), which is mediated by CaMKII, is not affected by the inhibition of autophosphorylation [7]. However, the finding that H_2O_2 increases autophosphorylation of CaMKII in cardiomyocytes [19] still suggests the role

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; LTF, long-term facilitation; ROS, reactive oxygen species; CDF, Ca^{2+}-dependent facilitation; $CaMKII_{Phospho}$, sustained CaMKII activity dependent on autophosphorylation; $CaMKII_{Oxi}$, sustained CaMKII activity dependent on oxidation; APD, action potential duration; SR, sarcoplasmic reticulum; DTT, dithiothreitol; AIP, autocamtide-2 related inhibitory peptide; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; TMRE, tetramethylrhodamine ethyl ester; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester.

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of autophosphorylation-dependent mechanisms in oxidative stress-induced autonomous CaMKII activity. However, the mechanistic details of how these two oxidation- and autophosphorylation-dependent mechanisms confer sustained autonomous CaMKII activity after transient oxidative stress, which mimics oxidative burst during reperfusion [4], are not clearly understood yet.

In this study, we demonstrate that H_2O_2-induced facilitation of $I_{Ca,L}$, which is dependent on CaMKII activity, is sustained even after washout of H_2O_2. Using $I_{Ca,L}$ facilitation as a functional assay for CaMKII activity, we investigated the mechanisms involved in sustained activity of CaMKII. Long-term facilitation (LTF) of $I_{Ca,L}$ was reversed by simultaneous inhibition of autophosphorylation and oxidation, suggesting that both mechanisms contribute to H_2O_2-induced sustained CaMKII activity. We then clarified that sustained CaMKII activity dependent on autophosphorylation ($CaMKII_{Phospho}$) requires sarcoplasmic reticulum (SR) Ca^{2+} release, whereas sustained CaMKII activity dependent on oxidation ($CaMKII_{Oxi}$) requires continuous ROS generation from mitochondria. Thus, the present findings provide the mechanistic details of how transient oxidative stress induces sustained CaMKII and LTF of $I_{Ca,L}$. We highlight the role of CaMKII as a pathological mediator of ROS and Ca^{2+} signaling in cardiomyocytes.

Materials and methods

Cardiomyocyte isolation

Rat ventricular myocytes were isolated as previously described [7]. Briefly, the hearts were removed from 6- to 8-week-old male Sprague–Dawley rats and connected to a Langendorff perfusion system. Initially, Ca^{2+}-free Tyrode's solution was perfused (10 ml/min) at 37 °C for 5 min, followed by 30 min perfusion with the same solution containing collagenase (0.4 mg/ml, Type II; Worthington Biochemical Co.). The remaining collagenase was then washed out with KB solution for 10 min. Finally, cardiomyocytes were obtained from the removed ventricles with gentle agitation. The isolated cells were stored in KB solution at room temperature to enhance survival of cardiomyocytes. Animals were carefully handled, anesthetized by a single-dose intraperitoneal injection of 70 mg/kg pentobarbital sodium, and sacrificed by cervical dislocation. All experimental procedures were conducted in accordance with the guidelines of the University Committee on Animal Resources at Seoul National University (Approval No. SNU-080107-8) and the *International Guiding Principles for Biomedical Research Involving Animals,* as issued by the Council for the International Organizations of Medical Sciences.

Voltage-clamp recording and analysis

$I_{Ca,L}$ was recorded in freshly isolated rat cardiomyocytes by applying depolarizing step pulses from the holding potential of −70 mV. Na^+ currents were inactivated by a prepulse to −40 mV for 200 ms before the second depolarization to 0 mV for 300 ms to activate $I_{Ca,L}$. To minimize the contamination of K^+ currents, we replaced K^+ with equimolar concentrations of Cs^+ in both the pipette and the external perfusion solutions. Signals were filtered at 2–5 kHz with a built-in low-pass filter of an Axopatch amplifier (Axon Instruments, USA) and digitized at 5–10 ksamples/s with an A/D converter (PCI-6040E; National Instrument, USA) and controlled by a custom-made software (R-clamp, by S.Y. Ryu) written with Delphi 6.0 (Borland Software Co., USA). Experiments were conducted either at room temperature (22 ± 2 °C) or at 36 °C.

Western blot analysis

The rat hearts were removed immediately and mounted on a Langendorff apparatus and perfused retrogradely through the aorta (10 ml/min) with specific solutions depending on the condition for 10 min at 37 °C: NT solution, NT solution containing 1 mM H_2O_2, Ca^{2+}-free Tyrode's solution, and Ca^{2+}-free Tyrode's solution containing 1 mM H_2O_2. After that the ventricles were cut into small pieces weighing about 300 g and homogenized into the same solution with which they were perfused in previous step. Each sample concentration of the total proteins was quantified using the Bradford assay. The same amount (100 g) of total proteins from each sample was separated on a 10% SDS–polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Corp). Membranes were blocked with 4% (w/v) bovine serum albumin in phosphate-buffered saline solution. Immunoreactivity was detected with the ECL Western analysis system (Amersham Pharmacia Biotech). CaMKII and phospho-CaMKII (p-CaMKII) antibodies purchased from Cell Signaling were used.

Mitochondrial ROS generation measurement

Cardiomyocytes were loaded with 5 μM MitoSOX red (Invitrogen, USA), a mitochondrial superoxide probe, for 1 h at room temperature. Cells were transferred to a chamber mounted on an inverted microscope (IX71; Olympus, Japan) and observed with a 20× objective (LCPlanFl, NA 0.4; Olympus). MitoSOX red fluorescence images were captured with a cooled CCD camera (Sensicam; PCO, Germany) and an imaging software (microManager [20]) using 470 nm excitation with a high-powered LED source and a driver (LED4C2 and DC4100; Thorlabs, Inc., USA) and 624/40 nm emission filter. The fluorescence intensity change of MitoSOX red is presented as $\Delta F = F - F_0$, where F is the fluorescence intensity at each time point and F_0 is the initial fluorescence intensity. The slope of MitoSOX red fluorescence change is considered the rate of mitochondrial ROS generation.

Total cellular ROS generation measurement

Cardiomyocytes were loaded with 5 μM CM-H_2DCFDA for 30 min at room temperature. DCFDA fluorescence images were obtained with 470 nm excitation and 535/40 nm emission.

Flavoprotein oxidation measurement

Flavoprotein autofluorescence images of cardiomyocytes were obtained with 470 nm excitation and 535/40 nm emission using a 40× water immersion objective (UApo/340, NA 1.15; Olympus).

Mitochondrial membrane potential measurement

Tetramethylrhodamine, ethyl ester (TMRE; Invitrogen) was loaded at 100 nM for 30 min at room temperature. TMRE fluorescence images were obtained with 530 nm excitation and 624/40 nm emission using a 40× objective.

Chemicals and solutions

The modified normal Tyrode's (NT) solution used in these experiments, including action potential recordings and fluorescence microscopy, contained (in mM) 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 5 HEPES and was titrated to pH 7.4 with NaOH. We replaced KCl with equimolar concentrations of CsCl and titrated to pH 7.4 with CsOH in the modified Cs^+-containing NT solutions for $I_{Ca,L}$ recordings. The pipette solution for $I_{Ca,L}$ recordings in the whole-cell configuration contained (in mM) 120 CsOH, 120 gluconic acid, 15-tetraethylammonium-Cl, 5 NaCl, 15 HEPES, 4 Mg-ATP, 4 Na-phosphocreatine, and 10 EGTA and was titrated to pH 7.3 with CsOH. For AP recordings in the whole-cell configuration, we used a pipette solution containing (in mM) 130 KOH, 130 gluconic acid, 5 NaCl, 15 Hepes, 4 Mg-ATP, 4 Na-phosphocreatine, and 0.5 EGTA, titrated to pH 7.3 with KOH. For Action potential recordings in nystatin (200 μg/ml) perforated patch-clamp mode, we used a pipette solution containing (in