Oxidative Stress–Induced Afterdepolarizations and Calmodulin Kinase II Signaling

Lai-Hua Xie, Fuhua Chen, Hrayr S. Karagueuzian, James N. Weiss

Abstract—In the heart, oxidative stress caused by exogenous H₂O₂ has been shown to induce early afterdepolarizations (EADs) and triggered activity by impairing Na current (I_{Na}) inactivation. Because H₂O₂ activates Ca²⁺/calmodulin kinase (CaMKII), which also impairs I_{Na} inactivation and promotes EADs, we hypothesized that CaMKII activation may be an important factor in EADs caused by oxidative stress. Using the patch-clamp and intracellular Ca (Ca_i) imaging in Fluo-4 AM–loaded rabbit ventricular myocytes, we found that exposure to H₂O₂ (0.2 to 1 mmol/L) for 5 to 15 minutes consistently induced EADs that were suppressed by the I_{Na} blocker tetrodotoxin (10 μmol/L), as well as the I_{Ca,L} blocker nifedipine. H₂O₂ enhanced both peak and late I_{Ca,L}, consistent with CaMKII-mediated facilitation. By prolonging the action potential plateau and increasing Ca influx via I_{Ca,L}, H₂O₂-induced EADs were also frequently followed by DADs in response to spontaneous (ie, non–I_{Ca,L}-gated) sarcoplasmic reticulum Ca release after repolarization. The CaMKII inhibitor KN-93 (1 μmol/L; n = 4), but not its inactive analog KN-92 (1 μmol/L, n = 5), prevented H₂O₂-induced EADs and DADs, and the selective CaMKII peptide inhibitor AIP (autocamtide-2–related inhibitory peptide) (2 μmol/L) significantly delayed their onset. In conclusion, H₂O₂-induced afterdepolarizations depend on both impaired I_{Na} inactivation to reduce repolarization reserve and enhancement of I_{Ca,L} to reverse repolarization, which are both facilitated by CaMKII activation. Our observations support a link between increased oxidative stress, CaMKII activation, and afterdepolarizations as triggers of lethal ventricular arrhythmias in diseased hearts. (Circ Res. 2009;104:79-86.)

Key Words: reactive oxidative species □ early afterdepolarization □ triggered activity □ arrhythmia □ CaM kinase

R eactive oxygen species (ROS) are generated as natural byproducts of normal oxygen metabolism and play important roles in cell signaling. However, under pathological conditions, such as heart failure and ischemia/reperfusion, ROS levels can become elevated and predispose the heart to arrhythmias.⁴-⁷ Oxidative stress induced by exposure to hydrogen peroxide (H₂O₂) and other agents has been shown to induce early afterdepolarizations (EADs), delayed afterdepolarizations (DADs), and triggered activity (TA).³–⁶ primarily attributed in previous studies to impaired inactivation of the Na current (late I_{Na}).⁴,⁵ However, H₂O₂ also affects other ion channels and transporters, including the L-type Ca current (I_{Ca,L}),⁷-⁹ K currents,¹⁰ ryanodine receptors (RyRs),¹¹ the sarcoplasmic reticulum (SR) Ca pump SERCA2a,¹² and the Na/Ca exchanger (I_{NCX}),¹²,¹⁵ which also may influence EADs and TA.

Recently, H₂O₂ has been shown to activate Ca/calcmodulin (CaM) kinase (CaMKIΙ) in cardiic myocytes and other cells,¹⁶-¹⁸ by direct oxidation of paired Met residues (Met281/282) in the regulatory domain. It is notable that binding of Ca²⁺/CaM is required to expose the redox sites in the regulatory domain in order for oxidation to persistently activate CaMKII.¹⁸ In addition, ventricular myocytes isolated from transgenic mice overexpressing CaMKIV have been shown to exhibit EADs and TA. Increased endogenous CaMKII activity accompanied EADs and ventricular tachycardia/ventricular fibrillation observed during telemetry in this mouse model.¹⁹ Alterations in I_{Ca,L} properties have been implicated as a major factor by which CaMKII activation promotes EADs,¹⁹,²⁰ but a recent study demonstrated that CaMKII activation also increases the late I_{Na} similar to H₂O₂. CaMKII inhibition has been shown to suppress EADs and TA. In a genetic heart failure model, for example, CaMKII inhibition prevented EADs and TA and improved mortality.¹⁹ The ability of pharmacological agents such as clofibrate to induce EADs in isolated rabbit ventricular myocytes was also suppressed by CaMKII inhibition.

Taken together, this evidence led us to hypothesize that activation of CaMKII by oxidative stress may be an important factor in arrhythmogenic effects of H₂O₂. Accordingly, we used isolated patch-clamped rabbit ventricular myocytes and...
Ca<sub>i</sub>, imaging to analyze the ionic mechanism(s) underlying H<sub>2</sub>O<sub>2</sub>-induced afterdepolarizations in the absence and presence of CaMKII inhibition. Our findings support a direct link between oxidative stress, CaMKII activation, and the genesis of TA caused by afterdepolarizations.

Materials and Methods

Cell Isolation

Ventricular myocytes were enzymatically isolated from adult rabbit hearts. Briefly, hearts were removed from adult New Zealand White rabbits (2 to 3 kg) anesthetized with intravenous pentobarbital, and hearts were then perfused retrogradely in Langendorff fashion at 37°C with nominally Ca-free Tyrode’s solution containing ~1.4 mg/mL collagenase (Type XIV, Sigma) for 25 to 30 minutes. After washing out the enzyme solution, the hearts were removed from the perfusion apparatus and swirled in a culture dish. The Ca concentration was slowly increased to 1.8 mmol/L, and the cells were stored at room temperature and used within 8 hours. The use and care of the animals were approved by the Chancellor’s Animal Research Committee at UCLA.

Intracellular Ca Measurement

Myocytes were loaded with the Ca indicator Fluo-4 by incubating them for ~30 minutes in bath solution containing 4 μmol/L Fluo-4 AM (Molecular Probes) and 0.016% (wt/wt) pluronic (Molecular Probes), after which the cells were washed and placed in a heated chamber on an inverted microscope. Ca<sub>i</sub> fluorescence was recorded using an Andor Ixon charge-coupled device camera (Andor Technology) operating at ~100 frames per second with a spatial resolution of 512×180 pixels. The fluorescence intensity was recorded in arbitrary units.

Patch-Clamp Methods

Myocytes were patch-clamped using the whole-cell configuration of the patch-clamp technique. For action potential (AP) recordings, patch pipettes (resistance, 2 to 4 MΩ) were filled with pipette solution containing (in mmol/L): 110 K-aspartate, 30 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 creatine phosphate, 0.05 cAMP, pH 7.2 with KOH. In some experiments, the CaMKII inhibitor peptide AIP (autocamtide-2–related inhibitory peptide) (2 to 10 μmol/L) (Biomol or Sigma) was added directly to the pipette solution. The cells were superfused with standard Tyrode’s solution containing (in mmol/L): 136 NaCl, 5.4 KCl, 0.33 Na<sub>2</sub>PO<sub>4</sub>, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose and 10 HEPES, pH 7.4 adjusted with NaOH. To isolate I<sub>ca,LT</sub>, patch pipettes (resistance 2 to 4 MΩ) were filled with pipette solution containing (in mmol/L): 110 Cs-Aspartate, 30 CsCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 creatine phosphate, 0.05 cAMP, pH 7.2 with KOH, and the cells were superfused with a modified Tyrode’s solution in which KCl was replaced by CsCl. H<sub>2</sub>O<sub>2</sub> (0.2 to 1 mmol/L) was added directly to the bath superfusate. Nifedipine (Sigma) and KN-93 and KN-92 (Biomol) were dissolved in DMSO (Sigma) and KN-93 and KN-92 (Biomol) were dissolved in DMSO as stock solution before diluting into the superfusate solution at the final concentration. The maximum DMSO concentration was <1/500 (vol/vol). Chemicals and reagents were purchased from Sigma unless indicated. Voltage or current signals were measured with an Axopatch 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1200 acquisition board driven by pCLAMP 8.0 software (Axon Instruments, Foster City, Calif). Action potentials were elicited with 2-ms, 2- to 4-nA pulses at pacing cycle lengths (PCLs) of 6 seconds. All experiments were performed at 34 to 36°C. Data are presented as means±SEM. Statistical significance was assessed using unpaired Student’s t tests, with P<0.05 considered significant.

Results

H<sub>2</sub>O<sub>2</sub>-Induced EADs and DADs in Adult Rabbit Ventricular Myocytes

APs were recorded from isolated rabbit ventricular myocytes using whole-cell current clamp mode. After AP duration (APD) and morphology reached steady state, cells were superfused with 0.2 to 1 mmol/L H<sub>2</sub>O<sub>2</sub>. The generation of EADs by H<sub>2</sub>O<sub>2</sub> was highly dependent on the PCL. Typically, at long PCLs (eg, 6 seconds), EADs occurred with every AP, and at short PCLs (eg, 1 seconds), no EADs occurred. In the
intermediate range, EADs occurred irregularly. Therefore, to elicit EADs most reliably, we used a PCL of 6 seconds throughout. EADs appeared after an average exposure time of 6.9 ± 1.1 minutes with 0.2 mmol/L H₂O₂ (n = 10) and 6.6 ± 0.8 minutes with 1 mmol/L H₂O₂ (n = 12). As shown in Figure 1A, EADs could be irregular, single, or multiple with an oscillating membrane potential before repolarization (Figure 1A and 1B). DADs were also observed after prolonged perfusion of H₂O₂ and occasionally triggered APs (Figure 1B, right). DADs often occurred following a previous AP with an EAD (Figure 1B, middle, and 1C), presumably because the prolonged APD allowed maintained I_{Ca,L} to overload the SR with Ca. Prolonged perfusion (15 to 20 minutes) of H₂O₂ eventually caused gradual depolarization of the resting membrane potential to less than −40 mV, and the cells became inexcitable.

Ionic Mechanisms of H₂O₂-Induced EADs and DADs

We next used pharmacological interventions to analyze the ionic and cellular mechanism(s) underlying H₂O₂-induced afterdepolarizations. As shown in Figure 2A, H₂O₂-induced EADs were reversibly suppressed by the selective Na current blocker tetrodotoxin (TTX) (10 μmol/L), which also shortened APD. Ranolazine (20 μmol/L), a more selective blocker of late I_{Na}, also eliminated H₂O₂-induced EADs and shortened APD (data not shown), consistent with previous reports by Song et al implicating late I_{Na} as playing a key role. Unlike H₂O₂, anemone toxin II (ATX), an agent that selectively delays the late phase inactivation of I_{Na}, failed to induce frank EADs, producing instead only small (1- to 2-mV) irregular oscillations during phase 2 of the AP. Note that APD was prolonged by ATX to a greater extent than after H₂O₂ (Figure 2B), yet no EADs were observed. This finding indicates that activation of late I_{Na} is not, by itself, sufficient to produce EADs under these experimental conditions, implying that other actions of H₂O₂ are also required.

Figure 2C shows that EAD amplitude after H₂O₂ depended on its takeoff potential during repolarization, such that the more repolarized the takeoff potential, the larger the EAD amplitude. This relationship parallels the voltage dependence of I_{Ca,L} reactivation and the I_{Ca,L} window current. Consistent with this interpretation, the selective I_{Ca,L} blocker nifedipine (10 μmol/L) eliminated EADs (Figure 2D), even though APD remained markedly prolonged because of the effect of H₂O₂ on late I_{Na} (as indicated by shortening of APD with subsequent application of 10 μmol/L TTX or 20 μmol/L ranolazine). Taken together, these results suggest that at least two actions of H₂O₂ are required for EAD formation: activation of late I_{Na} to reduce repolarization reserve (ie, prolong APD) and modification of I_{Ca,L} to enhance its reactivation properties so as to generate the EAD upstroke.

Whereas the effects of H₂O₂ on late I_{Na} have been well documented in previous studies, the reported effects of H₂O₂ on I_{Ca,L} have been variable. Therefore, we examined how H₂O₂ affected I_{Ca,L} in rabbit ventricular myocytes under our experimental conditions. Figure 3A and 3B shows the time course of the peak and residual pedestal (ped) I_{Ca,L} during a 300-ms voltage clamp pulse to 0 mV (voltage protocol shown in B). Voltage clamp pulse (above) and superimposed current traces showing I_{Ca,L} before (black) and ~5 minutes after perfusion of 1 mmol/L H₂O₂ (red). The difference current is shown in the bottom trace. C. Same as in B but with an AP clamp waveform replacing the square voltage clamp pulse.
at the end of a 300-ms voltage clamp to 0 mV in a representative myocyte during exposure to 1 mmol/L H₂O₂. Both peak and pedestal $I_{\text{Ca,L}}$ increased, from $7.3\pm0.8$ to $12.1\pm1.8$ pA/pF ($n=5$) and from $0.5\pm0.1$ to $3.2\pm0.3$ pA/pF ($n=5$), respectively. To examine how these changes affect $I_{\text{Ca,L}}$ during the repolarization phase of the AP, we performed AP clamp experiments before and after exposure to 1 mmol/L H₂O₂. Figure 3C shows that H₂O₂ induced a prominent $I_{\text{Ca,L}}$ hump during the late phase of the AP plateau, consistent with the timing of the EAD (compare with Figures 1 and 2). The recordings shown in Figure 3B and 3C represent the nifedipine-sensitive (subtracted) current, indicating that the increase of the inward current (including the hump in the late phase) is mostly attributable to $I_{\text{Ca,L}}$, although minor contamination by the Na/Ca exchange current ($I_{\text{NCX}}$) cannot be excluded.

The Role of Cai Cycling

After H₂O₂ exposure, we often observed a transient inward current ($I_{\text{ti}}$) following repolarization to −80 mV in a representative myocyte during exposure to 1 mmol/L H₂O₂. Both peak and pedestal $I_{\text{Ca,L}}$ increased, from $7.3\pm0.8$ to $12.1\pm1.8$ pA/pF ($n=5$) and from $0.5\pm0.1$ to $3.2\pm0.3$ pA/pF ($n=5$), respectively. To examine how these changes affect $I_{\text{Ca,L}}$ during the repolarization phase of the AP, we performed AP clamp experiments before and after exposure to 1 mmol/L H₂O₂. Figure 3C shows that H₂O₂ induced a prominent $I_{\text{Ca,L}}$ hump during the late phase of the AP plateau, consistent with the timing of the EAD (compare with Figures 1 and 2). The recordings shown in Figure 3B and 3C represent the nifedipine-sensitive (subtracted) current, indicating that the increase of the inward current (including the hump in the late phase) is mostly attributable to $I_{\text{Ca,L}}$, although minor contamination by the Na/Ca exchange current ($I_{\text{NCX}}$) cannot be excluded.

To further investigate the role of Cai cycling in H₂O₂-induced afterdepolarizations, we examined the effects of pre-loading myocytes with BAPTA-AM to buffer changes in Cai (Figure 5A). We also pretreated myocytes with thapsigargin (0.2 μmol/L) and ryanodine (10 μmol/L) to suppress SR Ca cycling (Figure 5B). Both interventions completely prevented EADs and DADs during exposure to 1 mmol/L H₂O₂ (Figure 5), indicating the intact Cai cycling plays a critical role. However,
APD remained prolonged under these conditions, probably because of reduced Ca-induced inactivation of \( I_{\text{Ca,L}} \) when the intracellular Ca transient was suppressed.26

The Role of CaMKII Signaling
Similar to the effects of \( \text{H}_2\text{O}_2 \), Ca\(^{2+}\)–dependent CaMKII activation has been shown to modify \( I_{\text{Na}} \) inactivation, enhance \( I_{\text{Ca,L}} \), and promote EADs.19–21 CaMKII could either be activated by Ca\(_{\text{i}}\), resulting from the effects of \( \text{H}_2\text{O}_2 \) on Ca\(_{\text{i}}\)-cycling proteins, or directly by oxidative stress, as shown recently in cardiac myocytes.16,18 The Ca\(_{\text{i}}\) dependence is still consistent \( \text{H}_2\text{O}_2 \)-mediated activation of CaMKII, because persistent activation of CaMKII by oxidation requires Ca-CaM binding to CaMKII to expose its redox sites.18 We examined the effects of CaMKII inhibition to test these possibilities. Pretreatment with the CaMK inhibitor KN–93 (1 \( \mu \)mol/L) prevented the emergence of EADs during exposure to \( \text{H}_2\text{O}_2 \) (1 mmol/L for up to 20 to 30 minutes) in 4 myocytes (Figure 6A), compared to nonpretreated myocytes in which \( \text{H}_2\text{O}_2 \) consistently induced EADs within 5 to 10 minutes of \( \text{H}_2\text{O}_2 \) exposure (Figures 1A and 6B). In addition, KN–93 also suppressed EADs after they were induced by \( \text{H}_2\text{O}_2 \) perfusion (Figure 6B).

In contrast, 1 \( \mu \)mol/L KN–92, an inactive analog of KN–93, was ineffective (Figure 7A and 7B), with EADs appearing after an average of 7.5 ± 1.2 minutes (\( n = 5 \)) after exposure to 1 mmol/L \( \text{H}_2\text{O}_2 \) (\( n = 5 \)). BAPTA-AM, however, remained effective at suppressing \( \text{H}_2\text{O}_2 \)-induced EADs in the presence of KN–92 (Figure 7B).

Because both KN–93 and KN–92 have nonspecific effects, such as blockade of Ca and K currents,22,27,28 we also examined the effects of the selective CaMKII inhibitor peptide AIP (2 \( \mu \)mol/L). In 8 myocytes, AIP added to patch pipette dialyzing the cytoplasm delayed the appearance of EADs during exposure to 200 \( \mu \)mol/L \( \text{H}_2\text{O}_2 \), from 6.6 ± 0.8 (\( n = 10 \)) to 13.9 ± 2.7 minutes (Figure 6C) (\( P < 0.05 \)). Moreover, AIP dialysis also prolonged APD in a concentration-dependent manner, presumably because of nonspecific peptide effects, such that higher AIP concentrations were ineffective at preventing \( \text{H}_2\text{O}_2 \)-induced EADs.

Discussion
In the present study, we investigated the mechanisms by which oxidative stress caused by exposure to \( \text{H}_2\text{O}_2 \) induces afterdepolarization and TA in rabbit ventricular myocytes. The novel findings are as follows: (1) \( I_{\text{Ca,L}} \) modification, in addition to \( I_{\text{Na}} \) modification, plays a crucial role in EAD generation by \( \text{H}_2\text{O}_2 \); (2) CaMKII activation, either directly via oxidative stress or indirectly via elevated Ca\(_{\text{i}}\) is critical for these effects; (3) by enhancing SR Ca loading and promoting spontaneous Ca\(_{\text{i}}\) waves, \( \text{H}_2\text{O}_2 \)-induced EADs also cause DADs, providing a direct link between these 2 types of afterdepolarizations, an effect that compounds the arrhythmogenic potential of oxidative stress.

Ionic Mechanisms of EADs and DADs Induced by \( \text{H}_2\text{O}_2 \)
Two conditions are required to generate an EAD. First, repolarization reserve must be reduced during phases 2 and 3.
blockers (Figure 2) showed that despite prolonging APD, generation of the EAD. The experiments using multiple ion channel successfully overcome by outward currents, accounting for the mechanisms of H2O2-induced EADs have implicated study, we show that in addition to modification of Iamplitude of I, subsequent partially suppressed by KN-93. APs at points a (control), b (~2 minutes after KN-92), c (KN-92+H2O2), and d (KN-93+H2O2) are shown below. B, Same as in A but applying BAPTA-AM to suppress EADs after KN-92+H2O2.

Figure 7. Failure of KN-92, an inactive analog of KN-93, to prevent H2O2-induced EADs. A, Same protocol as in Figure 6A but with KN-92 initially in place of KN-93. KN-92 failed to prevent EADs during exposure to 1 mmol/L H2O2, which were subsequently partially suppressed by KN-93. APs at points a (control), b (~2 minutes after KN-92), c (KN-92+H2O2), and d (KN-93+H2O2) are shown below. B, Same as in A but applying BAPTA-AM to suppress EADs after KN-92+H2O2.

of the AP, by an increase in inward current, a decrease in outward current, or both. Second, once repolarization reserve has been compromised, reactivation of ICa,L window current, and/or additional SR Ca release augmenting Ca-sensitive inward currents such as INCX, must be sufficiently powerful to reverse repolarization and generate the EAD upstroke. In ventricular muscle, inward currents influencing repolarization reserve include INa, ICa,L, and INCX; whereas outward currents include the rapid and slow delayed rectifier potassium currents (IKr and IK1), the transient outward current (Ito), and the inward rectifier potassium current (IK1). Previous studies analyzing the mechanisms of H2O2-induced EADs have implicated impaired INa inactivation as the primary mechanism reducing repolarization reserve. However, H2O2 has also been reported to affect other currents and transporters. In the present study, we show that in addition to modification of INa, modification of ICa,L by H2O2 plays a key role in EAD genesis. Not only did H2O2 substantially increase the peak amplitude of ICa,L, but it also impaired ICa,L inactivation (increase of pedestal current as shown in Figure 3A and 3B). The consequence was a large increase in the late phase of ICa,L, as seen during the AP clamp in Figure 3C, which was not successfully overcome by outward currents, accounting for the genesis of the EAD. The experiments using multiple ion channel blockers (Figure 2) showed that despite prolonging APD, INa modification was not by itself sufficient to induce EADs when ICa,L was blocked. Both INa and ICa,L modification could be attributed to CaMKII activation, because, as shown in Figure 6A, H2O2 failed to prolong APD significantly or cause EADs when CaMKII was blocked by KN-93.

Oxidative stress also modulates the properties of other Ca2+-cycling proteins, including ryanodine receptors11,12 and SERCA2a,13 which could potentially promote EAD genesis by modulating SR Ca release during repolarization. Indeed, some recent studies have shown that EADs and DADs can share a common mechanism under CaO2 overload conditions.29–31 Non–ICa,L–gated SR Ca2+ release during repolarization has been proposed to contribute to EAD genesis by activating Ca-sensitive inward currents.29 but it is difficult to unequivocally distinguish from SR Ca release triggered by reactivated L-type Ca channels.

It has been reported that voltage can directly activate SR Ca2+ release in cardiac myocytes, which is enhanced by cAMP.32,33 However, EADs were readily induced by H2O2 using cAMP-free pipette solution (data not shown; see also Song et al5), excluding an essential role of a cAMP-sensitive voltage-activated Ca release in EAD generation.

**Contribution of SR Ca Handling to the EADs and DADs Induced by H2O2**

CaMKII can phosphorylate RyR2, which may enhance SR Ca release—dependent TA.34 However, although it is well accepted that CaMKII phosphorylates RyR2, the functional consequences are controversial. Bers and colleagues found increased SR Ca leak from CaMKII-mediated RyR2 phosphorylation,34,35 but Yang et al36 reported a suppression of Ca sparks and Ca waves. The cause of this discrepancy remains to be determined. In addition, oxidative stress has direct effects on RyR and SR function. The interdependencies between SR Ca cycling, ICa,L, INCX, and repolarization reserve are complex and nonlinear, making it very challenging, if not impossible, to assign a simple mechanistic role of SR Ca cycling to EAD generation. For example, SR depletion could suppress EADs by any of the following interactive mechanisms: (1) preventing CaMKII activation by oxidative stress (because the Ca-CaM interaction is still required); (2) increasing repolarization reserve by suppressing INCX; (3) preventing Ca waves during the repolarization phase; or (4) altering activation/inactivation properties of other ionic currents.

In the presence of BAPTA, the AP has a higher rectangular plateau (Figure 5), which may also hinder the reactivation of ICa,L and EAD formation, consistent with a recent study showing that triangulation of the AP favors ICa,L reactivation.37

**The Role of CaMKII Signaling in H2O2-Induced EADs**

The enhancement of ICa,L by H2O2 in the present study is consistent with activation of CaMKII by H2O2, although direct redox modifications of L-type Ca channel subunits may also contribute. CaMKII is known to mediate ICa,L facilitation,38 and CaMKII activation has been shown to promote EADs in a variety of settings.19,22 Moreover, CaMKII activation has recently been reported to impair INa inactivation,21 consistent with a common underlying pathogenesis of H2O2-induced and CaMKII-induced EADs. Our finding that the CaMKII inhibitor KN-93 but not its inactive analog KN-92
suppressed EAD formation by \( \text{H}_2\text{O}_2 \) generally supports a common mechanism. On the other hand, both KN-93 and KN-92 have nonspecific effects, including substantial block of \( I_{\text{Ca,L}} \)^27 which is critical in EAD formation. However, the selective CaMKII peptide inhibitor AIP also significantly delayed the onset of EADs during exposure to 0.2 mmol/L \( \text{H}_2\text{O}_2 \). Theoretically, AIP should be equally effective at preventing CaMKII activation by oxidative stress as by elevated Ca, because both activation modes require initial interaction of the regulatory domain of CaMKII with Ca-CaM complexes to expose the redox and autophosphorylation sites on the catalytic domain of CaMKII.\(^{18} \) This also explains why BAPTA-AM prevents EADs during \( \text{H}_2\text{O}_2 \) exposure, by preventing Ca from rising sufficiently for Ca-CaM to interact with CaMKII and trigger the subsequent persistent activation by redox modification.

Based on these observations, the role of CaMKII activation in \( \text{H}_2\text{O}_2 \)-induced EADs can be summarized as follows: (1) CaMKII activation by \( \text{H}_2\text{O}_2 \) contributes to both impaired \( I_{\text{Na}} \) inactivation and \( I_{\text{Ca,L}} \) facilitation; (2) Both factors enhance cellular Ca loading by prolonging APD and increasing Ca influx via \( I_{\text{Ca,L}} \), promoting further CaMKII activation via Ca-CaM; (3) KN-93 synergistically suppresses EADs by preventing CaMKII activation by \( \text{H}_2\text{O}_2 \) and reducing Ca influx by directly blocking \( I_{\text{Ca,L}} \). Without concomitantly inhibiting CaMKII activation, partial block of \( I_{\text{Ca,L}} \) by KN-92 is not sufficient to suppress EAD formation; and (4) direct redox modification of ion channel proteins may also contribute, because selective CaMKII inhibition with AIP delayed the onset of, but did not entirely suppress, \( \text{H}_2\text{O}_2 \)-induced EADs. We do not have a ready explanation for why AIP showed less effectiveness than KN-93 in our present study. KN-93 also blocks the L-type Ca current on which EAD generation depends, which may account for its more complete potency. Alternatively, AIP may have had nonspecific effects which reduced repolarization reserve, because it modestly prolonged APD.

**EAD-Associated DADs**

Associations between EADs and DADs have been noted previously,\(^{6,31,39,40} \) but the underlying mechanisms have not been analyzed in detail. For example, in atrial myocytes, Song et al\(^{40} \) found that ATX-induced EADs were also associated with DADs. They postulated that DADs were attributable to excessive Ca loading as a result of APD prolongation by EADs, leading to spontaneous SR Ca release following repolarization. Here, we directly documented this postulated mechanism for \( \text{H}_2\text{O}_2 \)-induced EADs by using optical Ca imaging to detect the spontaneous Ca wave inducing a transient inward current causing the DAD (Figure 4). It is also conceivable that oxidative stress directly sensitizes the SR to non-\( I_{\text{Ca,L}} \)-gated SR Ca\(^{2+} \) release through direct effects on Ca\(^{2+} \)-cycling proteins such as RyR\(^{11,12} \) but this remains to be established. In either case, our findings suggest that both EADs and EAD-induced DADs may both contribute to TA during oxidative stress, amplifying the arrhythmogenic consequences.

**Clinical Implications**

Increased oxidative stress is believed to be an important factor predisposing the diseased heart to lethal arrhythmias. Here, we have shown how oxidative stress caused by exogenous \( \text{H}_2\text{O}_2 \) predisposes the heart to both EADs and DADs, and we have demonstrated a link to CaMKII activation, which is also involved in other aspects of heart failure.\(^{41,42} \) Other free radical sources have also been reported to produce similar results, ie, inducing EADs, DADs, and TAs.\(^{6} \) Although it is still under debate, \( \text{H}_2\text{O}_2 \) levels up to \( \approx 35 \mu \text{mol/L} \) have been reported in human blood plasma.\(^{43} \) It is also well known that ROS levels can increase during ischemia and reperfusion by as much as 100-fold\(^{44} \) and also with age by 7.5-fold.\(^{45} \) Because ROS are short-lived radicals, it is conceivable that the local concentrations near sites of production are considerably higher than circulating concentrations. Therefore, the concentrations (100 to 200 \( \mu \text{mol/L} \)) used in most of our experiments are reasonable approximations of the pathophysiologically relevant range. Targeting CaMKII signaling may have therapeutic potential to prevent arrhythmias in the failing heart, although negative inotropic actions\(^{46} \) may limit the use of CaMKII inhibitors.

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**Disclosures**

None.

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